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The expression characteristics of vitellogenin (VTG) in response to B(a)p exposure in polychaete *Perinereis aibuhitensis**

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Abstract In order to investigate the endocrine toxicity of B(a)p to marine polychaete *Perinereis aibuhitensis*, vitellogenin (VTG) cDNA from the *P. aibuhitensis* was isolated, recombinated and expressed for the first time. The full length *P. aibuhitensis* vitellogenin gene (PaVTG) was 5 325 bp, and encoded 1 692 amino acids. It contained the vitellogenin_N domain of unknown function (DUF1943), a von Willebrand factor type D domain, as well as a conserved KALGNAG motif. The expression of VTG gene and protein were mainly up-regulated after exposed to B(a)p at transcriptional and translational levels. PaVTG gene expression did not change significantly at day 4. At day 7 PaVTG expression was up-regulated in 0.5 μg/L and 5 μg/L B(a)p group. At day 14 PaVTG was significantly up-regulated in 0.5–10 μg/L B(a)p. The protein expression of PaVTG in 0.5 μg/L and 10 μg/L B(a)p group was up-regulated with time prolonging, but the expression in 5 μg/L and 50 μg/L B(a)p group exhibited first increased and then decreased trend. With the increasing of B(a)p concentration PaVTG mRNA and protein expression both firstly increased then decreased. In contrast to B(a)p exposure, estradiol did not induce PaVTG gene and protein expression, until late times of exposure (14 d). Overall, the results in this study indicate that PaVTG could be used as a potential indicator of the effects environmental estrogenic compounds.

Keyword: *Perinereis aibuhitensis*; vitellogenin; B(a)p; expression profiles

1 INTRODUCTION

The hormonal systems of animals are important for maintaining and regulating growth, development, reproduction, and other physiological processes. Endocrine-disrupting chemicals (EDCs) can interfere with the hormonal system, and disrupt synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body (Park and Kwak, 2010). In the past few decades, increasing attention has been paid to evaluating the adverse effects of EDCs in aquatic environments. The toxic effects of EDCs on aquatic animals are diverse, and include abnormal development of the reproductive tracts and disruption of the reproductive cycle, among others.

Benzo(a)pyrene (B(a)p) is a highly toxic polycyclic aromatic hydrocarbon (PAH). B(a)p is a representative PAH pollutant in aquatic toxicological experiments (Da Silva Rocha et al., 2012). In addition to carcinogenic characteristics, B(a)p seems to possess a negative impact on reproduction, as do other EDCs (Kanaly and Harayama, 2000; Laffon et al., 2006; Wu et al., 2017). For example, B(a)p can suppress the secretion of androstenedione and estradiol in flounder *Platichthys flesus L*. (Monteiro et al., 2000). Also,

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Miao et al. (2009) reported that 10 μg/L B(a)p caused a significantly higher rate of DNA fracture in the gonad, a significantly higher degradation rate of oocytes, and hampered accumulation of yolk granules.

Many researchers have documented the induction of vitellogenin (VTG) after exposure to xenoestrogens. Researchers have used VTG as a sensitive biomarker of estrogenic pollution (Marin and Matozzo, 2004; Matozzo et al., 2008). VTG is a precursor of yolk protein that is ubiquitous in oviparous animals. VTG provides nutritional and functional substances such as amino acids, fat, carbohydrates, vitamins, phosphorus, sulfur, and trace elements. These substances are essential for the development of oocytes, embryos, and early larvae, as well as to ensure survival of the young until they are self-supporting (Auttarat et al., 2006; Phiriyangkul et al., 2007; Matozzo et al., 2008). VTG is normally produced in female organisms, when exposed to exogenous estrogens, males and juveniles can also synthesize and secrete VTG (Marin and Matozzo, 2004). VTG production is regulated through the estrogen receptor pathway in vertebrates. The Organization for Economic Cooperation and Development (OECD) has developed a fish screening assay protocol that uses VTG as an endpoint for screening for EDCs with estrogenic activity (OECD, 2010; Wang et al., 2015). The presence of a VTG system in aquatic oviparous invertebrates raises the possibility that invertebrates can also be used for endocrine toxicity testing (Matozzo et al., 2008). Recently, several studies have focused on the measurement of VTG/vitellins (Vn) levels after xenoestrogen exposure in groups of aquatic invertebrates, such as mollusks (Zhang et al., 2012; Ni et al., 2014; Tran et al., 2016; Liu et al., 2017) and crustaceans (Huang et al., 2006; García and Heras, 2012; Jubeaux, 2012; Wen and Pan, 2015; Boulangé-Lecomte et al., 2017). Compared to the available information for mollusks and crustaceans, there is limited data about VTG induction in marine polychaetes. A significantly increase in VTG secretion was detected by enzyme linked immunosorbent assay (ELISA) in cultured eleocytes from mature *Nereis virens* females when they were incubated in vitro with 1 μg/L 17β-estradiol for three d (García-Alonso et al., 2006). Zheng et al. (2010) showed that exposure of female *Perinereis nuntia* to sub-lethal concentrations of cadmium caused a significant increase in the relative expression of VTG mRNA.

