Vitellogenin2: spermatozoon specificity and immunoprotection in mud crabs

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Abstract

As the precursor of vitellin (Vn), vitellogenin (Vg) has initially been considered as a female-specific protein involved in vitellogenesis, while it was also present in males induced by hormones or organs manipulation. Distinct from *vtg1* we previously found in female mud crab *Scylla paramamosain*, *vtg2* was intriguingly detected in male testis under normal physiological conditions in this study. Sequence analysis showed that *vtg2* and *vtg1* were actually two isoforms of Vg caused by different types of alternative splicing. PCR and *in situ* hybridization analysis revealed that *vtg2* was localized only in the testicular spermatozoa, while Vn was detected in both the spermatozoa of the testis and seminal vesicle. Therefore, we speculated that Vn was initially translated in testicular spermatozoa, then migrated with spermatozoa, and finally stored in the seminal vesicle, where spermatozoa gradually accomplished maturation. We presumed that *vtg2*/Vn might act as an immune-relevant molecule in the male reproduction system. In the subsequent experiment, the expression of *vtg2*/Vn in testis was significantly induced in response to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) injection at both transcriptional and translational levels. In the light of the results presented above, we deemed that *vtg2*/Vn is a novel candidate of immune-relevant molecules involved in immunoprotection during the spermatozoon maturation, and this research helps to open a new avenue for further exploring the role of Vg.

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Introduction

Vitellogenin (Vg) is the precursor of vitellin (Vn) in egg yolk, and expressed in the females of nearly all oviparous species, including fish, amphibians, reptiles, birds, most invertebrates, and monotremes (Robinson 2008). Vg is usually produced in an extraovarian tissue and then transported by the circulation system into ovary, where it is internalized into growing oocytes via receptor-mediated endocytosis (Arukwe & Goksory 2003, Finn & Fyhn 2010). Thereafter Vg undergoes several modifications, such as specific proteolytic cleavage, to become Vn providing nutrients and energy to the embryos development (Avarre *et al.* 2003, Subramoniam 2011, Zhang *et al.* 2016). It is well known that the sites of Vg synthesis include the liver of vertebrate species (Gerber-Huber *et al.* 1987, Pousis *et al.* 2011), the intestine of nematode (Sharrock 1983), the fat body of insects (Chen & Hillen 1983, Giorgi *et al.* 1989), and the hepatopancreas and ovary of crustaceans (Tsutsui *et al.* 2000, Jia *et al.* 2013).

Vg was initially regarded as one of a female-specific protein (Pan *et al.* 1969); however, it was also reported to be synthesized in some males. Under the induction

of some environmental hormones, Vg was synthesized in male fish by the estrogen (Del Giudice *et al.* 2012), and in male crab by the 4-nonylphenol (Ricciardi *et al.* 2008). Certain manipulations could also induce the synthesis of Vg, such as in andrectomized male terrestrial isopod *Armadillidium vulgare* (Suzuki *et al.* 1990) and male giant freshwater prawn *Macrobrachium rosenbergii* with X-organ sinus gland (XO-SG) complex removed (Wilder *et al.* 1994). Intriguingly, even under normal physiological conditions without any induction or treatment, Vg/Vn has also been reported in a few male species such as fish (Goodwin *et al.* 1992), sea urchins (Shyu *et al.* 1986, Unuma *et al.* 2010), insects (Bebas *et al.* 2008, Majewska *et al.* 2014) and crustaceans (Avarre *et al.* 2003, Niksirat *et al.* 2014, 2015, Liu *et al.* 2015). This suggests that, besides involving in yolk protein formation, Vg/Vn may play other roles independent of gender. In recent years, accumulating data have demonstrated that Vg fulfills important roles in innate immune responses in different species (Raikhel *et al.* 2002, Tong *et al.* 2010, Wu *et al.* 2015). Vg in fish acts as a multivalent pattern recognition receptor (PRR), a bactericidal molecule or an acute-phase protein in innate immunity. For instance, Vg has

multiple specificities capable of identifying bacterium, virus and an antimicrobial effector, as well as causing damage to the microbes and enhancing phagocytosis (Zhang et al. 2011a).

During the last decade, molecular methods have been used to investigate the function of Vg/Vn in many crustaceans (Tsukimura et al. 2001, Avarre et al. 2003). Most studies have been focused on the mechanism of Vg/Vn involved in the vitellogenesis and its hormonal control (Subramoniam 2011). On the other hand, partial Vg genes are detected in male marine shrimp *Penaeus semisulcatus* and Chinese mitten crab *Eriocheir sinensis* (Avarre et al. 2003, Liu et al. 2015) without any induction or treatment. This suggests that Vg may also exist in male crustacean under normal physiological condition. By far, little is known about whether the Vg in male has the same structure and/or physiological function with that in female. This study attempts to investigate the above issues.

The mud crab, *Scylla paramamosain*, is a gonochoristic species with distinct sexual dimorphism. The male reproductive system includes testis, vas deferens (including anterior vas deferens, seminal vesicle and posterior vas deferens) and ejaculatory duct (Xu et al. 2011). Spermatozoa in *S. paramamosain* lack a true flagellum and are nonmotile (Zhang et al. 2010) as described in other crustacean (Kouba et al. 2015). In testis, the mature spermatozoa are agglutinated by the testicular secretion, and then transferred to the vas deferens, which are packaged inside spermatophore. During mating, the spermatophore is transferred to the seminal receptacle of the female and is stored there until the beginning of fertilization process (Guan et al. 2003).

We have previously found *vtg1* (GenBank: KC734559) in female ovary and hepatopancreas of the mud crab *S. paramamosain*. In this study, we detected a novel Vg in the testis of the male mud crab, and obtained a full-length cDNA encoding Vg of *S. paramamosain* (*vtg2*). Genome sequence verified the male *vtg2* and female *vtg1* were two isoforms of Vg, caused by alternative splicing. Finally, we demonstrated the potential role of *vtg2/Vn* in the gonad of male mud crab might act as an immunoprotection factor in the process of spermatozoon maturation.

