Turk. J. Fish.& Aquat. Sci. 19(7), 561-570
http://doi.org/10.4194/1303-2712-v19_7_03

RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Molecular Characterization of Estrogen-Related Receptor (ERR) of *Macrobrachium rosenbergii* and its Differential Expression during Ovary Development Stages and NP Exposures

Jin-lei Liu[1], Hua-pu Chen[1,2], Miao-xin Zhao[1], Si-ping Deng[1], Guang-li Li[1], Chun-hua Zhu[1,*], Yu-cong Hong[1,3]

[1] Zhanjiang City Key Laboratory of Marine Ecology and Environment, Guangdong Research Center on Reproductive Control and Breeding Technology of Indigenous Valuable Fish Species, Fisheries College, Guangdong Ocean University, Zhanjiang 524088, China.
[2] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266071, China.
[3] Guangdong Zhen Yuan Aquatic Industry Co., Jieyang, 522010, China.

Article History
Received 21 December 2017
Accepted 25 June 2018
First Online 11 July 2018

Corresponding Author
Tel.: +86.0759 2382417
E-mail: guangligdou@163.com

Keywords
Environmental endocrine-disrupting chemicals
Xenoestrogen
Orphan nuclear receptor
Estrogen receptor

Abstract

Nonylphenol (NP) is a xenoestrogen whose estrogenic effects are mediated by the estrogen receptor (ER) in vertebrates. However, the ER appears to be absent in arthropods. Estrogen-related receptor (ERR) may be a candidate that responds to NP. To investigate the effects and mechanisms of NP in giant freshwater prawn *Macrobrachium rosenbergii,* a unique homolog of mammalian *ERR* was cloned from *M. rosenbergii* by rapid amplification of cDNA ends. The full length ERR cDNA consisted of 1897 bp with a 1374 bp open reading frame encoding 457 amino acid residues. RT-PCR analysis revealed that *ERR* was widely expressed in various tissues of male and female *M. rosenbergii*, including the brain ganglion, hepatopancreas, heart, and gonads. Interestingly, expression of *ERR* in ovaries was higher than that in testes. qPCR results showed high expression of *ERR* in ovaries at early developmental stages. NP exposure up-regulated the expression levels of *ERR* in the ovaries of *M. rosenbergii* in a time-dependent manner. The current study provides fundamental data to understand the response of ERR to NP exposure in *M. rosenbergii*.

Introduction

Estrogen-related receptor (ERR) is a member of the nuclear receptor superfamily. It was first isolated from a human cDNA library by Giguère in 1988 using a low stringency cross-hybridization strategy based on DNA-binding domain (DBD) homology sequences with the estrogen receptor (ER) (Giguère, Yang, Segui, & Evans, 1988). In mammals, there are three ERRs that are closely related and share similar DBDs to the ER with 68% amino acid identity. However, ERRs and ER possess moderate similarity (36%) in the ligand-binding domain (LBD), which can explain why ERRs are not responsive to estrogen (Bonnelye, Merdad, Kung, & Aubin, 2001). It has been demonstrated that ERRs are constitutive transcriptional factors that activate expression of target genes via interactions with co-activators in the absence of exogenous ligands (Hong, Yang, & Stallcup, 1999; Schreiber, Knutti, Brogli, Uhlmann, & Kralli, 2003).

Although the functions of ERRs are not well understood, they likely play important roles in animal reproduction, growth, development, and metabolism because their expression in embryonic and adult tissues occurs in very restricted spatiotemporal patterns (Giguère, 2002). In mammals, ERRs are involved in ER-dependent pathways related to the formation of bone and cancer development. For