The marine polychaete *Perinereis aibuhitensis* is widely distributed along the coasts of Southeast Asia in mudflats and estuarine sediments. Their life within estuarine sediments and mudflats puts them in continuous contact with sediment-associated contaminants. Furthermore, polychaetes may be important vectors for the transfer of sediment-associated contaminants to higher trophic levels, as they are high-quality bait used for many commercial fish, for shrimp, and for crab (Lewis and Watson, 2012). In addition, many kinds of polychaetes including *P. aibuhitensis* are known to accumulate significant amounts of organic matter from the environment, and steady-state body burdens are a function of biotransformation and elimination processes. Due those reason, several studies have focused on toxicity assessments using polychaetes. The study of Zhang et al. (2008) showed that *P. aibuhitensis* exposure to 0.15 and 0.2 mL/L of petroleum hydrocarbons could significantly inhibit *P. aibuhitensis* acetyl cholinesterase activity. Chen et al. (2012) found that exposure to petroleum hydrocarbons significantly induced cytochrome P450 mRNA expression in *P. aibuhitensis*. We also found that gene expression and enzyme activity of catalase and superoxide dismutase were positively correlated to the concentration of crude oil in *P. aibuhitensis* (Zhao et al., 2017). However, little information about the effect of EDCs on the endocrine system in this species has been documented.

In this study, we isolated the full length VTG cDNA from *P. aibuhitensis* and acquired recombinant protein. Then we investigated its expression after exposure to the xenoestrogen B(a)p at both transcriptional and translational levels. The results of this study will provide useful insight for the development of potential biomarkers of xenoestrogen pollutants using *P. aibuhitensis*.

2 MATERIAL AND METHOD

2.1 B(a)p exposure

Perinereis aibuhitensis (1.0–1.5 g wet weight) was collected from an estuary of Shuangtaizi in Panjin, Liaoning Province, in China. In preliminary experiment we found the gene expression of VTG in female worm was sensitive to B(a)p (Sigma, purity≥96%) exposure, so in this study we chose female worms as experimental animals. We could observe oocytes suspended in the coelomic cavity of sampled worms, and the development of reproductive cell in each worm was observed by microscope in order to choose the worm which was in the same stage

Table 1 The primers used in RACE and Real-time PCR

Primer		Sequence (5′→3′)
3′-RACE	VTG	AGCCTTGGAGTTGAGGTGGTGGTGA
5′-RACE	VTG	ATGGTGGGCTCCAAGAGTGGGATAAA
UPM (universal primer mix)	Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
	Short	CTAATACGACTCACTATAGGGC
β-actin	F	GGGCTACTCCTTCACCACCA
	R	CGAAGTCCAGAGCAACATAG
VTG	F	CAAGGAAATCCCAGTGCCAAC
	R	CCCTTCACCACCACCTCAACT

of gonad maturation. The animals were transferred to the laboratory and acclimated in tanks (60 cm× 45 cm×40 cm) containing filtered seawater (salinity 31–32, temperature 20±0.5°C) for a week before the initiation of experiments. Water was changed daily and continuously aerated. During acclimatization, *P. aibuhitensis* worms were fed with a powder mix containing kelp powder, gulfweed powder, fishmeal, yeast, and spirulina; worms were deprived from food during the B(a)p exposure experiment. Four concentrations of B(a)p (0.5, 5.0, 10, and 50 μg/L) were tested, according to the study of Song et al. (2011), and to our preliminary experiments. The worms were exposed to B(a)p dissolved in acetone (final acetone concentration 100 μg/L) for 14 d. Negative controls were unexposed worms, and worms exposed to acetone alone at 100 μg/L. Worms were also exposed to 50 μg/L estradiol, as an additional control group. The exposure experiment was carried out in 2-L beakers. Worms were randomly divided into seven treatment groups, and each group consisted of three replicates. Each replicate consisted of ten individuals. During the exposure experiment, seawater was renewed daily. The worms was dissected at 4, 7, and 14 d and then ground in liquid nitrogen and immediately stored at -80°C.

2.2 Cloning of *P. aibuhitensis* VTG full length cDNA

The production of gametes is related to the germinal epithelia located in the body wall of this worm, so we choose the body wall of *P. aibuhitensis* as sampling tissue. Total RNA was extracted from 100 mg of body wall using RNAiso Plus (TaKaRa Biotechnology Co. Ltd.) according to the manufacturer's instructions. The quality of RNA was evaluated by electrophoresis on 1% agarose gels. Total RNA (1 μg) was reverse transcribed into cDNA for rapid amplification of cDNA ends (RACE) using Clontech SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). Primers for VTG were designed using Primer Premier 5.0 software, based on the partial sequence obtained from transcriptome sequencing of *P. aibuhitensis* (unpublished data) (Table 1). The amplification method was conducted according to the manufacturer's instructions. The 3′ RACE amplification protocol was as follows: 25 cycles at 94°C for 30 s, 65.2°C for 30 s and 72°C for 3 min. The 5′ RACE amplification protocol was as follows: five cycles at 94°C for 30 s, 72°C for 3 min; followed by five cycles at 94°C for 30 s, 70°C for 30 s, 72°C for 5 min; and finally 25 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 3 min. The PCR products were analyzed by electrophoresis on 1% agarose gels and then purified using an agarose gel DNA purification kit (TaKaRa Biotechnology Co. Ltd.). The purified PCR products were ligated into the pMD18-T vector, and transformed into *E. coli* DH5α competent cells. Positive clones were sequenced by TaKaRa Biotechnology Co. Ltd.