Materials and methods

Animals

All experimental and surgical procedures were approved by an Institutional Animal Care and Use Committee. Adult male crabs *S. paramamosain* (120–200 g, with carapace width of 8.0–10.0 cm) were obtained from the local aquatic market in Xiamen, Fujian Province, China. Before dissection, crabs were reared in rectangular tanks with seawater at a temperature of 25–29°C and a salinity of 26 ppm for a

week, fed with the live clam *Ruditapes philippinarum*. All crabs used in experiments were under normal physiological conditions without any induction or treatment. For tissues sampling, crabs were placed on ice for anesthetization before tissues dissection.

Animals at intermolt stage were used for the immune challenges with injection of lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (Sigma) at a dose of 1 µg suspended directly in 20 µL crustacean saline (440 mM NaCl, 11.3 mM KCl, 13.3 mM CaCl₂, 26 mM MgCl₂, 23 mM Na₂SO₄, 10 mM HEPES, pH 7.4) for the test group and 20 µL saline for the control, similar to that described in the blue crab *Callinectes sapidus* (Tsutsui & Chung 2012). In each group, four crabs were sampled at 0, 0.5, 1, 2, 4, 8 and 16 h after injection (hpi) respectively, and testis and seminal vesicle were withdrawn for RNA and protein preparation. The control and experimental crabs were separately maintained in 75 L plastic aquarium tanks and cared following the protocols of previous research (Yang et al. 2014).

RNA extraction and cDNA synthesis

All tissues were removed immediately and flash frozen in liquid nitrogen. Total RNA was extracted using TRIzol RNA isolation reagent (Invitrogen) according to the manufacturer's instructions and the total RNA concentration, quality and integrity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Genomic DNA was removed by DNase I (Thermo Scientific) digestion and the first-strand cDNA was synthesized from 1 µg total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions.

Cloning the full-length cDNA and gDNA of *vtg2*

According to the nucleotide sequence of *vtg1* cDNA, five pairs of specific primers and two 3' RACE primers (Table 1) were designed to clone the *vtg2* cDNA. The PCR was performed in a 25 µL reaction volume containing 2.0 µL testis cDNA template, 0.5 mM each of primers and 0.2 U of Taq DNA polymerase (Takara Bio), using a thermal cycling profile of 3 min at 94°C for 1 cycle, 30 s at 94°C, 30 s at 50°C, 3 min at 72°C for 42 cycles; 10 min at 72°C and finally held at 10°C. PCR products were analyzed by 1.0 % agarose gel and sequenced.

The 3' terminal of *vtg2* cDNA was obtained by the rapid amplification of cDNA ends (RACE) methods with 3'-Full RACE Kit (Takara Bio) according to the manufacturer's instructions. Specific primers F6 and F7 are shown in Table 1. Finally, five fragments and 3' terminal sequences of *vtg2* were linked to form the full-length cDNA based on the overlapping sequences.

High-molecular-mass genomic DNA was isolated from four samples. In order to determine whether the *vtg2* and *vtg1* is the alternative splicing of the same mRNA, PCR was performed with five pairs of gene-specific primers F1 and R1, F2 and R2, F3 and R3, F4 and R4, F5 and R5 (Table 1) designed according to the full-length cDNA of *vtg2*. The PCR protocol was performed as described above.

Table 1 Primers used in this study.

Name	Sequence (5'–3')	Position	Purpose
F1	TGGCGAGTGC GAAGCC	1	Gene cloning
R1	TTCCACCAGTCTTTGACCATC	1600	Gene cloning and <i>in situ</i> hybridization
F2	CAGGAGCCTCCGTTACGACTA	1291	Gene cloning and <i>in situ</i> hybridization
R2	GCACATCTTGATACCGAACACTG	3335	Gene cloning
F3	TTGGCTACGACGCCTACATT	3045	Gene cloning
R3	AGCAGGAATGGCACCCTACTT	4974	Gene cloning
F4	GCTTTTATCTTTAGAGGGCAACG	4588	Gene cloning
R4	TCGATACCGATGAGGGAGAT	6417	Gene cloning
F5	TCGTCAACGAAATCGCTCAA	6044	Gene cloning
R5	GCGTTCCTGCCATTGCTC	7869	Gene cloning
F6	GGAGAGCCAAAGTGACGCAGA	7171	3' RACE
F7	GCGGGCCAACGACAGAATG	7831	3' RACE
F8	GAGCGAGAAATCGTTCGTGAC	–	Internal control
R8	GGAAGGAAGGCTGGAAGAGAG	–	Internal control
F9	GAAAGTGCTGGTGAAGAAGGAA	985	Real-time PCR
R9	ACACGACTGAAGAGATGATGCC	1148	Real-time PCR

Tissue distribution and expression profile of vtg2

Total RNA was isolated from various tissues including gill, stomach, hepatopancreas, thoracic ganglion, cerebral ganglion, testis, seminal vesicle, heart, muscle and hemocytes of four adult male crabs. The *vtg2* mRNA distribution in male tissues was carried out by PCR (protocol was described above) using primer F2 and R1 (Table 1). In order to compare the relative levels of expression of *vtg2* in the samples, the housekeeping gene *β-actin* (GenBank: GU992421) was also amplified with the same cDNA samples using the primers F8 and R8 (Table 1). The products were assessed by 1% agarose gel electrophoresis.

The fluorescent quantitative real-time PCR (qRT-PCR) was used to investigate the response of *vtg2* gene to LPS and LTA at mRNA level. Gene-specific primers, F9 and R9 (Table 1) for *vtg2* and F8 and R8 for β -actin, were used to amplify the corresponding products. The qRT-PCR was carried out with 2xSYBR Select Master Mix (Applied Biosystems) as per the manufacturer's instructions in a 7500 Fast Real-time PCR System (Applied Biosystems). All experiments were performed in triplicate.

database using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The deduced amino acid sequence was obtained using DNASTar BioEdit software. The molecular mass and theoretical isoelectric point were predicted using the compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). The amino acid sequences were aligned using ClustalW software. The signal peptide and other characteristic structures of Vtg2 were predicted by the UniProt program (<http://www.uniprot.org/uniprot/A0A0G2ST52>).