example, *ERRα* is highly expressed in bone formation zones and osteoblastic-like cells, and ERRα positively regulates osteopontin (Turner, Riggs, & Spelsberg, 1994; Bonnelye *et al.*, 1997). ERRs regulate estrogen-inducible gene *pS2*, a human breast cancer prognostic marker (Lu, Kiriyama, Lee, & Giguere, 2001). They also recognize estrogen response elements, suggesting that ERRs and ERs modulate identical target genes (Lu *et al.*, 2001). In addition to its role as a modulator in ER-dependent pathways, ERRα is related to energy metabolism in mammals (Luo *et al.*, 2003; Schreiber *et al.*, 2003; Carrier, Deblois, Champigny, Levy, & Giguère, 2004). In fish, ERRs might play an important role in developmental processes (Bardet *et al.*, 2004), and ERRα may be involved in sex differentiation and gonad development (Zhang *et al.*, 2008). In invertebrates, only one ERR has been discovered thus far (Sluder, Mathews, Hough, Yin, & Maina, 1999; Dehal *et al.*, 2002; Sullivan & Thummel, 2003; Thomson, Baldwin, Wang, Kwon, & LeBlanc, 2009). A study has shown that ERR may be related to male fertility in *Drosophila melanogaster* (Misra *et al.*, 2017). In crustaceans, a unique *ERR* gene has been found in the crab *Scylla paramamosain* (GenBank: GU290313.1) and *Portunus trituberculatus* (GenBank: KM189915.1), but reports on the function of ERR in crustaceans are rare.

Environmental endocrine-disrupting chemicals (EDCs) are exogenous compounds that interfere with the normal physiological processes of organisms. The EDC nonylphenol is widely distributed in aquatic environments, which is largely used in the production of detergent and resinous products (Kavlock *et al.*, 1996). In vertebrates, NP is considered to be a xenoestrogen that exerts or inhibits estrogenic effects in various organisms by mimicking and blocking the action of endogenous estrogen (Ashley, Moore, Stapleton, Velinsky, & Wilhelm, 2003). Similarly, NP exerts estrogenic effects and interferes with normal vitellogenesis in invertebrates (Hannas *et al.*, 2011). In mysid *Neomysis integer*, a low concentration of NP significantly increases the expression of vitellogenin (*VTG*) (Ghekiere, Verslycke, & Janssen, 2006). *VTG* expression in the hepatopancreas of the giant freshwater prawn *Macrobrachium rosenbergii* is also induced by NP in a dose-dependent manner (Ara & Damrongphol, 2014). In vertebrates, the estrogenic effects of NP are mediated by ER (Ashley *et al.*, 2003). However, in arthropods, ER appears to be absent (Thomson *et al.*, 2009). Therefore, it is unknown how NP exerts estrogenic effects in arthropods, and ERR may be a candidate to resolve this issue.

M. rosenbergii is an important commercial species as a food source and extensively cultured in southern and southeastern Asian countries as well as Northern Australia (Bonami & Widada, 2011). In recent years, there has been a limitation in its nurseries because of aquatic environmental deterioration. NP is one of the factors causing pollution in aquatic environments. Therefore, studies of the effects and mechanisms of NP in *M. rosenbergii* have become very important. In the current study, we cloned the *ERR* gene from *M. rosenbergii* and detected *ERR* expression levels in various tissues and ovary with or without NP exposure to provide fundamental data to understand the response of ERR to NP exposure in *M. rosenbergii*.

Materials and Methods

Experimental Animals and Tissue Collection

M. rosenbergii (10–20 g) were purchased from a local market in Zhanjiang, China. Seven tissues (brain ganglion, gonads, muscles, hepatopancreas, heart, eyestalks, and gills) from 6 males and 6 females were harvested and stored at -80°C for RT-PCR analysis and the ovaries was used to isolate the partial and full sequences of *ERR*. Ovaries at various developmental stages (0, I, II, and III) were collected in accordance with the external shape as described previously (Damrongphol, Eangchuan, & Poolsanguan, 1991) and then stored at -80°C for reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR) analyses or fixed in Bouin's solution to prepare tissue sections.