2.3 Sequence analysis

The sequences obtained from the PCR-cloned products were analyzed for similarity with other known sequences using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The deduced amino acid sequences were obtained via the Expert Protein Analysis System (http://www.us.expasy.org/tools). Alignment of multiple sequences was performed using CLUSTAL W software (http://www.ebi.ac.uk/clustalW). The functional sites and motifs in amino acid sequences were predicted using Motif Scan (http://www.hits.isbsib.ch/cgi-bin/PESCAN) and Expasy (http://www.au.expasy.org/prosite/) software. Phylogenetic analysis was conducted using Mega 5.0 software with the Neighbor-Joining algorithm. The tree topology was evaluated by 1 000 replication bootstraps.

2.4 Quantitative real-time PCR

SYBR Green quantitative real-time PCR was used to investigate the expression of the VTG gene in *P. aibuhitensis*. Gene-specific primers for PaVTG (Table 1) were designed according to the full length PaVTG cDNA sequence. *β-actin* was chosen as reference gene according to the previous studies (Chen et al., 2012). Standard curve testing was performed using serial 10-fold samples dilutions. The slopes of standard curve and the PCR efficiency were calculated to confirm the accuracy of real-time PCR data. PCR amplification was done in a 20-μL reaction mixture containing 10 μL of SYBR® Premix Ex Taq™ II (TaKaRa Biotechnology Co. Ltd.), 0.8 μL of each primer (10 mmol/L), 0.4 μL of Rox Reference Dye, 2.0 μL of cDNA, and 6 μL of diluted water. The amplification protocol was carried out at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, and 60°C for 34 s. Melting curve analysis of the amplification products was done at the end of each PCR assay to confirm the specificity of the PCR products. Each product generated a single discrete peak in the melting curve analysis, which demonstrated the specificity of the PCR products. The PCR efficiency of PaVTG and *β-actin* were 100.076% and 99.959%.

2.5 Expression and purification of recombinant PaVTG

The conserved domain of full length PaVTG cDNA (30-740 aa) was amplified by PCR, and the PCR product was purified and digested with Nde I and Hind III to generate fragments with overhanging ends that could be ligated into the multiple cloning site of the expression vector PET30a. The recombinant plasmid was transformed into *E. coli* BL21(DE3) cells, and PaVTG positive clones were confirmed by PCR, restriction enzyme digestion, and sequence analysis. The expression of PaVTG was induced by 1 mmol/L Isopropyl β-D-Thiogalactoside (IPTG) at 37°C for 4 h. Bacteria were suspended in phosphate-buffered saline (PBS) and lysed by sonication on ice, until the bacterial suspension was no longer viscous. Bacteria were then centrifuged at 12 000 r/min for 30 min. The expressed PaVTG protein accumulated in inclusion bodies, according to the analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The protein was collected by centrifugation at 13 000 r/min for 30 min and by disruption of the inclusion bodies in Tris-urea buffer (50 mmol/L Tris, 8 mol/L urea, pH 8.0). The resulting solution was centrifuged, and the supernatant was purified by using a Ni-NTA column. The purified protein was sent to GenScript (China, Nanjing) to prepare PaVTG-specific antibodies.

2.6 Western-blotting analysis

Purified PaVTG protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto a Polyvinylidene Fluoride (PVDF) membrane at 120 V at 4°C for 1 h. PVDF membranes protein were blocked with 5% nonfat dry milk in Tris-Buffered Saline and Tween 20 (TBST) at 4°C overnight. The membranes were washed three times with TBS for 5 min, and incubated with anti-PaVTG rabbit serum (1:4 000 dilution in TBST) for 1 h at 37°C. The membranes were washed three times, and incubated with goat anti-rabbit IgG (H+L) conjugated to horseradish peroxidase (HRP) (1:4 000 dilution, Proteintech, Chicago, IL, USA) for 1 h at 37°C. After three washes with TBST, the immunoreative bands were visualized using DAB substrate solution (OriGene, Wuxi, China).

2.7 Indirect Enzyme-linked immunosorbent assay

The expression of PaVTG protein in female *P. aibuhitensis* under B(a)p exposure was detected by ELISA. The protein of each sample was extracted using lysates (Beyotime, Shanghai, China), and diluted to a concentration of 0.2 μg/mL with PBST. Ninety-six well microtiter plates (Corning, Shanghai, China) were coated overnight with 100 μL of the sampling protein, plates were then blocked with 3% (w/v) BSA in PBS for 2 h at 37°C. After incubation, the plates were washed three times with PBST for 5 min. Anti-PaVTG rabbit serum (100 μL, 1:2 000 dilution) was added to each well and incubated at 37°C for 1 h. After incubation, the plates were washed with PBST three times for 5 min. HRP AffiniPure Goat Anti-Rabbit IgG (H+L) (Proteintech) was diluted 1:4 000 (v/v) in PBST, and 100 μL of the dilution were added to each well. After incubation at 37°C for 1 h, 100 μL of TMB substrate solution (Solarbio, Beijing, China) were added to each well. The plates were incubated at room temperature for 10 min in the dark. The reaction was stopped by adding 50 μL of ELISA terminator (Solarbio). Absorbance values at 450 nm were measured using a microplate reader (Perlong, DNM-9602A).