Each expression levels of gene were assayed and the cycle time (Ct) values were accessed from the PCR cycler software (7500 software v2.0.5). Fold change for the gene expression relative to control was determined by the $2^{-\Delta\Delta C_t}$ method. All data were given in terms of relative mRNA expressed as mean $\pm$ S.D. Statistical analysis was performed using IBM SPSS 20. The data obtained from qRT-PCR analysis were subjected to One-way Analysis of Variance (ANOVA) followed by Dunnett's two-sided *post hoc* test to determine the differences in the mean values among the treatments. Significance was set at $P < 0.05$.

Sequence analysis and statistics

The obtained *vtg2* cDNA and gDNA sequences were compared with other known *Vg* sequences in NCBI

Western blotting analysis

Western blotting technique was used to detect Vtg2 protein (Vn) in testis and seminal vesicle (the position was

A

TGTGGAGGTGGGAAGCCATCATGACTAGTGCACACAGTCTGCTACTGGCCCTGTGGCGCTGCTGTCTGCAAGTATATAAGGTTTCATTTTGAGGTACTCT 100
 ATAACCTCGGTGA AAAAGGATCAAAGTAAAGTATATCAATTTA TCCCTTTTCCCTTCAATTCACATCTGTAAAGCAATACCTCATCTCTTCTCTACTTTGGCTTC 200
 ATAGAGACGGATGGATACCATCTCTCA TGAAGAATTATGCTCAATAATCATCTACACAGTGGCCCATCATCTCTTAACATCAGTGTCTTTCCACAGGCGCG 300
 TATGGAGGCCACACCCAGGCGCTGTGGACGGAGTGCCCGTGTGGCGCCCTGCCAAGCTGTCTTTCACTCCAGGC AAAAATTAAGTGTACTTACTGTACTTCAACAGGAA 400
 AGTCCAAATGTACAGCTCAAAAGGCGTGTATGCGGCTATGCTGGAGACACAATGGGAAGAACCAAGTGTCTGCTGACTGTCTCGGCCGCTGGCAGGTGGGCAT 500
 CTCCTTCAAGGCGTCCAAGGTGATGGCAGACAGGTAAAGTCCCAACCAAGCTAGTCCAGTGGTCTTCACTCCACGCTACACAGGCCAACAGACCTCTCTTA 600
 TATTACTTCCCGCGTGTCTCTAGGATTGCTGTGGTCCGATAAAGTTGGAGAGGTACCCGCTGTGGTGGGATGACCGACGCGAGTGCAGCGCGGTGTGC 700
 AGTCATCCCGATGACGATACATGGGCAATCAACATGAAGAAGGCGGTGTTTGGCTCTGCAGATAAAGCTGCGGCTCTCTCCATAGCAATTCAGGAC 800
 TCAACATTAACAGAGTAAATACCGCTGTGGATGCGTCACTCGCAATCACATGATTAAGTCTCTGTTAAAGTGTACAGATGAGCTTAATTTGGGAAGGCCA 900
 AGATATCTAAAGCTTCTATTGCTGTGATAACAGACGGAGCTGCTGGGAAGTGCCCACTACTCTGAGGTCAAGCTGAAAGGTGCGCAAGGTGCTGTGAAG 1000
 AAGGAAAGAACCATCTGCTTTATCGCAAGACCAATTACTCTACCCGACAGAGATAAATCTCCACATCTG 1000

B

CGACACGGACTAGACAATGACTACTACTGCTAAACAGGTTTGGCTAATGTAAGATAGCAAAACATGTCTCTGAATTTTGAGAGACATGAGAAAAGAAAT 8240
AAAAAAAAAGTAAATAAATACATTAAACAAATAGATAAAATCAAAAATTTAAATTTAAAAAAAAAAAAAAAAAAAAAAAAAAAA 8316

Figure 1 Deduced amino acid sequence and 5' and 3' UTRs of *vtg2* of male *S. paramamosain*. (A) 5' UTR (nt 1-1069) of *vtg2* cDNA. Underlined sequences were identical with the first three intron of *vtg1* gDNA. (B) 3' UTR (nt 8141-8316) of *vtg2* cDNA. The polyadenylation signals were underlined.

Figure 2 Genome structure of *vtg2* in *S. paramamosain*.

(A) Exon-intron organization of *vtg2* gene. Blue boxes represent exons and joining lines introns. Red and green lines indicate start and stop codons respectively. Intron number (I1–I11) and size (78–380bp) are indicated at the top and bottom of the figure. (B) Localization of predicted protein structural domains: LPD-N (vitellogenin), DUF1943, DUF1081 and VWD.

described previously (Xu et al. 2011)) and to investigate the response of Vn in seminal vesicle to LPS and LTA at protein level. Total proteins from four samples were extracted with RIPA (Solarbio, Beijing, China), then centrifuged at 5000 *g* at 4°C for 10 min, the supernatant was collected and stored at –80°C before use. Protein concentrations were determined using the BCA Protein Assay Kit (Solarbio, Beijing, China).

Each protein sample was homogenized at 50 µg and subjected to 8% SDS-PAGE, and then transferred to a PVDF membrane at 100V for 1 h in transfer buffer (192 mM glycine, 25 mM Tris, 20% methanol). After blocking with 5% BSA in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4) for 2 h at room temperature, the membrane was incubated with rabbit anti-Vn serum (1:5000) of the swimming crab *Portunus trituberculatus* (a gift from Professor Yongxu Cheng, Shanghai Ocean University, China. The preparation of antibody was presented in the reference (Zhang et al. 2011b)) overnight at 4°C. After washing in TBST (10 mM Tris, 150 mM NaCl, 0.2% Tween 20, pH 7.8) for three times, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (H+L) (1:5000, Multisciences, Hangzhou, China) for 1 h at room temperature. For the control group, the first antibody was Rb a beta-actin (1:2000, Bioss, Beijing, China). Finally, the membrane was washed and then the signal was developed using a SuperEnhanced Chemiluminescence Detection Kit (Appligen, Beijing, China), and the protein bands were visualized by a gel imaging and analysis system (ChemiDoc XRS) (Bio-Rad).

Figure 3 Distribution of *vtg2* mRNA in different tissues of male *S. paramamosain*.

Histology

Histological staining

Testis and seminal vesicle were dissected from adult male crabs. They were fixed in Bouin's fixative for 12 h at 4°C, dehydrated, and embedded in paraffin. Sections of 7 µm thicknesses were deparaffinized, rehydrated and then stained with hematoxylin and eosin.