Total RNA Extraction and First-Strand cDNA Synthesis

Total RNAs from tissues stored at -80 °C were prepared using Trizol reagent (Invitrogen, USA). The concentration of total RNA was measured by a UV spectrophotometer (Nanodrop 2000c; Thermo, USA), and quality was checked by the $OD_{260/280}$ (1.8–2.0) and agarose gel electrophoresis (bands of 28S and 18S rRNAs were clear). One microgram of total RNA from each sample was used to synthesize first-strand cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan).

Cloning of Full Length cDNA

Two pairs of degenerate primers (Table 1), which were designed based on the conserved sequences of other species phylogenetically close to *M. rosenbergii*, were used to amplify a partial sequence of *ERR* through nested PCR. The amplification was performed in a 25 µl volume under the following conditions: 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 15 min. PCR products were detected by agarose gel electrophoresis, and the expected fragment was purified using a DNA Fragment purification kit (Dongsheng Biotech, China). The purified product was subcloned into the pMD™19-T Vector (Takara, Japan) for sequencing. The full-length sequence of *ERR* was obtained using the SMART™ rapid amplification of cDNA ends (RACE) cDNA Amplification Kit (Clontech, USA). First-strand cDNA for 5'- and 3'-RACE templates was synthesized by reverse transcription in accordance

Table 1 Primers used for ERR cloning in *Macrobrachium rosenbergii*

Purpose	Primer name	Sequence (5'-3')	Melt temperature (°C)	Product size (bp)
Partial ERR	F_1	GTGTCGATTTCCACAAGTGTC	58	1076
	R_1	CACKSAAMAATCASYAAMAGT		
	F_2	GTACAGACGGACGTCTGATTCTC	58	569
	R_2	CCATYTWATCMGTSMAGGMAA		
ERR 3'-RACE	F_3	CTTTGGGACTTGCCTACCGTTCTATG	55	835
	F_4	GTGTTGTTGAACGATTGGAACAG	55	709
ERR 5'-RACE	R_3	GACACTTGTGGAAATCGACAC	55	705
	R_4	GATGTTTCCCTGGATAGTTCT	55	620

with the manufacturer's protocols. Nested PCR was performed to isolate 5'- and 3'-end sequences using specific primers (Table 1) that were designed according to the desired partial ERR sequence. The reaction was carried out in a 25 µl volume under the following conditions: 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 15 min. RACE-PCR products were detected and sequenced.

Bioinformatic Analysis of *ERR*

Sequence analysis and homology searching were performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Three fragments were assembled by DNAMAN 4.0. The open reading frame (ORF) and deduced amino acid sequence were determined by ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The signal sequence was predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The molecular mass and isoelectric point were predicted by the Compute pI/Mw tool (http://cn.expasy.org/tools/pi_tool.html). Potential functional motifs of the protein sequence were predicted using the PROSITE database (http://prosite.expasy.org/). Representative ERR homologous sequences were acquired from GenBank, and multiple sequence alignments were performed by Clustal X. A phylogenic tree was constructed using the neighbor-joining method bootstrapped with 1,000 replications in MEGA5.0.

NP Treatments

Adult female prawns (10.5±1.4 g) were purchased from the local market in Zhanjiang, China. Before experiments, all prawns were reared in a cement pool filled with freshwater (24±2°C) for 1 week. The pool was aerated with a photoperiod of 12 h/12 h light/dark. During acclimatization, prawns were fed with compound feed for shrimp twice every day. Experiments were performed in 450 L tanks containing shelters. The corresponding volume of 10 mg/mL NP (diluted with acetone) was added to 300 L aerated freshwater to prepare the treatment solutions with concentrations of 5, 25, or 125 µg/L NP. The control group was not treated with NP. Female prawns (stage I) that were healthy and exhibited normal body coloration were randomly assigned to control, low concentration (5 µg/L), medium concentration (25 µg/L), and high concentration (125 µg/L) groups. Each group included duplicates with 20 prawns. The culture conditions were the same as those during acclimatization. One-fifth of the water volume was exchanged every 2 days while maintaining the NP concentration of each group. After exposure to NP for 2 weeks, the prawns were sacrificed on ice and the ovaries (stage II) from six prawns (three prawns from each duplicate) in each group were dissected, immediately frozen in liquid nitrogen, and then stored at -80°C for subsequent qPCR analysis. To investigate the effects of long term NP exposure on *M. rosenbergii*, the optimal NP concentration was chosen. Female prawns (stage I) that were healthy and exhibited normal body coloration were selected and randomly divided into control and treatment groups as duplicates with 50 prawns each. The experimental conditions were the same as described above. Ovaries from six prawns (three prawns from each duplicate) were collected after 0 (stage I), 6 (stage I), 12 (stage II), 18 (stage II), 24 (stage II), and 30 days(stage III) as the method described above, and stored at -80 °C for qPCR analysis.

