Ecdysone receptor in the mud crab *Scylla paramamosain*: a possible role in promoting ovarian development

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Abstract

In arthropods, it is known that ecdysteroids regulate molting, limb regeneration, and reproduction through activation of the ecdysone receptor (EcR). However, the ecdysteroid signaling pathway for promotion of ovarian development in crustaceans is still unclear. In this study, three cDNA isoforms of EcR were cloned from the mud crab *Scylla paramamosain*. qRT-PCR revealed that the *SpEcR* mRNA was abundant in the eyestalk, ovary and epidermis. During ovarian development, the *SpEcR* transcripts increased from stage I (undeveloped stage) and reached a peak at stage IV (late vitellogenic stage) before dropping to a lower level at stage V (mature stage). Meanwhile, levels of 20-hydroxyecdysone (20E) in the hemolymph, detected by HPLC-MS, displayed a similar pattern of increase with ovarian development. Results from *in situ* hybridization indicated that *SpEcR* mRNA was present in the follicular cells during vitellogenesis. Results from *in vivo* experiments revealed that 20E at 0.2 µg/g body weight significantly stimulated the expression of *SpEcR* and *vitellogenin* (*SpVg*) in female crabs during the early vitellogenic stage but not during the previtellogenic stage. This was confirmed by results from *in vitro* experiments which indicated that *SpEcR* and *SpVg* expression levels were significantly upregulated in early vitellogenic ovarian explants incubated with 5.0 µM 20E at 3 and 6 h but not in previtellogenic ovarian explants. Finally, results from *in vitro* gene silencing experiments indicated that the expression of *SpEcR* and *SpVg* in the ovary was significantly inhibited by *SpEcR* dsRNA. All these results together indicated that in *S. paramamosain*, 20E, and *SpEcR*, located in the follicular cells, play important roles in the promotion of ovarian development via regulating the expression of *SpVg*.

Key Words

- ecdysone receptor
- ovarian development
- 20-hydroxyecdysone
- *SpEcR* dsRNA
- *Scylla paramamosain*

Journal of Endocrinology
(2015) 224, 273–287

Introduction

Ecdysteroids, a group of polyhydroxylated ketosteroids, are important steroid hormones found in arthropods, with the primary function of regulating molting. However, they are also known to be involved in the regulation of ovarian development and reproduction of arthropods (Horigane *et al.* 2008, Tarrant *et al.* 2011). In crustaceans,

it is generally known that ecdysteroids are first synthesized in the Y-organ (molting gland), and subsequently released into hemolymph where they are hydroxylated to become 20-hydroxyecdysone (20E), a biologically active form of ecdysteroid (Lachaise *et al.* 1993, Subramoniam 2000). Ecdysteroids need to bind to the ecdysone receptor (EcR),

which belongs to the nuclear receptor family, to activate DNA regulatory element (Bortolin *et al.* 2011, Gaertner *et al.* 2012). In crustaceans, EcR can form a heterodimer with another nuclear receptor known as retinoid X receptor (RXR), which is orthologous with ultraspiracle (USP) in insects, to regulate the downstream genes in the ecdysteroid signaling pathway (Wu *et al.* 2004, Kim *et al.* 2005a,b, Hopkins *et al.* 2008, Hill *et al.* 2013).

In insects, due to alternative splicing, a number of EcR isoforms have been reported and most of them differ mainly in the N-terminal region, which is related to regulation of transcriptional activation (Lafont 2000, Bortolin *et al.* 2011). However, recently alternatively spliced regions in the hinge domain and the ligand-binding domain have also been identified in various crustaceans, including the fiddler crab *Uca pugilator* (Chung *et al.* 1998a,b, Durica *et al.* 2002), the kuruma prawn *Marsupenaeus japonicus* (Asazuma *et al.* 2007), the American clawed lobster *Homarus americanus* (Tarrant *et al.* 2011), the freshwater prawn *Macrobrachium nipponense* (Shen *et al.* 2013), and the blue crab *Callinectes sapidus* (Techa & Chung 2013). It has been proposed that different isoforms of EcR had their unique domains, which influence each receptor's ability to activate or repress gene expression, and hence exert different physiological functions (Hopkins *et al.* 2008, Tan & Palli 2008, Schwedes *et al.* 2011).

It is well known that physiological roles of ecdysteroids and EcR include regulation of molting, development, limb regeneration, and reproduction in arthropods (Hopkins 1989, Durica & Hopkins 1996, Ogura *et al.* 2005, Durica *et al.* 2006, Asazuma *et al.* 2007). In the fruit fly *Drosophila melanogaster*, EcR mutants in females caused defects in oogenesis; the spectrum of oogenic defects includes the presence of abnormal egg chambers and disappearance of vitellogenic stages, indicating that EcR is required during the ovarian maturation of the species (Carney & Bender 2000). It has also been reported that the knockdown of the EcR gene by RNA interference (RNAi) significantly reduced the level of vitellogenin (Vg) mRNA in the red flour beetle *Tribolium castaneum*, indicating that EcR is required for primary oocyte maturation, ovarian growth, and the migration of the follicle cells of this specie (Parthasarathy *et al.* 2010, Xu *et al.* 2010). In crustaceans, ecdysteroids can induce the expression of the Vg gene and the concentrations of ecdysteroids increase during the initial stages of oogonial and spermatogonial mitoses (Subramoniam 2000, Tiu *et al.* 2010). In crustaceans, ovary has been regarded as the site of synthesis of Vg (Yano & Chinzei 1987, Browdy *et al.* 1990); however, it has also been reported that Vg is

synthesized in the hepatopancreas and then transported to the ovary (Soroka *et al.* 2000, Okuno *et al.* 2002). Furthermore, Tiu *et al.* (2006) reported that both the ovary and hepatopancreas made equal contributions to the production of Vg transcripts in the tiger shrimp *Penaeus monodon*. Thus, expression of Vg may occur at multiple sites with species-specific expression patterns. However, so far little is known about the possible roles of EcR in synthesis of Vg in the ovary of crustaceans.