In situ hybridization

A 310bp fragment of *vtg2* was amplified by PCR using primer F2 and R1 (Table 1) and cloned into pGEM-T vector (Promega) used as probe templates. The antisense and sense riboprobes were synthesized using a DIG-RNA Labeling Kit (Roche). The probes were stored at –80°C until used.

Testis and seminal vesicle of adult male crab were quickly dissected out and then immediately fixed in 4% paraformaldehyde (Solarbio, Beijing, China) in PBS overnight at 4°C. Tissues were dehydrated, embedded in paraffin, and then sectioned at 7 µm thickness and sections were mounted on glass slides coated with poly-L-lysine (Solarbio, Beijing, China). Sections were deparaffinized, rehydrated and rinsed in PBS. Then the following protocols were carried out according to our previous description (Yang et al. 2014).

Immunohistochemistry

Sections were incubated with 3% H₂O₂ in PBS for 10 min at room temperature to deplete endogenous peroxidase activity. After washing in PBS (3 × 5 min), sections and

Figure 4 Localization of *vtg2* mRNA by *in situ* hybridization in testis (A, B and C) and seminal vesicle (D, E and F) of male *S. paramamosain*. A and D: hematoxylin-eosin staining; B and E: antisense probe; C and F: sense probe. Arrows in A and B indicated spermatozoa. SG, seminal granule; SP, spermatophore.

Figure 5 Detection of Vn in male reproductive system of *S. paramamosain*. Lanes 1: total protein of testis; Lanes 2: total protein of seminal vesicle. Molecular mass markers were indicated on the left.

smears were blocked in 5% BSA (Solarbio, Beijing, China) for 20 min at room temperature, and incubated with Vn antibody diluted 1:1000 for 2 h at 37°C. Meanwhile, at control groups, anti-Vn serum was replaced by PBS. After washing in PBS for another three times, sections and smears were incubated with HRP-conjugated goat anti-rabbit IgG (Maixin, Fuzhou, China) for 15 min at room temperature. Sections and smears were washed with PBS and then colored with 3,3'-diaminobenzidine (Solarbio, Beijing, China). Images were taken using an Olympus BX51 microscope (Olympus) equipped with an UPlanFL 20×/0.5 numeric aperture and UPlanFL 40×/0.75 numeric aperture objective lens. Images were captured using an Olympus DP71 digital camera system and processed using Image-Pro Plus software.

Results

Cloning and sequence analysis of *vtg2* cDNA and gDNA

According to the specific primers (Table 1) designed based on the *vtg1* cDNA, the full-length cDNA of *vtg2* was cloned from testis template (GenBank: KJ923433). The total length *vtg2* cDNA is 8316 bp, 5'-UTR is 1069 bp (Fig. 1A), and 3'-UTR is 176 bp containing two polyadenylation signals AATAAA and a poly(A) tail (Fig. 1B). The predicted ORF encoded a protein of 2356 amino acids with a deduced molecular mass of 265.68 kDa and a theoretical isoelectric point (PI) of 6.81, while it had no signal peptide. Blast of the Vtg2 with other sequences from NCBI revealed that it shared high scoring amino acid identities to Vg in other crab species, i.e. 96% to *S. paramamosain*, 81% to *C. sapidus* and 80% to *P. trituberculatus*. One surprising result is that in different crabs, the 5'-UTR of *vtg2* matched with the gDNA rather than the cDNA.

In order to explore the structure of Vtg2, a 10.329 kb gDNA (GenBank: KU987908) was obtained by PCR, and it turned out that the *vtg2* gDNA sequence was the same with the *vtg1* gDNA. Unlike 14 introns in the *vtg1*, *vtg2* only contains 11 introns (I1–I11) and 12 exons (Fig. 2A). These two genes share a common gDNA sequence. Compared to *vtg2* cDNA, the gDNA intron sequences are 106, 93, 124, 352, 244, 137, 380, 78, 160, 246 and 114 bp respectively. These introns are in the nucleotide positions of 1601, 1747, 3472, 3724, 3882, 4470, 4846, 5520, 6047, 6422 and 6701 in *vtg2* cDNA respectively. BLAST search of the Vtg2 found that it has four structural domains typical of vitellogenin: the lipoprotein N-terminal domain (LPD-N), the domain of unknown function 1943 (DUF1943), the domain of unknown function 1801 (DUF1801) and the von Willebrand factor type D domain (VWD) (Khalil et al. 2011) (Fig. 2B).

Localization of *vtg2* mRNA

The expression of *vtg2* mRNA in different tissues (gill, stomach, hepatopancreas, thoracic ganglion, cerebral ganglion, testis, seminal vesicle, heart, muscle and hemocytes) of male crabs was detected using PCR. As a result, the distribution was observed exclusively in the testis (Fig. 3).

Then, *in situ* hybridization was employed to investigate the specific distribution of *vtg2* mRNA in male reproductive system, including testis and seminal vesicle, which is an accessory gland closely linked to testis. The positive signals were localized in the testicular spermatozoa when an antisense probe was used (Fig. 4B), while no signals was observed when a sense probe was used (Fig. 4C). However, no transcript was detected in the spermatophore of seminal vesicle was detected

Figure 6 Localization of Vn by immunohistochemistry in testis (A, B and E) and seminal vesicle (C, D and E) of *S. paramamosain*. A and C: hematoxylin-eosin staining; B and D: positive immunohistochemistry; E and F: negative immunohistochemistry. SC, spermatocyte; SD, seminiferous duct; SG, seminal granule; SP, spermatophore; SZ, spermatozoa.

whatsoever (Fig. 4E and F). Hematoxylin–eosin staining indicated the morphology of spermatogenic cells in testis (Fig. 4A) and spermatophore in seminal vesicle (Fig. 4D).

Detection of Vn

We also detected the distribution of Vn in the male reproductive system, including the testis and the seminal vesicle. The result revealed that there was a weak band of ~250 kDa visualized in the total protein of seminal vesicle, while no band was observed in testis (Fig. 5).

Then we further explored the localization of Vn in both testis and seminal vesicle. Immunohistochemical study showed that the positive signals were localized in the testicular spermatozoa and the

spermatophore of seminal vesicle in adult male crab (Fig. 6).