2.8 Statistical analysis

The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression level of PaVTG mRNA. Data are expressed as mean±standard deviation (S.D.). The differences between samples exposed to different concentrations of B(a)p at each sampling time, were determined using one-way analysis of variance (ANOVA) followed by Tukey's test, using SPSS19.0 software. P values ≤ 0.05 were considered statistically significant.

3 RESULT

3.1 Molecular characterization of PaVTG

The full length cDNA of PaVTG has a length of 5 325 bp, including a 199-bp 3′ untranslated region (UTR), a 47-bp 5′ UTR, and a 5 079-bp open-reading frame. The open-reading frame predicted a protein of 1 692 amino acids (aa) with a predicted molecular mass of 185.27 kDa, and a theoretical isoelectric point of 8.66 (Fig.1). The 3′ UTR of PaVTG contains a putative polyadenylation consensus signal (AATAAA). The cDNA sequence of the PaVTG gene has been deposited in NCBI with accession number KF212194.1. The predicted amino acid sequence of VTG contained various functional motifs, such as the lipoprotein amino-terminal region (vitellogenin_N domain) (27–643 aa), a domain of unknown function (DUF domain) (681–974 aa), and a von Willebrand and factor type D domain (1 394–1 553 aa). A conserved amino acid motif KALGNAG in the vitellogenin-domain (544–550 aa), and a consensus RXXR cleavage sequence motifs (RKQR and RASR) were also found. SignalP analysis indicated that the signal peptide was positioned at the first 23 aa. The sequence of the *P. aibuhitensis* VTG protein had no phosvitin or polyserine domains, which are involved in receptor binding, and in phosphate and metal ion transport, in insects and vertebrates. Clustal W analysis revealed that the predicted amino acid sequence of PaVTG shared 59% identity with VTG protein from polychaete *Platynereis dumerilii* and had 21% to 27% homology with VTG protein from other species. And the sequence matched with 2.28% VTG sequence of *perinereis* sp. acquiring by pyrosequencing method (Phoonsamran et al., 2017). Figure 2 also showed that the PaVTG sequence had the closest relationship to VTG from *P. dumerilii*. The two sequences were located in the same branch, and then clustered together with mollusks. The VTG sequences from lophotrochozoan were located in the same branch, and clustered with tardigratal sequence.

3.2 PaVTG gene expression in female *P. aibuhitensis* after exposure to B(a)p

The expression pattern of PaVTG in female *P. aibuhitensis* in response to B(a)p exposure is shown in Fig.3. The expression of PaVTG in acetone group did not have significant difference with that in control group, which indicated the solvent used to dissolve B(a)p had no effect in these processes. Estradiol failed to induce PaVTG gene expression at day 4 and 7, but it enhanced the expression of PaVTG at day 14 ($P<0.05$). With the increase of B(a)p concentration PaVTG mRNA expression was firstly increased then decreased. B(a)p induced PaVTG gene expression in a time-dependent manner. At day 4 of exposure, PaVTG gene expression in B(a)p-exposed worms did not change significantly, relative to the unexposed controls ($P>0.05$). On day 7 of exposure to 0.5 μg/L and 5 μg/L B(a)p, PaVTG expression was up-regulated in worms , but only the gene expression in 0.5 μg/L B(a)p group had significant difference with control group ($P<0.05$). At day 14 of exposure, PaVTG was significantly up-regulated in worms exposed to 0.5–10 μg/L B(a)p ($P<0.05$), but not in worms exposed to 50 μg/L B(a)p.

3.3 Expression and purification of the recombinant protein

The PaVTG recombinant protein was expressed successfully as a Histidine-tag fusion protein with an expected molecular mass of 79.5 kDa. The recombinant protein was expressed at a relatively higher level when bacteria were incubated at 37°C for 4 h with 1 mmol/L IPTG (Fig.4). Most of the recombinant protein existed in inclusion bodies, and was extracted with urea. The protein was purified by Ni-NTA resin column and refolded. Only a single band at the appropriate position corresponding to the molecular mass was showed in Fig.5, and it was indicated the specificity of antibody.

3.4 VTG protein expression in female *P. aibuhitensis* exposed to B(a)p

An indirect ELISA method was used to detect the protein expression of PaVTG in female *P. aibuhitensis* exposed to B(a)p (Fig.6). The expression of PaVTG protein in acetone and estradiol group did not have significant difference with that in control group.