The mud crab *Scylla paramamosain* is a large portunid crab species distributed widely from tropical to warm temperate coasts of China and other Indo-Pacific countries and is an important species for fisheries and aquaculture (Ye *et al.* 2011). Female mud crabs with mature ovaries fetch substantially higher prices because their ripe ovaries are considered a delicacy. Given the function of ecdysteroids in the control of crustacean reproduction (Subramoniam 2000), the investigation of the role of ecdysteroids and EcR in regulation of ovarian development of the mud crab *S. paramamosain* is likely to provide results relevant to the manipulation of ovarian maturation in aquaculture. Hence, in this study, the expression patterns of EcR transcripts in the ovary of *S. paramamosain* (*SpEcR*) were investigated and their mRNA was localized via *in situ* hybridization. Meanwhile, 20E titers were measured in the hemolymph during ovarian development using HPLC-MS. Finally, the effect of 20E on expression of *SpEcR* and *SpVg* was investigated in female crabs at different stages of ovarian development while *in vitro* experiments were also conducted on ovarian explants to measure the changes in the mRNA levels of *SpEcR* and *SpVg* when exogenous 20E and double-stranded EcR (*EcR dsRNA*) were added respectively.

Materials and methods

Tissue sampling and RNA isolation

All experimental animals and procedures used in this study have been approved by the university animal ethical committee.

Healthy female *S. paramamosain* were purchased from a local fish market in Xiamen, China. They were transported back to Xiamen University and acclimated in cement tanks filled with seawater (temperature 26–28 °C; salinity 26 ppt) with aeration for at least 3 days before any experiments. On the basis of results from previous studies (Shangguan *et al.* 1991, Huang *et al.* 2014), the ovarian development of *S. paramamosain* can be divided into five stages. That is stage I (undeveloped stage): the

ovary appears translucent and contains oogonium only; stage II (previtellogenic stage): the ovary is milky white, 0.5–4.0 mm in size, and the oocyte is small; stage III (early-vitellogenic stage): the ovary size increases to 5–20 mm, appears yellow/orange in color, and the oocyte contains yolk granules; stage IV (late-vitellogenic stage): the ovary is orange, 25 mm in size; the oocyte is about 240 µm in diameter and contains larger yolk granules; and stage V (mature stage): the ovary reaches its largest size and the diameter of the oocyte reaches about 260 µm with cell nucleus atrophy. On the basis of the above-mentioned ovarian staging system, female crabs with ovarian development at stage II were selected for sampling of tissues from the stomach, hepatopancreas, ovary, muscle, heart, epidermis, gill, hemocyte, eyestalk, thoracic ganglion, and brain. Meanwhile, ovary specimens from female crabs at each ovarian developmental stage were also collected for gene expression analysis. All tissues sampled were immediately frozen in liquid nitrogen and stored at -80°C for later nucleic acid extraction.

Total RNA was extracted from the tissue samples using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). The extracts were then treated with DNase I to eliminate genomic contamination. The RNA reliability was estimated by agarose gel electrophoresis and quantified using a ND-1000 nanoDrop u.v. spectrophotometer (nanoDrop Technologies, Inc., Wilmington, NC, USA). A 2 µg sample of total RNA was

reverse transcribed using the reversed First-strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) and stored at -20°C .

Cloning and sequencing of *SpEcR*

Total RNA extracted from the stage II ovary was reversely transcribed for the template cDNA. Degenerate primers *EcR* F and *EcR* R (Table 1), designed on the basis of results of multiple alignment of the conserved DNA-binding domain, were used to amplify a partial sequence (461 nucleotides) encoding the *EcR* protein of *S. paramamosain*. The full sequences of *EcR* were completed by 3' and 5' RACE using the 3', 5' Full RACE kit (Takara, Shiga, Japan). Specific primers *EcR* 3' and *EcR* 5' (Table 1) were designed based on the initial sequences. PCR products were separated on 1% agarose gel and visualized using a u.v. transilluminator. The expected DNA fragments were gel-purified and ligated to pMD19-T vectors (Takara), and then transformed into competent cells of *Escherichia coli*. In order to avoid PCR artifacts, three positive recombinant clones were sequenced in both directions with sequencing primers *M13*-47 and *RV*-M (Table 1) (Sangon Biotech Co., Ltd, Shanghai, China). The similarity analysis was performed using the Blast program at the National Center for Biotechnology Information, US (<http://www.ncbi.nlm.nih.gov/blast/>). Sequence alignment was performed using ClustalW Software.

Table 1 Summary of primer pairs used for the study

Primers	Primer sequence (5'–3')	Purpose
<i>EcR</i> F	TTYTTCCGKMGVTCVATCAC	Fragment amplification of <i>EcR</i>
<i>EcR</i> R	TCWGWGTGGHWGYTCAWAST	Fragment amplification of <i>EcR</i>
<i>EcR</i> 3'	ACTCTTCGTTTCTGTCGCAA	3' amplification of <i>Rxr</i>
<i>EcR</i> 5'	GGTTGCGACAGAAACGGAAGA	5' amplification of <i>Rxr</i>
<i>YEcR</i> F	AAGAACAAAAGACTCCCACCAT	Real-time quantitative PCR for <i>EcR</i>
<i>YEcR</i> R	TCTCTCACTTACAGCCGACAGG	Real-time quantitative PCR for <i>EcR</i>
<i>TEcR</i> F	TATGAGTTTGTTGGCTTGGGAG	Riboprobe amplification for <i>EcR</i>
<i>TEcR</i> R	GGTGGGAGTCTTTGTTCTTGAGT	Riboprobe amplification for <i>EcR</i>
<i>T7</i>	TAATACGACTCACTATAGGG	Riboprobe amplification for <i>EcR</i>
<i>EcR</i> F2	ATTCACGGGGTCTCATCATCTC	Full-length confirmation for <i>EcR</i>
<i>EcR</i> R2	TTGTAAGGACGGCATAACCAGC	Full-length confirmation for <i>EcR</i>
<i>Vg</i> F	GAGTGATGATGGAGGTGTCCTG	Real-time quantitative PCR for <i>Vg</i>
<i>Vg</i> R	GACCTTGAGCGATTCTGGTGACGA	Real-time quantitative PCR for <i>Vg</i>
<i>Gapdh</i> F	AATGCCATCACAATAGAAAAATC	Real-time quantitative PCR for <i>Gapdh</i>
<i>Gapdh</i> R	GGAACAATCAACTACCACACC	Real-time quantitative PCR for <i>Gapdh</i>
<i>SEcR</i> F	CATGACATCGTTAGTGGGATTC	Amplification for <i>EcR</i> dsRNA
<i>SEcR</i> R	GTAATCCTTCTTATCCTTGCTCG	Amplification for <i>EcR</i> dsRNA
<i>Gfp</i> F	TGGGCGTGGATAGCGGTTTG	Amplification for <i>GFP</i> dsRNA
<i>Gfp</i> R	GGTCGGGGTAGCGGCTGAAG	Amplification for <i>GFP</i> dsRNA
<i>M13</i> -47	CGCCAGGGTTTCCCAGTCACG	Colony PCR
<i>RV</i> -M	GAGCGGATAACAATTCACACA	Colony PCR
β -actin F	GAGCGAGAAATCGTTCGTGAC	Internal control
β -actin R	GGAAGGAAGGCTGGAAGAGAG	Internal control

Gene expression profiling by qRT-PCR

To determine the abundance of *SpEcR* transcripts in various tissues and in ovaries at different development stages, qRT-PCR was performed using an ABI 7500 FAST (Applied Biosystems). A pair of primers, *YEcr F* and *YEcr R* (Table 1), designed based on the sequence of the common domain (1391–1568 bp) of different isoforms, were used to amplify a product of 189 bp. Two β -actin primers, *β -actin F* and *β -actin R* (Table 1), were used to amplify a 183 bp fragment as the internal control (Huang *et al.* 2012, Shen *et al.* 2013).