Expression profile of vtg2 by LPS and LTA

In order to study the immune function of *vtg2*, the expression of *vtg2* in testis was investigated by qRT-PCR after LPS and LTA injection in *S. paramamosain*. Injection of LPS resulted in a significant downregulation of *vtg2* expression at 0.5 hpi (Fig. 7). However, its expression level reached a peak at 2 dpi, then decreased at 4 dpi, and recovered to normal level at 8 and 16 dpi. Unlike the LPS group, injection of LTA induced a marked increase in *vtg2* expression, and the expression rapidly reached a significant expression level at 0.5 dpi. The maximum expression (2.5-fold) was observed at 1 dpi, followed by a decrease (Fig. 7).

The effect of injection of LPS and LTA on the expression of Vn in seminal vesicle was then investigated. Results showed that injection of LPS induced a little increase of Vn at 1 hpi, and then a significant one at 8 and 16 hpi respectively (Fig. 8). In contrast, injection of LTA caused a rapid and significant increase at 1 and 16 hpi (Fig. 8).

Figure 7 Expression of *vtg2* in testis at different time intervals in response to LPS and LTA in *S. paramamosain*. Each bar represents the mean value from four determinations with standard error. The testes were collected at 0, 0.5, 1, 2, 4, 8 and 16 h post-injection. Statistically significant differences ($n=3$; * $P<0.05$; # $P<0.05$) between the treated groups and controls are indicated by * at LPS group, # at LTA group respectively.

Figure 8 Western bolt detected Vn in seminal vesicle at different time intervals in response to LPS and LTA in *S. paramamosain*. The protein of C, P and T groups were collected at 0, 0.5, 1, 2, 4, 8 and 16 h post-injection. C, control group; P, LPS injection; T, LTA injection.

These results demonstrated that both LPS and LTA were able to trigger a significant increase in seminal vesicle Vn, agreeing with our above-mentioned observation of the *vtg2* mRNA in testis.

Discussion

This study is the first to report that Vg/Vn is present in spermatozoon of the mud crab *S. paramamosain*, under normal physiological conditions. Vg/Vn had spermatozoa specificity and could be induced after injection of LPS and LTA. Since, we presume that the Vtg2/Vn in spermatozoa probably acts as an immune factor involved in immunoprotection in *S. paramamosain*.

Molecular characterization of *vtg2*

In this study, a complete cDNA of *vtg2* with 8316bp was cloned from male mud crab without any induction or treatment. Comparing the sequence of *vtg2* from male with the *vtg1* gDNA (GenBank: KU987908) from the female crab, we found that they were similar, but *vtg2* cDNA had three more sequence fragments of 224, 88 and 117bp at 5'-UTR (Fig. 1A, underlined sequences). Subsequently, a 10.329kb gDNA of *vtg2*, containing 11 introns (I1–I11) and 12 exons (Fig. 2), was cloned. By analysis of *vtg2* and *vtg1* gDNA, we were surprised to find that *vtg1* and *vtg2* share a common gDNA sequence, and they were actually two isoforms of Vg caused by different types of alternative splicing in the mud crab *S. paramamosain*. This finding was confirmed in some reported species, in which Vg usually had several isoforms encoded by a multigene family. For instance, there were four isoforms of Vg in Africa frog *Xenopus laevis* (Wahli et al. 1979), seven in zebrafish *Danio rerio* (Wang et al. 2005), two in the harvester ant *Pogonomyrmex barbatus* (Corona et al. 2013) and two in the sand shrimp *Metapenaeus ensis* (Kung et al. 2004). As to *vtg2* of mud crab *S. paramamosain*, the first three fragments composed one exon in *vtg2* gDNA, but they were introns in *vtg1* gDNA. This phenomenon is called intron retention, which is a type of alternative splicing (Kung et al. 2013). Intron retention had been reported in crustacean hyperglycemic hormone (CHH) gene of crayfish *Procambarus clarkii*, and the cDNA of *CHH2* retained a truncated segment of intron II of *CHH2* gene (Kung et al. 2013). Intron retention could regulate the expression of genes, such as the model plant *Arabidopsis thaliana* (Ner-Gaon et al. 2004) and human genome (Ge & Porse 2014). To date, many Vgs have been obtained from some vertebrates and invertebrates, and almost all Vgs encoded by multiple genes display a similar structure (Hayward et al. 2010, Sun et al. 2013). Usually, Vg contains three conserved domains: the lipoprotein N-terminal domain (LPD-N), the domain of unknown function 143 (DUF1943) and the von Willebrand factor type D domain (VWD) (Sun & Zhang 2015). In the

mud crab, structure analysis revealed that the Vtg2 also contained these three domains, and displayed a similar structure to other species. Meanwhile, it possessed the fourth domain DUF1081 with unknown function.

Localization of *vtg2*/Vn

So far, little is known about Vg in male crustaceans. Vg mRNA fragment has been detected in male juveniles of Chinese mitten crab *E. sinensis* via whole-body transcriptome analysis (Liu et al. 2015). Although a partial Vg genome sequence was present in hepatopancreas, Vg mRNA was not found in male shrimp *Penaeus semisulcatus* (Avarre et al. 2003). In this study, *vtg2* was detected in male, which was only transcribed in the testis by PCR. *In situ* hybridization further confirmed that *vtg2* mRNA was uniquely presented in the testicular spermatozoa (Figs 2 and 3). In addition, the distribution of *vtg2* mRNA showed some difference with other male invertebrates. In previous studies, Vg was transcribed in the testis and intestine of the male sea urchins *Strongylocentrotus purpuratus* (Unuma et al. 2010), and in the intestine, coelomocytes and testis of the male sea urchins *Pseudocentrotus depressus* (Shyu et al. 1986). In insects, the Vg mRNA was only synthesized in testicular somatic cyst cells in male moth *Spodoptera littoralis* and fruit fly *Drosophila melanogaster* (Bebas et al. 2008, Majewska et al. 2014). As to Vn detected in male testis in this study, it was a single polypeptide component of ~250kDa (Fig. 4), approximating the deduced molecular mass of 265.68kDa of Vtg2, and was present only in seminal vesicle other than testis. This result was different from the detection of Vn in the female mud crab, which was composed of five polypeptides (unpublished). The composition difference of Vn in male and female mud crabs might be due to different cleavages. A similar phenomenon was also observed in the giant freshwater prawn *M. rosenbergii* (Wilder et al. 1994). Furthermore, the localization of Vn was in testis and seminal vesicle (Fig. 6) via immunohistochemistry, which differed from its exclusive presence in the seminal vesicle via the Western blotting (Fig. 5). One possible reason for this difference was that the testis also had numerous other types of cell in addition to spermatozoa (Fig. 6A), therefore the concentration of Vn in testis was too low to be detected by Western blotting. Taken together, both of the *vtg2* mRNA and Vn were present in testicular spermatozoa in mud crabs.