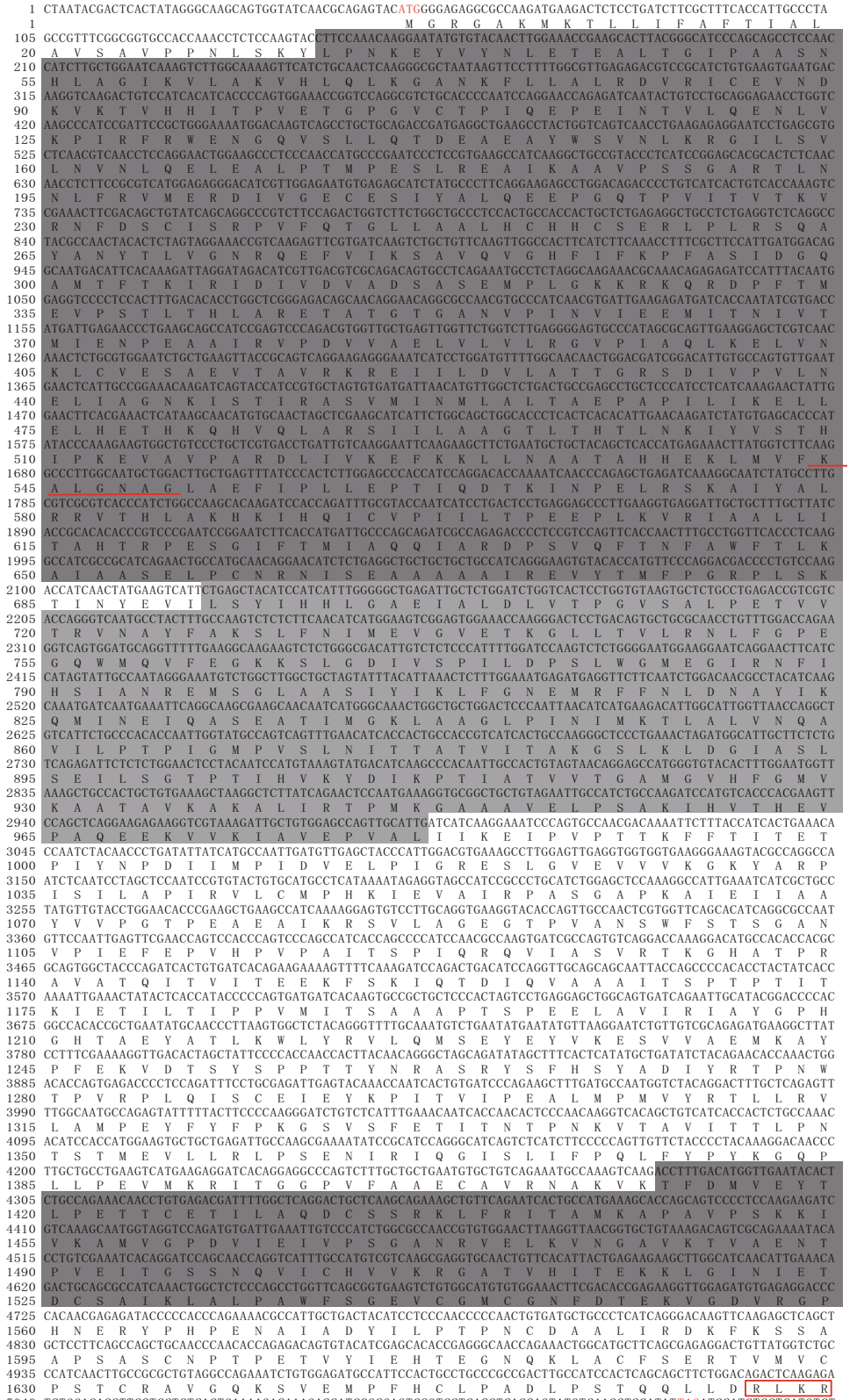

Fig.1 The full length cDNA and deduced amino acid sequence of *PaVTG*

The start and stop codons are red and the putative polyadenylation consensus signal (AATAAA) is underlined. Vitellogenin_N domain (138–1 994 aa), DUF (2 121–2 990 aa) and VWD domain (4 281–4 724 aa) are shadowed. R-X-K/R-R splice site is boxed in red. The conserved motif KALGNAG is underlined in red.