PCR was performed in a 20 μ l reaction volume containing 10 μ l of SYBR premix, 0.8 μ l of each primer (10 μ M), 2 μ l of cDNA template, and 6.4 μ l of MilliQ-water and following the instructions of the manufacturer of SYBR Premix EX Taq (Takara). The PCR conditions were as follows: 94 °C for 10 min; 45 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s. All samples were analyzed in triplicate.

Quantification of 20E in the hemolymph using HPLC-MS

The chemicals used for this experiment, methanol, acetonitrile, 20E, and Makisterone A, were all purchased from Sigma–Aldrich. First, from female crabs at each ovarian developmental stage, 0.3 ml hemolymph was collected through the arthrodial membrane of their last walking leg. The hemolymph was then homogenized in 5 ml methanol with 125 ng Makisterone A added as internal standard and centrifuged for 15 min at 9600 g. The supernatant was collected and concentrated to 200 μ l using a rotary evaporator. The concentrated extract was eluted on 3 ml Waters Oasis HLB extraction cartridge (Waters Corporation, Milford, MA, USA) preconditioned with 4 ml methanol and 5 ml water. The extraction cartridge was then washed by 1 ml of 10% acetonitrile in water, and eluted with 100% acetonitrile. The collected eluant was dried by nitrogen and re-dissolved in 0.3 ml methanol.

An Agilent 1200-LC system coupled to a 3200Q TRAPMS detector equipped with an ESI interface (Agilent Technologies, Shanghai, China) was used to determine the concentration of 20E, which was eluted through a Zorbax 300SB-C18 column (4.6 mm $\times$ 250 mm) with a solvent mixture of 90% acetonitrile and 10% water at a flow rate of 1 ml/min for 4 min. The column thermostat was maintained at 25 °C. 20E was detected in the positive mode (m/z =481) with the Makisterone A acting as the internal standard (negative mode, m/z =493). MS parameters

included curtain gas (CUR) at 20 psi; a nebulizer pressure (GAS1) of 50 psi; and a temperature of 400 °C.

Localization of *SpEcR* in the ovary by *in situ* hybridization

Digoxigenin-labeled cRNA riboprobes were synthesized with a DIG-RNA labeling kit (Roche) using a 300 bp length template of *SpEcR*, which was subcloned into the pGEM-T easy vector (Promega). The ovarian tissues at different stages of vitellogenesis were obtained and immediately fixed in 4% paraformaldehyde (PFA) in PBS for one night. The fixed ovarian tissues were dehydrated through a series of increasing concentrations of ethanol, then cleared with xylene and infiltrated with liquid paraffin at 55 °C before finally being embedded in paraffin blocks. The blocks were trimmed and sliced to 7 μ m using a microtome. For conventional histological observation, the tissue sections were deparaffinized, hydrated, and stained with hematoxylin and eosin (H&E). For *in situ* hybridization, the paraffin sections were deparaffinized, hydrated, and then washed twice with 1 $\times$ PBS, followed by 0.1 M glycine for 10 min and in 0.3% Triton X-100 for 10 min. The sections were digested with protease K (10 μ g/ml) for 20 min at 37 °C. After refixation with 4% PFA, the serial sections were hybridized overnight at 57 °C with riboprobe (1 ng/ μ l) and then washed with 50% deionized formamide diluted to different concentrations of SSCT (with 0.1% Tween-20) solution (2 $\times$ SSCT and 0.2 $\times$ SSCT). The hybridized tissue sections were incubated with anti-DIG alkaline-phosphatase-conjugated antibody (Roche) and signals were visualized with the colorimetric substrates nitroblue tetrazolium/4-bromo-4-chloro-3-indolylphosphate (Yang *et al.* 2013). The riboprobe template for *SpEcR* was generated by PCR from ovary cDNA using the specific primers *TEcr F* (1079–1100 bp), *TEcr R* (1387–1410 bp), and *T7* (Table 1). The specific primers were designed based on the sequence of the common domain of different isoforms. Photographs were taken using an Olympus multifunction microscope (Olympus BX51, Tokyo, Japan).

In vivo effect of 20E on *SpEcR* and *SpVg* expression

Six female crabs at the early vitellogenic stage (carapace width: 128.3 $\pm$ 5.1 mm, body weight: 377.9 $\pm$ 19.2 g) and another six at the previtellogenic stage (carapace width: 92.8 $\pm$ 3.8 mm, body weight: 130.8 $\pm$ 11.2 g) were equally divided into control and treatment groups. The crabs assigned to the treatment group received 100 μ l 20E injection at 0.2 μ g/g body weight through the arthrodial

membrane at the base of the last pereopods, while control crabs received the same volume of carrier. The crabs were transferred to two concrete tanks ($L \times W \times D = 8 \times 3 \times 0.7$ m) with half of the tank bottom covered with 10 cm sand as the substrate. The tanks were filled with filtered seawater and aerated continuously. The crabs were cultured at 24–26 °C and a salinity of 26 ppt and fed with live clams (*Ruditapes philippinarum*) at a ration of approximately 30% of the crab body weight per day. A daily 100% water exchange was carried out. All the crabs were sampled at 24 h after the injection to extract the total RNA of the ovary. The first-strand cDNA synthesis and qRT-PCR were performed according to the procedures described in the ‘Tissue sampling and RNA isolation’ and ‘Gene expression profiling by qRT-PCR’ sections.