Immunoprotection function of *vtg2*/Vn

In crayfish *Astacus astacus*, Vg/Vn was considered to be related to the cholesterol efflux in post-mating spermatophore, which is necessary for the spermatozoon capacitation (Niksirat et al. 2015). In the testis of some other male invertebrates, Vg/Vn were found in the somatic cyst cells of the moth *S. littoralis* and fruit fly *D. melanogaster*

to provide nutrition to spermatozoon (Bebas et al. 2008, Majewska et al. 2014), and in the nutritive phagocytes of the sea urchins *S. purpuratus* acting as a zinc carrier (Unuma et al. 2010). However, unlike the distribution in the somatic cells of testis in these three species, Vg/Vn was observed in the testicular spermatozoa in the mud crab *S. paramamosain*. Hence, the specific localization of vtg2/Vn arouses the issue that it might perform other functions, probably as an immune-relevant molecule (Raikhel et al. 2002, Tong et al. 2010, Wu et al. 2015). In order to explore the potential immune function of vtg2 in male mud crab *S. paramamosain*, the vtg2/Vn expressions were investigated at both transcriptional and translational levels. As we observed, vtg2/Vn expression was inducible at both levels, after injection of LPS and LTA into male crabs (Figs 7 and 8). This result was similar to the previous study of Vg in male zebra fish *D. rerio* (Tong et al. 2010). In the innate immune system, Vgs have repeatedly been reported to act as multivalent PRR, a bactericidal molecule or an acute-phase protein (Zhang et al. 2011a). As specific pathogen-associated molecular patterns (PAMPs), both LPS and LTA can be recognized by PRRs to initiate immune responses (Akira et al. 2006, Kumar et al. 2009). LPS from Gram-negative bacteria and LTA from Gram-positive bacteria are elicitors, which can specifically activate immune-related genes in insect and fish (Altincicek et al. 2008, Tong et al. 2010). Both LPS and LTA can activate the prophenoloxidase and antimicrobial peptide expression in the tobacco hornworm *Manduca sexta* (Rao et al. 2010) as well as Vg in male zebra fish *Danio rerio* (Tong et al. 2010). Consequently, we presume that the Vtg2/Vn in spermatozoa probably act as an immune factor for protection instead of as a nutritional source in the somatic cells of testis (Unuma et al. 2010). In addition to Vtg2, another immune factor, scygonadin, has been isolated from the seminal plasma in the mud crab *S. paramamosain* (Xu et al. 2011). Hence, some immunoprotection factors are necessary for spermatozoon maturation.

In the light of the results presented here, we speculate that Vtg2/Vn is a novel candidate of immune-relevant molecules involved in immunoprotection in the process of spermatozoon maturation, and this viewpoint helps to open a new avenue for further exploring the role of Vg.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Akira S, Uematsu S & Takeuchi O 2006 Pathogen recognition and innate immunity. *Cell* **124** 783–801. (doi:10.1016/j.cell.2006.02.015)
- Altincicek B, Knorr E & Vilcinskis A 2008 Beetle immunity: identification of immune-inducible genes from the model insect *Tribolium castaneum*. *Developmental and Comparative Immunology* **32** 585–595. (doi:10.1016/j.dci.2007.09.005)
- Arukwe A & Goksory A 2003 Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comparative Hepatology* **2** 4. (doi:10.1186/1476-5926-2-4)
- Avarre JC, Michelis R, Tietz A & Lubzens E 2003 Relationship between vitellogenin and vitellin in a marine shrimp (*Penaeus semisulcatus*) and molecular characterization of vitellogenin complementary DNAs. *Biology of Reproduction* **69** 355–364. (doi:10.1095/biolreprod.102.011627)
- Bebas P, Kotwica J, Joachimiak E & Giebulowicz JM 2008 Yolk protein is expressed in the insect testis and interacts with sperm. *BMC Developmental Biology* **8** 1–12. (doi:10.1186/1471-213X-8-1)
- Chen TT & Hillen LJ 1983 Expression of the vitellogenin genes in insects. *Gamete Research* **7** 179–196. (doi:10.1002/ISSN1554-3919)
- Corona M, Libbrecht R, Wurm Y, Riba-Grognus O, Studer RA & Keller L 2013 Vitellogenin underwent subfunctionalization to acquire caste and behavioral specific expression in the harvester ant *Pogonomyrmex barbatus*. *PLoS Genetics* **9** e1003730. (doi:10.1371/journal.pgen.1003730)
- Del Giudice G, Prisco M, Agnese M, Verderame M, Rosati L, Limatola E & Andreuccetti P 2012 Effects of nonylphenol on vitellogenin synthesis in adult males of the spotted ray *Torpedo marmorata*. *Journal of Fish Biology* **80** 2112–2121. (doi:10.1111/jfb.2012.80.issue-5)
- Finn RN & Fyhn HJ 2010 Requirement for amino acids in ontogeny of fish. *Aquaculture Research* **41** 684–716. (doi:10.1111/are.2010.41.issue-5)
- Ge Y & Porse BT 2014 The functional consequences of intron retention: alternative splicing coupled to NMD as a regulator of gene expression. *BioEssays* **36** 236–243. (doi:10.1002/bies.201300156)
- Gerber-Huber S, Nardelli D, Haefliger JA, Cooper DN, Givel F, Germond JE, Engel J, Green NM & Wahli W 1987 Precursor-product relationship between vitellogenin and the yolk proteins as derived from the complete sequence of a *Xenopus* vitellogenin gene. *Nucleic Acids Research* **15** 4737–4760. (doi:10.1093/nar/15.12.4737)
- Giorgi F, Bradley JT, Vignali R & Mazzini M 1989 An autoradiographic analysis of vitellogenin synthesis and secretion in the fat body of the stick insect *Bacillus rossius*. *Tissue and Cell* **21** 543–558. (doi:10.1016/0040-8166(89)90007-4)
- Goodwin AE, Grizzle JM, Bradley JT & Estridge BH 1992 Monoclonal antibody-based immunoassay of vitellogenin in the blood of male channel catfish (*Ictalurus punctatus*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **101** 441–446. (doi:10.1016/0305-0491(92)90025-M)
- Guan WB 2003 Studies on the structure and function of male and female reproductive system and spermatophore artificial transfer of the mud crab *Scylla serrata* (Forskål). Dissertation 2003. Xiamen University, Xiamen, China.
- Hayward A, Takahashi T, Bendena WG, Tobe SS & Hui JH 2010 Comparative genomic and phylogenetic analysis of vitellogenin and other large lipid transfer proteins in metazoans. *FEBS Letters* **584** 1273–1278. (doi:10.1016/j.febslet.2010.02.056)
- Jia X, Chen Y, Zou Z, Lin P, Wang Y & Zhang Z 2013 Characterization and expression profile of Vitellogenin gene from *Scylla paramamosain*. *Gene* **520** 119–130. (doi:10.1016/j.gene.2013.02.035)
- Khalil SMS, Donohue KV, Thompson DM, Jeffers LA, Ananthapadmanaban U, Sonenshine DE, Mitchell RD & Roe RM 2011 Full-length sequence, regulation and developmental studies of a second vitellogenin gene from the American dog tick, *Dermacentor variabilis*. *Journal of Insect Physiology* **57** 400–408. (doi:10.1016/j.jinsphys.2010.12.008)
- Kouba A, Niksirat H & Bláha M 2015 Comparative ultrastructure of spermatozoa of the redclaw *Cherax quadricarinatus* and the yabby *Cherax destructor* (Decapoda, Parastacidae). *Micron* **69** 56–61. (doi:10.1016/j.micron.2014.11.002)