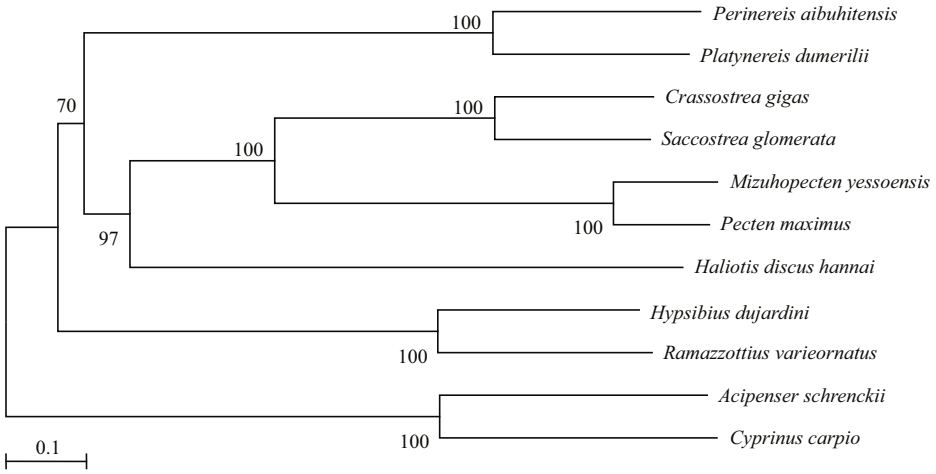

Fig.2 Phylogenetic neighbor-joining tree of VTG

The other VTG sequence were as follows: *Platynereis dumerilii* APP91162.1; *Crassostrea gigas* EKC30345.1; *Saccostrea glomerata* ANB82451.1; *Mizuhopecten yessoensis* AGE13945.1; *Pecten maximus* CAQ06469.2; *Perinereis aibuhitensis* AHY02164.1; *Hypsibius dujardini* OQV17559.1; *Haliotis discus hannai* BAF98238.1; *Ramazzottius varieonatus* GAU88667.1; *Acipenser schrenckii* AOK96644.1; *Cyprinus carpio* BAD51933.1.

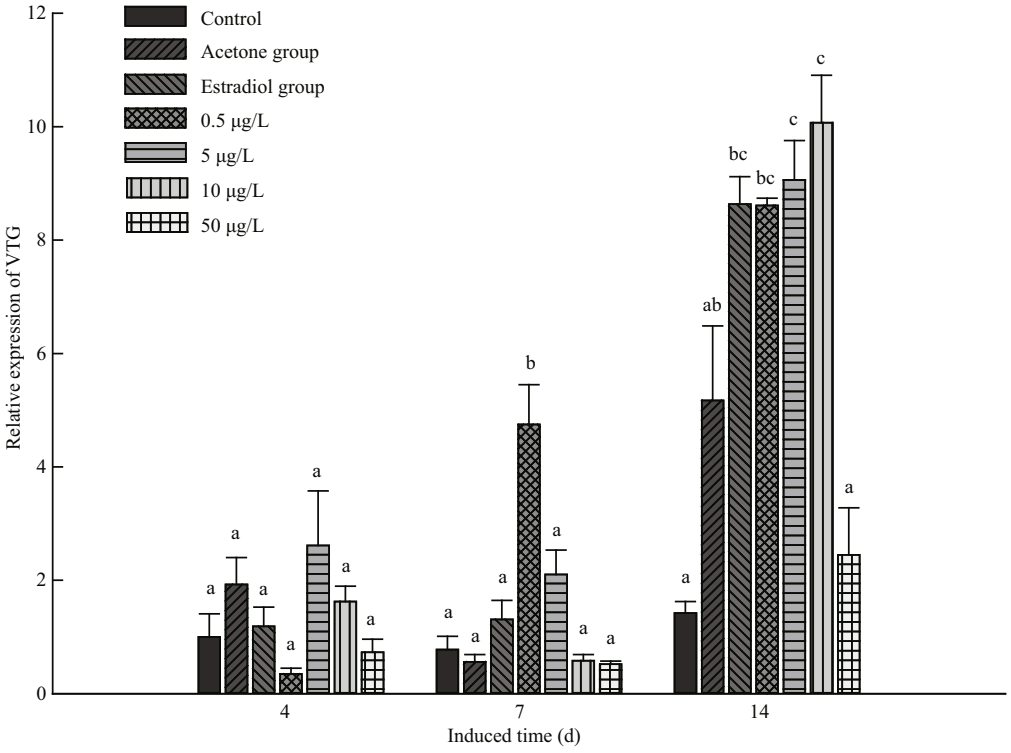

Fig.3 *PaVTG* expression in female *P. aibuhitensis* under B(a)p exposure

Value were mean±S.D. (n=6). The significance difference between groups was analyzed using one-way ANOVA and Tukey's multiple-comparison test. Bars with dissimilar letters indicate significant difference between different groups at the same sampling time.

Expression of the PaVTG protein was slightly up-regulated by B(a)p at day 4, but there were no significant differences between groups (P>0.05). Compared to the protein expression in day 4, the concentration of the PaVTG protein in day 7 in each B(a)p concentration group was increased especially in 5 μg/L B(a)p group (P<0.05). On day 14 of exposure, the concentration of the PaVTG protein was decreased significantly in worms exposed to 5 μg/L and 50 μg/L B(a)p, but the concentration of the PaVTG protein was sightly increased in 0.5 μg/L and 10 μg/L B(a)p.

4 DISCUSSION

In order to investigate the estrogenic effect of B(a)p to *P. aibuhitensis*, the full length cDNA of VTG in *P. aibuhitensis* was identified and characterized for the first time. The sequence conserved domains

predicted PaVTG protein was identified as a member of the lipid transport protein family (Zheng et al., 2012). In our study, a DUF domain was also found in PaVTG, which has been rarely detected in insects and vertebrates (Smolenaars et al., 2007). This domain family possesses a structure consisting of several large open beta sheets (Thompson and Banaszak, 2002). The function of this domain has not been identified yet. The predicted sequence of PaVTG had no phosvitin or polyserine domains, which are involved in receptor binding, and in phosphate and