In vitro effect of 20E on *SpEcR* and *SpVg* expression

The female crabs were sterilized in 70% ethanol after immobilization on ice for 15 min. Early vitellogenic ovarian tissues were dissected from the crabs and then rinsed nine times with saline solution modified for crabs (hereafter referred to as ‘crab saline solution’): 440 mM NaCl, 11.3 mM KCl, 13.3 mM CaCl_2 , 26 mM MgCl_2 , 23 mM Na_2SO_4 and 10 mM HEPES (pH 7.4), and contained penicillin G (300 IU/ml) and streptomycin (300 µg/ml, Sigma–Aldrich Chemical Co.). After the ovarian tissues were cut into small pieces of about 50 mg, each tissue fragment was placed in a well of a 24-well culture plate with 0.5 ml of medium 199 and 2 µl 20E added at a designated concentration. Three concentrations of 20E – 0.05, 0.5, and 5 µM – were first prepared in medium 199. The ovarian tissue fragments placed in 0.5 ml of medium 199 with 2 µl crab saline solution or ethanol were meanwhile set up as controls. Every treatment was triplicated and the culture plates were incubated at 25 °C. Total RNAs from the fragments were extracted 3 h after 20E was added. In addition, to assess the effects of 20E over time, 5 µM 20E was added to both previtellogenic and early-vitellogenic ovary explants. The ovary explants were sampled at 1, 3, 6, and 9 h after incubation with 20E to observe changes in the expression of *SpEcR* and *SpVg*. The first-strand cDNA synthesis and qRT-PCR were performed as described in ‘Tissue sampling and RNA isolation’ and ‘Gene expression profiling by qRT-PCR’ sections.

dsRNA synthesis and *in vitro* gene silencing

The 453 bp region of *SpEcR* was amplified using the primers *SEcR* F and *SEcR* R (Table 1), which were designed

on the basis of the common sequence (104–556 bp) of the three *SpEcR* isoforms. Another 454 bp of green fluorescent protein (*Gfp*) gene, as an exogenous control gene, was amplified with *Gfp* F and *Gfp* R from the pSicoR-EGFP vector. The PCR products were inserted into pGEM-T easy vectors to clone the DNA templates for *in vitro* transcription. The dsRNAs were synthesized using the purified DNA templates amplified by the T7 and SP6 polymerase. The remaining DNA templates were removed with RNase-free DNase I.

The subsequent *in vitro* experiment on gene silencing with the synthesized dsRNA was similar to that described in ‘*In vitro* effect of 20E on *SpEcR* and *SpVg* expression’ section. There were four treatments: treatment group 1 received 5 µg *Gfp* dsRNA; treatment groups 2 and 3 initially received 5 µg *SpEcR* dsRNA, while treatment group 4 received neither *Gfp* dsRNA nor *SpEcR* dsRNA. After 8 h of culture with dsRNA, the medium 199 in treatment group 3 was cleared away with a pipette before an equal amount of medium 199 with 5 µM 20E was added. At the same time, 5 µM 20E was added to the treatment group 4. Three hours after addition of 20E, the ovarian tissue fragments from treatment group were sampled for the extraction of total RNAs for cDNA synthesis and qRT-PCR analysis.

Statistical analyses

The qRT-PCR data obtained were calculated using $2^{-\Delta\Delta\text{Ct}}$ method as described by Livak & Schmittgen (2001) before subjecting them to statistical analysis. One-way ANOVA and Student’s *t*-test were performed to determine the statistically significant differences among treatments, which was set at the $P < 0.05$ level. Before the comparison, Kolmogorov–Smirnov and Cochran tests were performed to test the normality and homogeneity of variances respectively. All statistical analysis was performed using the SPSS 11.5 Software (SPSS).

Results

SpEcR sequence identification

Three full-length *SpEcR* cDNAs, *SpEcR1* (2197 bp, GenBank accession number JQ821372.1), *SpEcR2* (2116 bp, GenBank JQ821373.1), and *SpEcR3* (2197 bp, GenBank JQ821374.1) were cloned. Both *SpEcR1* and *SpEcR3* contained 1299 bp open reading frames encoding 432 amino acids (aa), but they were differentiated between amino acids 227 and 275. Sequence alignment of these

TTTCACCACTGTATCT 16
 CCCCTCAGTGAACACATCAATTAGATTATCAGCAACTCTTACATTGTAATTCACGGGGTCTCAT 82
 CATCTCATCACCGTGGCTTTTCATGACATCGTTAGTGGGATTCATCAACACGGATCACAAACGGC 148
 ATGGCATGGAATCTGAAGGGGAGATGACATGTGCGGCCCTCTTCAGTGAGCAACTACAGCGCC 214
 M A L E S E G R D D M S P P S S V S N Y S A
 GACTCCTTCGGCGACCTGAAAAAGAAAAAGGCCCATTCGCCGCCAGCAGGAGGATGTGTCTA 280
 D S F G D L K K K K G P I P R Q Q E E L C L
 GTGTGCGGGACAGGGCGTCAGGCTACTACACGCACTACCTGCGAGGGATGCAAGGCTTC 346
 V C G D R A S G Y H Y N A L T C E G C K G F
 TTCGGGAGATCCATCACAAGATGCTGTATATCAGTGTAAATATGGTGCAACTGTGAAATGGAT 412
 F R R S I T K N A V Y Q C K Y G G N C E M D
 ATGTATATGCGACGCAATGTCAAGATGTGCGCTCAAGAAGTGTCTTGGTGTGGGATGCGACCA 478
 M Y M R R K C Q E C R L K K C L G V G M R P
 GAATGTGTTGTGCCAGAGTCTCAGTGGTGGTGAAGAGAGAGCAGAAGAAGTACGAGACAAGGAT 544
 E C V V P E S Q C V V K R E Q K K L R D K D
 AAGAGGATTACCCAACTCAAGGCTCCCGTAGCTGAGGAAAAGGCCGTTCCAACAGTCCAGTG 610
 K K D Y P S Q G S P V A E E K A V P T S P V
 AGTAATGATGTAAATCCAAAGGCTCACCCTGCATGTGATATGCAAGTTCCTGTTGAC 676
 S N D C K S K G P S P A C D M Q F K N L V D
 ACTCTAGCATCGTTCAAACCTCTATGTCGCTGGGGCCAGATCAATGTCAAGCCACTCACCGCA 742
 T P S I V Q T P M S A G A R S N V K P L T R
 GAGCAGGAGGAGCTGATCAACACATAGTCTACTACCAAGAAGCAGTTGAGCAGCAACTGAAGCA 808
 E Q E E L I N T L V Y Y Q E Q F E Q P T E A
 GACATCAAAAGATAAGATTAACTTCGATGGTGAAGATACAGTGACATGAGATTACGGCACATA 874
 D I K K I R F N F D G E D T S D M R F R H I
 ACCGAGATGACGATCTCACAGTTCAGCTCATAGTGAATTCCTCAAGCAACTACCAGGTTTCGGC 940
 T E M T I L T V Q L I V E F S K Q L P G F G
 ACACCTTCAGCGAGAAGACGATTACCTGCTCAAGGCTTGCTATCAGAAGTCATGATGCTCCGA 1006
 T L Q R E D Q I T L L K A C S S E V M M L R
 GCAGCCCGGTTCTATGATGCCAAGACAGATGCTGTTGTTGAAATACCTTGCCCTACACAAA 1072
 A A R F Y D A K T D C I V F G N T L P Y T Q
 ACATCTTATGAGTTTGTGCTGGCTGGGAGAATCATCAAAATCTCTTCGTTTCTGTCGCAACCTT 1138
 T S Y E F A G L G E S S Q I L F R F C R N L
 TGAAGATGAAAGTTGATAATGCTGAGTATGCACTTGTCTGCCATAATTATTTACAGAGAGG 1204
 C K M K V D N A E Y A L L S A I I I F S E R

