Ecdysone receptor in the mud crab

Scylla paramamosain: a possible role in promoting ovarian development

Jie Gong1, Haihui Ye1,2, Yinjie Xie1, Yanan Yang1, Huiyang Huang1, Shaojing Li1 and Chaoshu Zeng2,3

1College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, China
2Collaborative Innovation Center for Development and Utilization of Marine Biological Resources, Xiamen 361102, China
3College of Marine and Environmental Sciences, James Cook University, Townsville, Queensland 4811, Australia

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.

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 & Hopkins 2002), the kuruma prawn Marsupe- naeus 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 Peneaus 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
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 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 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 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>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence (5’–3’)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcR F</td>
<td>TTYTTCCGKMQVTCVATCAC</td>
<td>Fragment amplification of EcR</td>
</tr>
<tr>
<td>EcR R</td>
<td>TCWGWTGHHGGYTCAWAST</td>
<td>Fragment amplification of EcR</td>
</tr>
<tr>
<td>EcR’ F</td>
<td>ACTCTCCGTTTCTGTGCAGAA</td>
<td>3’ amplification of Rrx</td>
</tr>
<tr>
<td>EcR’ R</td>
<td>GGTGGACACAGAAGGGAAGAG</td>
<td>5’ amplification of Rrx</td>
</tr>
<tr>
<td>YEcR F</td>
<td>AAGAACAAAGAATCCACCCATT</td>
<td>Real-time quantitative PCR for EcR</td>
</tr>
<tr>
<td>YEcR R</td>
<td>TCTCTCACCCATACCCGACAGG</td>
<td>Riboprobe amplification for EcR</td>
</tr>
<tr>
<td>TEcR F</td>
<td>TATGAGTTTTGGCTTGGGAG</td>
<td>Riboprobe amplification for EcR</td>
</tr>
<tr>
<td>TEcR R</td>
<td>GTGGGAGGCTTGTGGTTGAGTG</td>
<td>Riboprobe amplification for EcR</td>
</tr>
<tr>
<td>T7</td>
<td>TAATACGACTCATAAGGG</td>
<td>Full-length confirmation for EcR</td>
</tr>
<tr>
<td>EcR F2</td>
<td>ATTACGCGGGGCTCATCATCTC</td>
<td>Full-length confirmation for EcR</td>
</tr>
<tr>
<td>EcR R2</td>
<td>TTGTAAGGAGCGCATACACC</td>
<td>Full-length confirmation for Vg</td>
</tr>
<tr>
<td>Vg F</td>
<td>GAGTGTGATAGGAGGTCTCTTG</td>
<td>Real-time quantitative PCR for Vg</td>
</tr>
<tr>
<td>Vg R</td>
<td>GACCTGTAGGCTTCTGTGAGCA</td>
<td>Real-time quantitative PCR for Vg</td>
</tr>
<tr>
<td>Gapdh F</td>
<td>AATGCCCATACACAAAGAAAATC</td>
<td>Real-time quantitative PCR for Gapdh</td>
</tr>
<tr>
<td>Gapdh R</td>
<td>GGAACATACAAACACTACACACC</td>
<td>Real-time quantitative PCR for Gapdh</td>
</tr>
<tr>
<td>SecR F</td>
<td>CATAGACATGCTAGGATTACCC</td>
<td>Amplification for EcR dsRNA</td>
</tr>
<tr>
<td>SecR R</td>
<td>GTAATCCCTTTATCTTCTGCTCG</td>
<td>Amplification for EcR dsRNA</td>
</tr>
<tr>
<td>Gfp F</td>
<td>TGCGGCGGTAGATGCCGTGG</td>
<td>Amplification for GFP dsRNA</td>
</tr>
<tr>
<td>Gfp R</td>
<td>GGGTGCGGGTCACGCGAGAG</td>
<td>Amplification for GFP dsRNA</td>
</tr>
<tr>
<td>M13-47</td>
<td>GCACAGGTTTCCAGTCAGC</td>
<td>Colony PCR</td>
</tr>
<tr>
<td>RV-M</td>
<td>GACGCGGTAACAATTTCCACA</td>
<td>Colony PCR</td>
</tr>
<tr>
<td>β-actin F</td>
<td>GAGCCGAGAAATCTGTTGAGC</td>
<td>Internal control</td>
</tr>
<tr>
<td>β-actin R</td>
<td>GGAAGGAAGGCTGGAGAGAG</td>
<td>Internal control</td>
</tr>
</tbody>
</table>
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 μl reaction volume containing 10 μl of SYBR premix, 0.8 μl of each primer (10 μM), 2 μl of cDNA template, and 6.4 μl 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 μl 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×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 μm 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× 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 μg/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/μl) and then washed with 50% deionized formamide diluted to different concentrations of SSCT (with 0.1% Tween-20) solution (2× SSCT and 0.2× SSCT). The hybridized tissue sections were incubated with anti-DIG alkaline-phosphatase-conjugated antibody (Roche) and signals were visualized with the colorimetric substrates nitroblue tetrozolium/4-bromo-4-chloro-30-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 ± 5.1 mm, body weight: 377.9 ± 19.2 g) and another six at the previtellogenic stage (carapace width: 92.8 ± 3.8 mm, body weight: 130.8 ± 11.2 g) were easily divided into control and treatment groups. The crabs assigned to the treatment group received 100 μl 20E injection at 0.2 μg/g body weight through the arthrodial
membrane at the base of the last pereiopods, while control crabs received the same volume of carrier. The crabs were transferred to two concrete tanks (L×W×D=8×3×0.7 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 CaCl2, 26 mM MgCl2, 23 mM Na2SO4 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−ΔΔCt 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
isoforms indicated that there was an 81 bp alternative deletion between nucleotide positions 620 and 700 in the D domain of \textit{SpEcR2} while a 147 bp substitution between nucleotide positions 827 and 973 differentiated \textit{SpEcR1} and \textit{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, EcRF2 and EcRR2, 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 \textit{SpEcR} isoforms had a domain organization typical of a nuclear hormone receptor (Fig. 2).