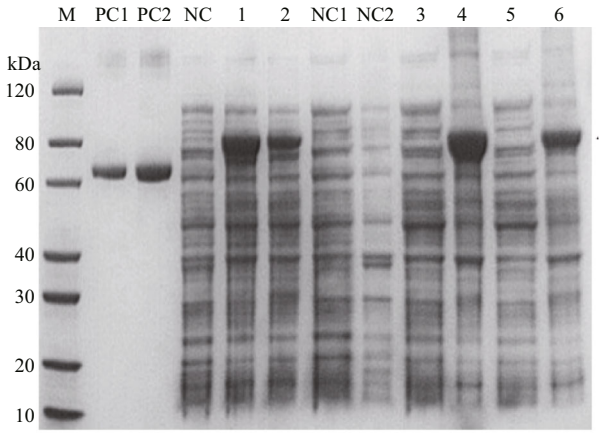

Fig.4 Expression analysis of the recombinant protein using SDS-PAGE

Lane M: Protein Ladder (PAGE MASTER protein standard, Genscript M00516); lane PC1 and PC2: BSA (1 μg and 2 μg); lane NC: total extract from DE3 before induction with IPTG; lane 1 and 2: total extract from DE3 after induction with 1 mol/L IPTG at 15°C for 16 h and 37°C for 4 h; lane NC1 and NC2: the supernatant and the precipitate of recombinant expression product before induction with IFTG; lane 3 and 4: the supernatant and the precipitate of recombinant expression products after induction at 15°C for 16 h; lane 5 and 6: the supernatant and the precipitate of recombinant expression products after induction at 37°C for 4 h.

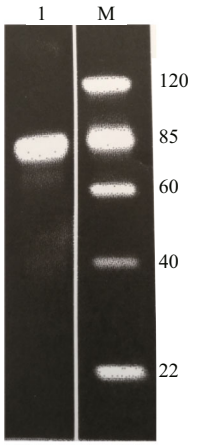

Fig.5 Western blotting analysis of the antigenicity of polyclonal antibody

M: Protein Ladder (PAGE MASTER protein standard, Genscript M00516); lane 1: protein extracted from worm; lane 2: recombinant protein.

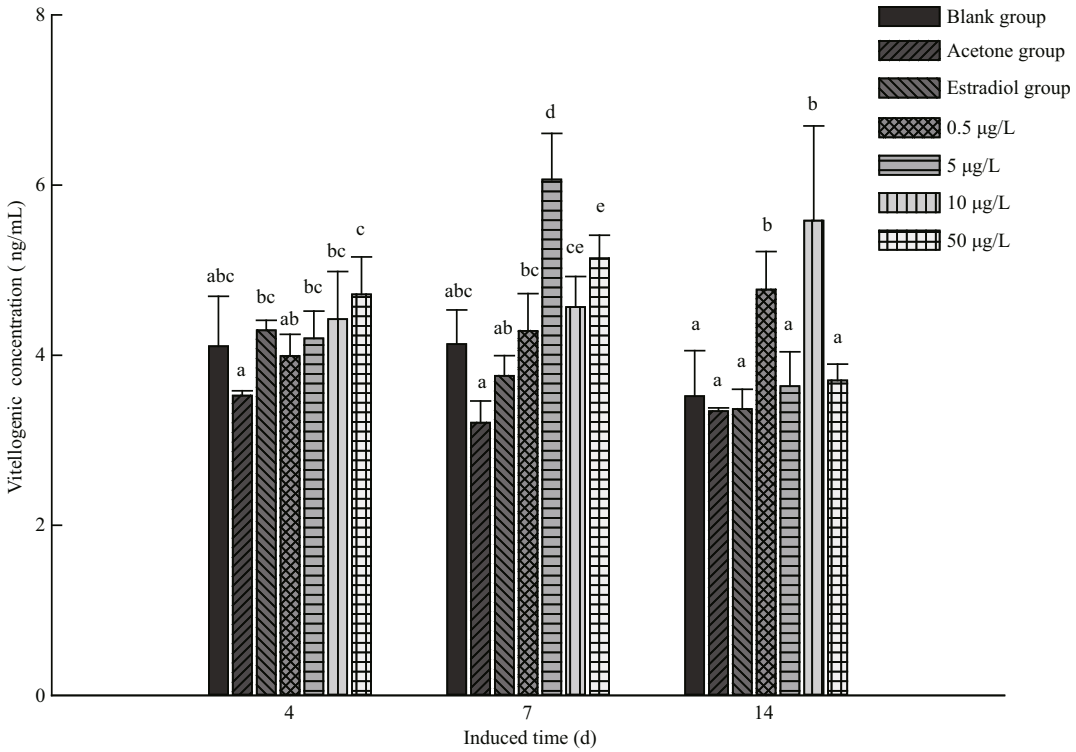

Fig.6 Levels of vitellogenin in body wall determined by indirect ELISA in adult female *P. aibuhitensis* samples

Bars showing different alphabets are significantly different at $P<0.05$.

metal ion transport in insects and vertebrates. This phenomenon has also been found in most crustaceans (Hwang et al., 2010). The absence of phosvitin and polyserine domains indicates that a different mechanism for VTG receptor binding may exist in the marine polychaete *P. aibuhitensis*. Moreover, absence of phosvitin in aquatic invertebrates makes the egg yolk protein more susceptible to oxidative damage produced by pollutants (Matozzo et al., 2008).