Figure 1

Nucleotide and deduced amino acid sequences of the *SpEcR* gene of the mud crab, *Scylla paramamosain*. The nucleotides are numbered at the right and an 81 bp alternative deletion is underlined. The shadowed sequence is

CCAAACTAAAGAACTCCAAAAGGTGAAAAGCTTCAAGAAATATACCTCGATGCAATGAGGGCC 1270
 P N L K E L Q K V E K L Q E I Y L D A L R A
 TACGTGTGTAATCAGAGGTTCTCGGCCCTGGCATGGTGTGCAAAAGTTGCTTAATATCTCCTACT 1336
 Y V C N Q R F P R P G M V F A K L L N I L T
 GAGTTGCGAACCTTGAAACTTGAACCTCAGAGGTATGCTTCTCCCTCAAACCTCAAGAACAAAAA 1402
 E L R T L G N L N S E V C F S L K L K N K R
 CTCCCACTATTTTGGCTGAGATCTGGGATGTTGCTGGTGTGCTGAGCCTCAGCCACCTCTGGGTG 1468
 L P P F L A E I W D V A G C
 CCATATTACCGAGGTAATGTGGCGCAAGAAGTGTGAGCCCTTTGGGGGTGGTGGATGGACT 1534
 CTACACTATGTACCTGTGCGGTGTAAGTGAGAGACTGTGTATGTTGTGCGCAGCAGCTTCACACTGC 1600
 CACCCAGGGGCCCTTTACGACCTGACGTGTTATGGTTCAGGCCACCACTGTGACAGTGTGT 1666
 GTAGCACTCCATTAGTGAATGATGAGTGTATAATGATGACCTTTAGATGTGAAGGGCAGCAGTAT 1732
 GGGCATTGTAGCCATTTTATTAACACATTTTATAAACCTTCAGATATAATGAAAGTGGTGTGCA 1798
 TATTGACTGTTTCATATACATATACCTACATGTATGTGTAATAATGTTTTTGTCTGTATATTTTT 1864
 TGATATAATATTAAGTAAAGGGCTACATTGCACTGTTGCTCAGACACTGCGCAAGCCACATT 1930
 CTGCGAGGCAACGAACCTCCCGTGCCTTGTACGGTCAAGCTCTGTTCTTACACTATTCTCAA 1996
 GGTGCTCTCGGGGACTGAGGAGAGGGGAATCTAAACACAGGAACAGTGGCATGCTCTCTGATCC 2062
 ATATTTTATATGAATCTTGTAAATTAATGAGCTGGTATGCCGCTCTTACAAGTAGTAACTAT 2128
 TGAATAATATGTAAATATTACTGTAATGTATTAACTGCTATGAAATGTAATAAAAAAAAAAAAA 2194
 AAA 2197

GATTACGAAATAGTGATGACGACTCGGATGCCAAGTTTAAACACATC 874
 D Y E I S D D D S D A K F K H I
 ACAGACATGACCATCTCAGTGCAGCTGATCGTAGAGTTTCCAAGCGCTCGCCGGCTTTGAC 940
 T D M T I L T V Q L I V E F S K R L P G F D
 ACACACTCAGAGGATCAGATTACACTGCTA 973
 T L L R E D Q I T L L

a 147 bp substitution between amino acids 226 and 275, and the bottom right corner of the figure shows the substituted sequence.

isoforms indicated that there was an 81 bp alternative deletion between nucleotide positions 620 and 700 in the D domain of *SpEcR2* while a 147 bp substitution between nucleotide positions 827 and 973 differentiated *SpEcR1* and *SpEcR3* in the LBD domain (Fig. 1). The three cDNA isoforms had the same 750 bp 3'-UTR with a poly A tail and a 148 bp 5'-UTR. Full-length confirmation primers, *EcR* F2 and *EcR* R2, were designed to test the veracity of the sequence and successfully amplified all the isoforms. Finally, alignment algorithms revealed similar homology in the DNA binding domain (DBD) and LBD with other species of crustaceans and insects. A comparison of all the deduced amino acid sequences indicated that all three *SpEcR* isoforms had a domain organization typical of a nuclear hormone receptor (Fig. 2).

Tissue-specific expression and expression profiles of *SpEcR* transcripts during ovarian development

As shown in Fig. 3, *SpEcR* was found in all 11 tissues examined, i.e. muscle, heart, thoracic ganglion, hemo-

cyte, brain, gill, stomach, hepatopancreas, eyestalk, ovary, and epidermis. However, the expression levels of *SpEcR* gene were significantly higher in the eyestalk, ovary, and epidermis than in other tissues ($P < 0.05$).

To test the correlation of *SpEcR* expression level with ovarian development, the relative abundance of transcripts were determined by qRT-PCR at different stages of ovarian development. The expression of *SpEcR* increased gradually with ovarian development from stage I and was significantly higher at both stage III and IV ($P < 0.05$). It reached a peak level at stage IV but dropped substantially at stage V (Fig. 4).

Hemolymph 20E concentration during ovarian development

20E titers in the hemolymph largely showed a similar trend to the expression of *SpEcR* as they increased with the development of the ovary from stage I and reached peak values at stage III and IV before dropping to a low level at stage V (Fig. 5). Statistical analysis confirmed that similar

Figure 2

Alignment of amino acid sequences of *SpEcR* from *S. paramamosain* with *EcR* orthologs from other crustacean species. Deduced amino acid sequences are aligned by the ClustalW alignment program. GenBank accession numbers: *Carcinus maenas EcR* (CmEcR) (AAR89628.1), *Gecarcinus lateralis EcR* (GIEcR) (AAT77808.1), *Crangon crangon EcR* (CcEcR) (ACO44665.1), *Celaca pugilator EcR* (CpEcR) (AAC33432.2), *S. paramamosain EcR1* (JQ821372.1), *S. paramamosain EcR2* (JQ821373.1), and

to the expression profile of *SpEcR* transcripts, hemolymph 20E concentrations at stage III and IV were significantly higher than that at stage I ($P < 0.05$).