- Kumar H, Kawai T & Akira S 2009 Pathogen recognition in the innate immune response. *Biochemical Journal* **420** 1–16. (doi:10.1042/BJ20090272)
- Kung SY, Chan SM, Hui JHL, Tsang WS, Mak A & He JG 2004 Vitellogenesis in the sand shrimp, *Metapenaeus ensis*: the contribution from the hepatopancreas-specific vitellogenin gene (MeVtg2). *Biology of Reproduction* **71** 863–870. (doi:10.1095/biolreprod.103.022905)
- Kung PC, Wu SH, Nagaraju GPC, Tsai WS & Lee CY 2013 Crustacean hyperglycemic hormone precursor transcripts in the hemocytes of the crayfish *Procambarus clarkii*: novel sequence characteristics relating to gene splicing pattern and transcript stability. *General and Comparative Endocrinology* **186** 80–84. (doi:10.1016/j.ygcen.2013.03.010)
- Liu Y, Hui M, Cui Z, Luo D, Song C, Li Y & Liu L 2015 Comparative transcriptome analysis reveals sex-biased gene expression in juvenile Chinese mitten crab *Eriocheir sinensis*. *PLoS ONE* **10** e0133068. (doi:10.1371/journal.pone.0133068)
- Majewska MM, Suszczynska A, Kotwica-Rolinska J, Czerwik T, Paterczyk B, Polanska MA, Bernatowicz P & Bebas P 2014 Yolk proteins in the male reproductive system of the fruit fly *Drosophila melanogaster*: spatial and temporal patterns of expression. *Insect Biochemistry and Molecular Biology* **47** 23–35. (doi:10.1016/j.ibmb.2014.02.001)
- Ner-Gaon H, Halachmi R, Savaldi-Goldstein S, Rubin E, Ophir R & Fluhr R 2004 Intron retention is a major phenomenon in alternative splicing in *Arabidopsis*. *Plant Journal* **39** 877–885. (doi:10.1111/j.1365-3113x.2004.02172.x)
- Niksirat H, Andersson L, James P, Kouba A & Kozák P 2014 Proteomic profiling of the signal crayfish *Pacifastacus leniusculus* egg and spermatophore. *Animal Reproduction Science* **149** 335–344. (doi:10.1016/j.anireprosci.2014.07.024)
- Niksirat H, James P, Andersson L, Kouba A & Kozák P 2015 Label-free protein quantification in freshly ejaculated versus post-mating spermatophores of the noble crayfish *Astacus astacus*. *Journal of Proteomics* **123** 70–77. (doi:10.1016/j.jprote.2015.04.004)
- Pan ML, Bell WJ & Teller WH 1969 Vitellogenic blood protein synthesis by insect fat body. *Science* **165** 393–394. (doi:10.1126/science.165.3891.393)
- Pousis C, De Giorgi C, Mylonas CC, Bridges CR, Zupa R, Vassallo-Agius R, de la Gandara F, Dileo C, De Metrio G & Corriero A 2011 Comparative study of liver vitellogenin gene expression and oocyte yolk accumulation in wild and captive Atlantic bluefin tuna (*Thunnus thynnus* L.). *Animal Reproduction Science* **123** 98–105. (doi:10.1016/j.anireprosci.2010.10.005)
- Raikhel AS, Kokoza VA, Zhu JS, Martin D, Wang SF, Li C, Sun GQ, Ahmed A, Dittmer N & Attardo G 2002 Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. *Insect Biochemistry and Molecular Biology* **32** 1275–1286. (doi:10.1016/S0965-1748(02)00090-5)
- Rao XJ & Yu XQ 2010 Lipoteichoic acid and lipopolysaccharide can activate antimicrobial peptide expression in the tobacco hornworm *Manduca sexta*. *Developmental and Comparative Immunology* **34** 1119–1128. (doi:10.1016/j.dci.2010.06.007)
- Ricciardi F, Matozzo V & Marin MG 2008 Effects of 4-nonylphenol exposure in mussels (*Mytilus galloprovincialis*) and crabs (*Carcinus aestuarii*) with particular emphasis on vitellogenin induction. *Marine Pollution Bulletin* **57** 365–372. (doi:10.1016/j.marpolbul.2008.02.023)
- Robinson R 2008 For mammals, loss of yolk and gain of milk went hand in hand. *PLoS Biology* **6** e77. (doi:10.1371/journal.pbio.0060077)
- Sharrock WJ 1983 Yolk proteins of *Caenorhabditis elegans*. *Developmental Biology* **96** 182–188. (doi:10.1016/0012-1606(83)90321-4)
- Shyu AB, Raff RA & Blumenthal T 1986 Expression of the vitellogenin gene in female and male sea urchin. *PNAS* **83** 3865–3869. (doi:10.1073/pnas.83.11.3865)
- Subramoniam T 2011 Mechanisms and control of vitellogenesis in crustaceans. *Fisheries Science* **77** 1–21. (doi:10.1007/s12562-010-0301-z)
- Sun C & Zhang S 2015 Immune-relevant and antioxidant activities of vitellogenin and yolk proteins in fish. *Nutrients* **7** 8818–8829. (doi:10.3390/nu7105432)