### Tissue-specific expression and expression profiles of \textit{SpEcR} transcripts during ovarian development

As shown in Fig. 3, \textit{SpEcR} was found in all 11 tissues examined, i.e. muscle, heart, thoracic ganglion, hemolymph, brain, gill, stomach, hepatopancreas, eyestalk, ovary, and epidermis. However, the expression levels of \textit{SpEcR} gene were significantly higher in the eyestalk, ovary, and epidermis than in other tissues ($P < 0.05$). To test the correlation of \textit{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 \textit{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 \textit{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
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 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...
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 μM. Statistical analysis showed that when 20E was added at both 0.5 and 5 μM, the mRNA expression levels of SpEcR and SpVg in the ovarian tissues were significantly higher than those for the crab saline control (P<0.05; Fig. 7).

In order to determine whether SpEcR dsRNA treatment had a non-specific silencing effect or led to tissue lethality, 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 μM, 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 μM 20E only again showed dramatically higher levels of SpEcR and SpVg expression than the controls (P<0.05; Fig. 10).

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 μM. Statistical analysis showed that when 20E was added at both 0.5 and 5 μM, 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 μM 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).

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 μM. Statistical analysis showed that when 20E was added at both 0.5 and 5 μM, 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 μM 20E was subsequently used as the dose for a time course
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
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, Techà & 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 almost 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 involved in the feedback regulation of MIH secretion. Therefore, the ecdysteroids in the epidermis of S. paramamosain might play a similar role. More research is needed to confirm this hypothesis.
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, Techia 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, Styryshave et al. 2008). However, more recent research on the fruit fly D. melanogaster (Terashima & Bownes 2004) and the shore crab Carcinus maenas (Styryshave 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 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 µM 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 ± 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).

**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 ± S.E.M. (n = 3). Values with different letters above the bars are 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 (Martin 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

Figure 11
Effects of *SpEcR* dsRNA on the relative abundance of *SpGaphd* 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 ± S.E.M. (n=3). Values with the same letter above the bars are not significantly different (P>0.05).
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 41476619 and 31472261), and the Fundamental Research Funds for the Central Universities (no. 2011120111).

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

References


Chang ES, Sage BA & O’Connor JD 1976 The qualitative and quantitative determinations of ecdysones in tissues of the crab, Pachygrapsus crassispinis, following molt induction. General and Comparative Endocrinology 30 21–33. (doi:10.1016/0016-6480(76)90062-9)


Received in final form 4 December 2014
Accepted 6 January 2015
Accepted Preprint published online 6 January 2015