Localization of *SpEcR* in the ovary by *in situ* hybridization

Paraffin sections stained with H&E revealed that the ovaries of *S. paramamosain* consisted of many ovarian lobules, and oocytes at different developmental stages could be readily distinguished (Fig. 6C, F, and I). For

S. paramamosain EcR3 (JQ821374.1). The DNA-binding domain is indicated by the bracket and the starting point of the ligand-binding domain is indicated by an arrow. The P-box and D-box residues, which are important for the binding to hormone response element, are shaded, and the AF-2 ligand-dependent activation region is boxed. The A/B domain and D domain are marked above the sequence.

ovaries at the previtellogenic and early-vitellogenic stages, clusters of follicular cells were found, often along the periphery of the ovarian lobules (Fig. 6C and F). With ovarian development, the follicular cells gradually spread to surround oocytes at the late-vitellogenic stage (Fig. 6I). Correspondingly, *in situ* hybridization of *SpEcR* mRNA showed that in the previtellogenic and early-vitellogenic ovaries, *SpEcR* mRNA was localized in the follicular cells distributed along the periphery of the ovarian lobules rather than inside oocytes (Fig. 6A and D) while in the

Figure 3

The qRT-PCR analysis of *SpEcR* expression in various tissues of *S. paramamosain*. Expression of the β -actin gene was used as a control. The relative abundances of *SpEcR* transcripts are shown as mean $\pm$ S.E.M. ($n=3$). Mu, muscle; Ht, heart; TG, thoracic ganglion; Hy, hemocyte; Br, brain; Gi, gill; St, stomach; Hp, hepatopancreas; Es, eyestalk; Ov, ovary; Ep, epidermis. Asterisks indicate significant differences from value for the stomach ($P<0.05$).

late-vitellogenic ovaries, *SpEcR* signals were detected in the follicular cells surrounding the oocytes (Fig. 6G). No positive signal was detected with the sense *SpEcR* riboprobe as the control (Fig. 6B, E, and H).

In vivo effects of 20E on *SpEcR* and *SpVg* expression

Injection of 20E into female crabs at the early-vitellogenic stage induced significantly higher relative levels of both *SpEcR* and *SpVg* transcripts as compared with those of the control ($P<0.05$). In contrast, no significant difference of the expression level of *SpEcR* and *SpVg* was detected in crabs that had received injections of 20E at the previtellogenic stage ($P>0.05$; Fig. 7).

In vitro effects of 20E on *SpEcR* and *SpVg* expression

As shown in Fig. 8, the mRNA expression levels of both *SpEcR* and *SpVg* in ovarian explants concurrently increased with the increase of added 20E concentration from 0.05 to 5 μ M. Statistical analysis showed that when 20E was added at both 0.5 and 5 μ M, the mRNA expression levels of *SpEcR* and *SpVg* in the ovarian tissues were significantly higher than those for the crab saline and ethanol control (Fig. 8; $P<0.05$).

On the basis of the above results, 5 μ M 20E was subsequently used as the dose for a time course

experiment on the effects of 20E on ovarian explants at the previtellogenic stage and the early-vitellogenic stage. The results indicated that at all the four sampling times, expression levels of both *SpEcR* and *SpVg* in previtellogenic ovarian explants treated with 20E was very similar to those for the crab saline control (Fig. 9A and B). In contrast, for early-vitellogenic ovarian explants, the addition of 20E elevated the transcript levels of *SpEcR* at all the sampling points with significantly higher levels than those for the crab saline control at both 3 and 6 h ($P<0.05$). Similarly, the expression of *SpVg* increased with 20E treatment in early vitellogenic ovarian explants at all sampling times with significant differences detected at 1, 3, and 6 h ($P<0.05$; Fig. 9C and D).

Gene silencing with *SpEcR* dsRNA

To further confirm the roles of *SpEcR* during ovarian development of *S. paramamosain*, dsRNA was employed to target the *SpEcR* gene. The result indicated that even with the addition of 20E at the dose of 5 μ M, incubation of ovarian explants with *SpEcR* dsRNA led to a significant suppression of both *SpEcR* and *SpVg* expression when compared with the *Gfp*-treated control ($P<0.05$). On the other hand, ovarian explants incubated with 5 μ M 20E only again showed dramatically higher levels of *SpEcR* and *SpVg* expression than the controls ($P<0.05$; Fig. 10).

In order to determine whether *SpEcR* dsRNA treatment had a non-specific silencing effect or led to tissue lethality,

Figure 4

Expression profile of *SpEcR* transcripts at different stages of ovarian development in *S. paramamosain* as determined by qRT-PCR. Expression of the β -actin gene was used as a control. The relative abundances of *SpEcR* transcripts are shown as mean $\pm$ S.E.M. ($n=3$). Values with different letters above the bars are significantly different ($P<0.05$).

Figure 5
20E concentration in hemolymph during ovarian development of *S. paramamosain* detected by HPLC-MS. The 20E titers are shown as mean $\pm$ S.E.M. ($n=3$). Values with different letters above the bars are significantly different ($P<0.05$).

the expression level of the housekeeping gene, *SpGapdh*, was synchronously detected (Das & Durica 2013, Yang *et al.* 2013). It was found that the addition of *SpEcR* dsRNA did not have any significant effect on the expression of the *SpGapdh* gene in ovarian explants as compared with that of the *Gfp*-treated controls (Fig. 11), indicating that the silencing effect of *SpEcR* dsRNA treatment was not nonspecific or that the treatment did not lead to tissue lethality.

Discussion

In this study, three *SpEcR* isoforms were identified and sequenced from *S. paramamosain* and, as determined by sequence comparison, these *SpEcR* isoforms showed high level of similarity with EcR sequences of other arthropods reported previously. Moreover, the results of multiple alignments indicated that the DBD and LBD of

Figure 6
Localization of *SpEcR* mRNA by *in situ* hybridization in the ovaries of *S. paramamosain*. Arrows indicate the specific *SpEcR* mRNA signals with the antisense riboprobe in ovaries at the previtellogenic stage (A), the early-vitellogenic stage (D), and the late-vitellogenic stage (G).

Sense riboprobe was used as a negative control (B, E, and H). Corresponding normal histological sections (C, F, and I). Nu, nucleolus; FC, follicular cell; Oc, oocyte. The scale bars represent 50 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-14-0526>.