- Sun C, Hu L, Liu S, Gao Z & Zhang S 2013 Functional analysis of domain of unknown function (DUF) 1943, DUF1944 and von Willebrand factor type D domain (VWD) in vitellogenin 2 in zebrafish. *Developmental and Comparative Immunology* **41** 469–476. (doi:10.1016/j.dci.2013.07.005)
- Suzuki S, Yamasaki K & Katakura Y 1990 Vitellogenin synthesis in andrectomized males of the terrestrial isopod, *Armadiidium vulgare* (Malacostracan Crustacea). *General and Comparative Endocrinology* **77** 283–291. (doi:10.1016/0016-6480(90)90312-A)
- Tong Z, Li L, Pawar R & Zhang S 2010 Vitellogenin is an acute phase protein with bacterial-binding and inhibiting activities. *Immunobiology* **215** 898–902. (doi:10.1016/j.imbio.2009.10.001)
- Tsukimura B 2001 Crustacean vitellogenesis: its role in oocyte development. *American Zoologist* **41** 465–467. (doi:10.1093/icb/41.3.465)
- Tsutsui N & Chung JS 2012 A novel putative lipoprotein receptor (CasLpR) in the hemocytes of the blue crab, *Callinectes sapidus*: cloning and up-regulated expression after the injection of LPS and LTA. *Fish and Shellfish Immunology* **32** 469–475. (doi:10.1016/j.fsi.2011.11.013)
- Tsutsui N, Kawazoe I, Ohira T, Jasmani S, Yang WJ, Wilder MN & Aida K 2000 Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus*. *Zoological Science* **17** 651–660. (doi:10.2108/zsj.17.651)
- Unuma T, Nakamura A, Yamano K & Yokota Y 2010 The sea urchin major yolk protein is synthesized mainly in the gut inner epithelium and the gonadal nutritive phagocytes before and during gametogenesis. *Molecular Reproduction and Development* **77** 59–68. (doi:10.1002/mrd.21103)
- Wahli W, Dawid IB, Wyler T, Jaggi RB, Weber R & Ryffel GU 1979 Vitellogenin in *Xenopus laevis* is encoded in a small family of genes. *Cell* **16** 535–549. (doi:10.1016/0092-8674(79)90028-X)
- Wang H, Tan JT, Emelyanov A, Korzh V & Gong Z 2005 Hepatic and extrahepatic expression of vitellogenin genes in the zebrafish, *Danio rerio*. *Gene* **356** 91–100. (doi:10.1016/j.gene.2005.03.041)
- Wilder MN, Okumura T, Suzuki Y, Fusetani N & Aida K 1994 Vitellogenin production induced by eyestalk ablation in juvenile giant fresh water prawn *Macrobrachium rosenbergii* and trial methyl farnesoate administration. *Zoological Science* **11** 45–53.
- Wu B, Liu Z, Zhou L, Ji G & Yang A 2015 Molecular cloning, expression, purification and characterization of vitellogenin in scallop *Patinopecten yessoensis* with special emphasis on its antibacterial activity. *Developmental and Comparative Immunology* **49** 249–258. (doi:10.1016/j.dci.2014.12.004)
- Xu WF, Qiao K, Huang SP, Peng H, Huang WS, Chen B, Chen FY, Bo J & Wang KJ 2011 Quantitative gene expression and in situ localization of scygonadin potentially associated with reproductive immunity in tissues of male and female mud crabs, *Scylla paramamosain*. *Fish and Shellfish Immunology* **31** 243–251. (doi:10.1016/j.fsi.2011.05.009)
- Yang Y, Bao C, Liu A, Ye H, Huang H & Li S 2014 Immune responses of prophenoloxidase in the mud crab *Scylla paramamosain* against *Vibrio alginolyticus* infection: in vivo and in vitro gene silencing evidence. *Fish and Shellfish Immunology* **39** 237–244. (doi:10.1016/j.fsi.2014.05.014)
- Zhang ZP, Cheng H, Wang YL, Wang SH, Xie FJ & Li SJ 2010 Acrosome reaction of sperm in the mud crab *Scylla serrata* as a sensitive toxicity test for metal exposures. *Archives of Environmental Contamination and Toxicology* **58** 96–104. (doi:10.1007/s00244-009-9326-7)
- Zhang S, Wang S, Li H & Li L 2011a Vitellogenin, a multivalent sensor and an antimicrobial effector. *International Journal of Biochemistry and Cell Biology* **43** 303–305. (doi:10.1016/j.biocel.2010.11.003)
- Zhang Y, Wu XG, Yang F, Liu ZJ & Cheng YX 2011b Purification of vitellin and ELISA determination of vitellin of swimming crab (*Portunus trituberculatus*). *Journal of Fisheries of China* **35** 1146–1157.
- Zhang Q, Lu Y, Zheng H, Liu H & Li S 2016 Differential immune response of vitellogenin gene to *Vibrio anguillarum* in noble scallop *Chlamys nobilis* and its correlation with total carotenoid content. *Fish and Shellfish Immunology* **50** 11–15. (doi:10.1016/j.fsi.2016.01.001)

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