Most studies concerning VTG induction in aquatic invertebrates have been conducted in economically important species, such as mollusks and crustaceans. Little information about changes in VTG levels in polychaetes (which form the dominant sediment dwelling fauna of most mud flat and estuaries) has been documented. In order to evaluate the possible estrogenic effect of B(a)p on *P. aibuhitensis*, we included an estradiol group in this study for comparison. Figures 4 and 6 show that estradiol induced PaVTG gene and protein expression on day 14 of exposure. These results contradict previous studies on the marine polychaete *N. virens*. García-Alonso et al. (2006) showed that VTG secretion was increased in the presence of 17β-estradiol in cultured eleocytes from mature female (but not immature) worms of *N. virens*. Wu et al. (2017) investigated the VTG gene expression level in female *Perinereis nuntia*, the results showed that VTG mRNA level increased significantly in 2.5 and 25 μg/L B(a)p groups after inducing for 60 d. Conflicting reports about VTG expression after estradiol exposure have also been reported in bivalves. Gagné et al. (2001) showed enhanced levels of a VTG-like protein in the haemolymph of mussels *Elliptio complanata* after they were injected with estradiol. However, Puinean et al. (2006) did not observe any significant change in VTG expression in gonad homogenates from mussels *Mytilus edulis* after a 10-day exposure to estradiol. Similarly, exposure to estradiol failed to induce any significant change in VTG levels in *E. complannata* mussels (Won et al., 2005). These conflicting reports in bivalves suggest the presence of a steroid regulatory mechanism that may be different in distinct bivalve species, or distinct stages of gamete development. Alternatively, these conflicting results may indicate that VTG in bivalves is under the control of other hormones (Matozzo et al., 2008). *P. aibuhitensis* and *N. virens* belong to the same family (*Nereidae*), the reproductive control strategy that has been most studied. There is consensus in that there is single hormonal system in *Nereidae*, the supra-oesophageal ganglion that secretes a juvenile hormone (nereidine) performing a range of functions (Lewis and Watson, 2012). The process of oogenesis and growth in *Nereidae* is related to the contents of nereidine, so the titre of nereidine may be different at different gamete developmental stages. So the difference between our study and García-alonso's study may be due to the difference in developmental stage.

There was a small but not statistically significant increase in PaVGT expression at the gene and protein levels on day 4 of B(a)p exposure. Gene and protein expression of PaVTG increased over time during B(a)p exposure, reaching a maximum at day 14. The lack of PaVTG induction at the beginning of B(a)p exposure may reflect potential cross talk between biological pathways, such as the detoxification pathway (Zhang et al., 2012). Previous studies have shown that B(a)p can be transformed into more hydrophilic intermediate metabolites through the reaction of cytochrome P450 detoxification enzymes. The existence of this mechanism has been confirmed in marine polychaetes. Polychaetes are known to accumulate significant amounts of PAH from the environment. Steady-state body PAH burdens are a function of biotransfomation and elimination processes (Jørgensen et al., 2008). Chen et al. (2012) identified a full-length cDNA coding for Cytochrome P450 subfamily 4 (CYP4) in *P. aibuhitensis*, and found that PAH could induce its expression on day 4 of exposure, in a time and dose-dependence manner. Enhanced CYP4 expression during B(a)p exposure may explain why PaVTG expression is not influenced by B(a)p in *P. aibuhitensis* at early time points. The possible relationship between the expression of CYP4 and PaVTG needs to be confirmed. Besides, why PaVTG expression is not influenced by B(a)p may be related to the estrogenic effect of intermediate metabolites of B(a)p. B(a)p can produce 1-,3-,7- and 9-OH-B(a)p,4,5-7,8- and 9,10-diOH-B(a)p and other intermediate metabolites with the participation of CYP450 enzymes (Fertuck et al., 2001). B(a)p intermediate metabolites, such as 3- and 9-OH-B(a)p, have been proved to possess estrogenic effects, but hydroxyl group is not necessary for exhibiting estrogenic or antiestrogenic activity (Charles et al., 2000; Hayakawa et al., 2007).

According to our preliminary experiments, females were more sensitive to B(a)p than males. Therefore, in this study only the female worms were investigated for gene and protein expression of PaVTG. The higher expression of PaVTG in female *P. aibuhitensis* may

be related to the existence of mechanisms for reducing the toxicity of pollutants. Volz and Chandler (2004) reported that the VTG concentration in female copepods *Amphiascus tenuiremis* was significantly induced by a 12-d exposure to phenylpyrazole insecticide, but no significant induction was observed in males. The authors suggested that lipovitellin induction in females may be a resistance mechanism for reducing the toxicity of lipophilic environmental contaminants by sequestration into lipoproteins. Besides, the absence of a phosvitin domain in the PaVTG sequence may also indicate that the egg yolk protein in this species is more susceptible to oxidative damage produced by pollutants. Further studies are needed to confirm this issue.

5 CONCLUSION

In conclusion, this is the first report regarding VTG gene expression profiles after exposure of *P. aibuhitensis* to EDCs, such as B(a)p. The B(a)p induced up-regulation of PaVTG gene and protein expression suggests that VTG could be used as an potential indicator of the effects of estrogenic compounds on polychaete reproduction.

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