Figure 7

Effects of 20-hydroxyecdysone (20E) injection (0.2 µg/g body weight) on the expressions of *SpEcR* and *SpVg* in the ovary of *S. paramamosain*. Experimental crabs were sampled at 24 h post-injection. Expression of the β -actin gene was used as a control. The relative abundances of transcripts are shown as mean $\pm$ s.e.m. ($n=3$). Values with different letters above the bars are significantly different ($P<0.05$).

EcR proteins were highly conserved (Fig. 2), which is in agreement with the function domains for EcR (Bortolin *et al.* 2011). In insects, differences among EcR isoforms mainly exist in the A/B domain caused by alternative splicing, and their expressions are regulated by different promoters (Nakagawa & Henrich 2009, Watanabe *et al.* 2010). Unlike in insects, all three *SpEcR* isoforms from *S. paramamosain* had a same A/B domain, indicating that they were probably transcribed under the regulation of a same promoter.

The *SpEcR* isoforms of *S. paramamosain* were differentiated by one deletion site in the D domain and one substitution site in the LBD, which is similar to what has been reported for other crustaceans (Asazuma *et al.* 2007, Techa & Chung 2013). In fact, the results of recent research on the genomic organization of the EcR gene in the fiddler crab *U. pugilator* (*Up gDNA*) further verified the existence of the D domain and LBD isoforms (Durica *et al.* 2014). Interestingly, the LBD substitutive sequences of *SpEcR* from *S. paramamosain* were almost the same as the two alternative LBD exons of *Up gDNA*, and the D domain deletion sequence of *SpEcR* was also similar to its D domain alternative exon. These results indicated that the three isoforms of *SpEcR* might result from alternative splicing of a single-gene locus and multiple variant sites might be a characteristic of EcR isoforms in crustaceans (Durica *et al.* 2014). However, it was not clear whether these isoforms

had different functions and this warranted further investigation. In particular, future studies should attempt to localize and quantify the levels of different isoforms in different physiological processes to determine their roles individually.

While the *SpEcR* transcripts were detected in all 11 tissues examined, substantially higher expression levels were found in eyestalk, ovary, and epidermis. It is well known that as the molting hormones in crustaceans, ecdysteroids are heavily involved in the physiological control of molting (Styrishave *et al.* 2008). Chung *et al.* (1998b) have reported that the expression level of *UpEcR* increased in the hypodermis before molting, which is probably associated with the physiological changes occurring during the molting process. High levels of expression of *SpEcR* mRNA found in the epidermis indicated that ecdysteroids might act to stimulate the epidermis in the regulation of molting and development of the crab species through binding to the increased level of *SpEcR*. On the other hand, it is well documented that molt-inhibiting hormone (MIH) is secreted by the sinus gland located in the eyestalks to regulate molting in crustaceans (Lachaise *et al.* 1993). Thus, high levels of *SpEcR* expression in eyestalk implied that they might be involved in the feedback regulation of MIH secretion.

In crustaceans, the ecdysone signaling pathway is also known to involve in the regulation of female reproduction (Subramoniam 2000). During ovarian maturation of crustaceans, high levels of ecdysteroids are reportedly

Figure 8

Effect of 20E on the expressions of *SpEcR* and *SpVg* gene in the ovarian explants of *S. paramamosain*. The explants were sampled at 3 h post-20E addition. Expression of the β -actin gene was used as a control. The relative abundances of transcripts are shown as mean $\pm$ s.e.m. ($n=3$). Values with different letters above the bars are significantly different ($P<0.05$).

Figure 9

The temporal patterns of *SpEcR* and *SpVg* expression in ovarian explants of *S. paramamosain* at both previtellogenic (A and B) and early-vitellogenic (C and D) stages treated with 5 μ M 20E. The explants were sampled at 1, 3, 6, and 9 h post-20E addition. Expression of the β -actin gene was used as a

control. The relative abundances of transcripts are shown as mean $\pm$ S.E.M. ($n=3$). (A and C) The relative mRNA expression levels of *SpEcR*. (B and D) The relative mRNA expression levels of *SpVg*. Asterisks indicate significant differences compared with the control ($P<0.05$).

transported from the hemolymph into the ovary (Okumura *et al.* 1992, Tseng *et al.* 2002) and this can induce high levels of expression of *EcR*. Indeed, in the first report on *EcR* gene expression in reproductive tissues of crustaceans, Durica *et al.* (2002) also found that the *EcR* transcription differed during ovarian maturation in *U. pugilator* and indicated that the ovary was a potential target for hormonal control. Furthermore, in the swimming crab *Portunus trituberculatus*, a higher level of expression of *EcR* was reported in the ovaries of crabs after copulation as compared with those of immature ovaries (Mu *et al.* 2014). Therefore, higher *SpEcR* mRNA level detected in the ovary of *S. paramamosain* in this study probably indicated that they were involved in the regulation of ovarian development and reproduction. In fact, in the ovary of fruit fly *D. melanogaster*, *EcR* has been reported to regulate the transcripts of a set of genes, including ecdysone-induced protein 75B and 74, early in the ecdysone signal pathway and the expressions of these genes in turn regulated the development/degeneration of the immature eggs (Schwedes & Carney 2012). Ye *et al.* (2010) also reported that in *S. paramamosain*, the yolk protein accumulated in parallel with ovarian maturation. The concurrent upregulation of *SpEcR* transcripts in the ovary with ovarian development implied that they probably participated in the regulation of the process of yolk protein accumulation. However, there are also reports indicating that the expression level of *EcR* does not differ significantly during ovarian development in other crabs (Durica *et al.* 2014, Techa *et al.* 2014). These results may be caused by the possible species-specific mechanisms of regulation of *EcR* during ovarian development.

Similar to *SpEcR*, 20E concentration of *S. paramamosain* also showed a trend of increasing in parallel with ovarian development. This result appeared to confirm previous reports for other crustaceans and indicated that

ecdysteroids are involved in promoting development of the ovary (Subramoniam 2000, Tiu *et al.* 2010). However, concentrations of 20E reached their highest level at stage III and did not further increase at stage IV as in the case of *SpEcR* mRNA. In crustaceans, it is generally known that ecdysteroids are initially produced in the Y-organ, later they were hydroxylated to become active 20E, which plays important roles in gamete production and maturation by promoting vitellogenesis (Chang *et al.* 1976, Styris have *et al.* 2008). However, more recent research on the fruit fly *D. melanogaster* (Terashima & Bownes 2004) and the shore crab *Carcinus maenas* (Styris have *et al.* 2008) has indicated that in addition to Y-organ the ovary might be another site of production of ecdysteroids. Therefore, there were two possible explanations for the result that 20E did not further increase at stage IV. First, with the accumulation

Figure 10

Effects of *SpEcR* dsRNA and 20E on the relative transcript abundance of *SpEcR* and *SpVg* in ovarian explants of *S. paramamosain*. Expression of the β -actin gene was used as a control. The relative abundances of transcripts are shown as mean $\pm$ S.E.M. ($n=3$). Values with different letters above the bars are significantly different ($P<0.05$).

Figure 11

Effects of *SpEcR* dsRNA on the relative abundance of *SpGapdh* transcripts in ovarian explants of *S. paramamosain*. Expression of the β -actin was gene used as a control. The relative abundances of transcripts are shown as mean $\pm$ S.E.M. ($n=3$). Values with the same letter above the bars are not significantly different ($P>0.05$).

of the ecdysteroids at stage II and III, sufficient ecdysteroids had probably already been accumulated in the ovary for promoting subsequent ovarian development. The alternative explanation would be that the ovary of *S. paramamosain* might also produce ecdysteroids, which substituted for some of the 20E needed. Meanwhile, the significant decrease in the level of 20E at stage V when vitellogenesis was over could be a result of some ecdysteroids being transformed into inactive conjugates (Subramoniam 2000). These conjugates may release a variety of free ecdysteroids through enzymatic hydrolysis during early-embryonic development when the Y-organ is yet to be formed (Styrishave *et al.* 2008).

In this study, *SpEcR* mRNA was mainly localized in the follicular cells of the ovary and the spatial distribution of *SpEcR* changed dynamically during vitellogenesis. The distribution pattern identified by *in situ* hybridization indicated that during vitellogenesis, the *SpEcR* contained in the follicular cells distributed along the periphery of the ovarian lobules initially might be activated first by ecdysteroids. It has been reported that in crabs, the follicular cells transport nutrient reserves into oocytes when ovarian maturation begins (Yang *et al.* 2007). In *S. paramamosain*, the follicular cells reportedly moved inward and gradually surrounded the oocytes during early vitellogenesis to allow yolk granules and fat droplets in their cytoplasm to be transported into oocytes by pinocytotic activity during late-vitellogenic stages (Cheng *et al.* 2002). In other arthropods, it has also been

reported that ecdysteroids could induce the migration of the follicular cells (Adiyodi & Subramoniam 1983). Consequently, the pathway by which *SpEcR* promotes ovarian development in *S. paramamosain* probably involves regulation of the movement of the follicular cells and promotion of the transfer of nutrient reserves from the follicular cells into oocytes. In other vertebrates, the follicular cells have also been reported to secrete steroid hormones, such as estradiol and progesterone, to activate oocyte maturation via paracrine mechanisms and such a process is considered necessary for ovarian development of some vertebrates (Swanson *et al.* 1989, Lubzens *et al.* 2010). The spatiotemporal expression patterns of *SpEcR* found in the ovary of *S. paramamosain* implied that it might also be the case that the ecdysteroids of *S. paramamosain* regulated oocyte maturation by stimulating the paracrine action of the follicular cells.

Both *in vivo* and *in vitro* experiments with 20E further confirmed the roles of ecdysteroids and *SpEcR* in the regulation of ovary development in *S. paramamosain*. It was found that increased levels of 20E could lead to concurrent up-regulation of the expression of *SpEcR* and *SpVg* in the early-vitellogenic ovary. In studies of the mosquito *Aedes aegypti*, it was reported that several transcription-binding sites, including *EcR* and *USP*, exist in the 5' upstream promoter region of the *Vg* gene that are essential for responses to 20E (Martín *et al.* 2001, Raikhel *et al.* 2002). After *EcR* had bound with 20E, an *EcR* heterodimer was formed that combined with the 5' promoter region of the *Vg* gene to promote its transcription (Tiu *et al.* 2010). It is well known that the ovarian development in decapod crustaceans is characterized by the maturation of the ovary, with a gradual increase in its size as a result of uptake of the yolk protein precursor, *Vg*, of the final product vitellin (*Vn*) (Yano & Hoshino 2006, Tiu *et al.* 2009). Thus, the clear stimulating effects of 20E on *SpVg*, observed both *in vivo* and *in vitro*, indicated that *SpVg* might be an ecdysteroid-responsive gene whose expression could be promoted by 20E, and that the ecdysteroid signaling pathway was involved in ovarian development via regulation of the expression of *SpVg*. Similar results have also been reported for other crustaceans. For example, 20E has been found to stimulate the expression of *Vg* in ovarian explants of the tiger shrimp *P. monodon* and in hepatopancreas explants of the American clawed lobster *H. americanus* (Tiu *et al.* 2006, 2010). Interestingly, 20E was found to not significantly affect the levels of *SpEcR* and *SpVg* mRNA in the previtellogenic crabs, and a similar result was obtained from *in vitro* experiments. This might be explained by the fact that at

the previtellogenic stage had not yet begun, hence ovarian explants were insensitive to exogenous ecdysteroids.

Results of recent research have indicated that gene knockdown using dsRNA is a powerful tool for investigating gene functions in crustaceans (Das & Durica 2013, Yang *et al.* 2014). For instance, in the fiddler crab *U. pugilator*, the silencing of *EcR* and *Rxr* during early limb regeneration could lead to the blastema failing to develop and downregulation of ecdysteroid levels in the hemolymph (Das & Durica 2013). Similarly, the injection of *EcR*-dsRNA into white leg shrimp *Litopenaeus vannamei* decreased the expression of ecdysteroid signaling response genes (Qian *et al.* 2014). It has also been reported that in the shore crab *C. maenas*, the *in vitro* *Rxr*-dsRNA treatment of ovarian tissue led to significantly inhibited expression of both *Rxr* and *Vg* (Nagaraju *et al.* 2011). In this study, the results of silencing experiments clearly indicated that the addition of *EcR*-dsRNA to the ovarian explants downregulated the expression of both *SpEcR* and *SpVg*, which occurred even when exogenous 20E was concurrently added. This result provided evidence indicating that *SpVg* is a responsive gene of ecdysteroid signaling and that *SpEcR* is involved in promoting ovarian development in the mud crab *S. paramamosain*.

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.

Funding

This research was supported by the National Natural Science Foundation of China (nos 41476119 and 31472261), and the Fundamental Research Funds for the Central Universities (no. 2011121011).

Acknowledgements

The authors sincerely thank the anonymous reviewers for valuable comments on the manuscript.

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Received in final form 4 December 2014

Accepted 6 January 2015

Accepted Preprint published online 6 January 2015