Histological Observation

As previously described (Chen *et al.*, 2015), ovarian tissues fixed in Bouin's solution for 24 h were transferred to 70% ethanol and subsequently dehydrated and embedded in paraffin. The ovarian tissues were serially sectioned at 5 µm thicknesses and stained with hematoxylin and eosin. Ovarian stages were verified under a light microscope (Nikon IQ50).

RT-PCR Analysis of *ERR* Expression

ERR expression in the cerebral ganglion, gonads, muscles, hepatopancreas, heart, eyestalks, and gills of male and female prawns, and ovaries at various developmental stages were detected by RT-PCR. The reactions were performed on the Bio-Rad PCR System

(Bio-Rad, CA, USA) in a 25 µl volume including 12.5 µl of 2× Taq Mix (Dongsheng Biotech, China). 18S rRNA (see Table 2 for primers) as an internal reference gene was used to standardize the quantity of template cDNA. Reaction conditions were denaturation at 94°C for 3 min, followed by 27 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Equal amounts of cDNA and specific primers (Table 2) were used to detect expression patterns of *ERR* in the various tissues by RT-PCR. Reaction conditions were denaturation at 94°C for 3 min, followed by 36 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. RT-PCR products were detected by 1.5% agarose gel electrophoresis with ethidium bromide.

qPCR Analysis

ERR expression in ovaries at various developmental stages and under NP exposure was detected by qPCR performed on the Bio-Rad Realtime PCR System (Bio-Rad) using SYBR®Green Realtime PCR Master Mix (TOYOBO, Japan). *18S rRNA* was tested as a housekeeping gene. The specific primers for *ERR* and *18S rRNA* are listed in Table 2. The amplifications were carried out in a 20 µl reaction volume containing 10 µl of 2× SYBR® Green Realtime PCR Master Mix, 0.8 µl of each primer (10 µM), 2 µl cDNA template, and 6.4 µl nuclease-free water. The cycling conditions were as follows: denaturation at 94°C for 1 min, followed by 40 cycles of 94°C for 5 s, 55°C for 10 s, 72°C for 20 s, and 84°C for 10 s (fluorescence data collection), and then melting curve analysis to confirm amplification of a single product. Each sample was run in triplicate and negative controls without template were included at the same time. After amplification, fluorescence data were converted to threshold cycle values (Ct), and relative expression levels of *ERR* were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Relative expression levels are presented as means±standard error of the mean (SEM). Statistical differences were determined by one-way analysis of variance followed by Duncan's multiple range test using SPSS 13.0. A value of $p<0.05$ was chosen to indicate significant differences.

Results

Cloning and Characterization of ERR cDNA

A 772 bp fragment that had the highest similarity to *ERR* of *P. trituberculatus* (91%) and *S. paramamosain* (88%) was obtained by using degenerate primers. The full length *ERR* consisted of 1897 bp including a 143 bp 5'-untranslated region (UTR), 1374 bp ORF, and 380 bp 3'-UTR with a poly(A) tail (Figure 1), which was submitted to the GenBank database (No. KU899089). The ORF encoded 457 amino acid residues with a predicted molecular mass of 50.75 kDa and isoelectric point of 8.08. SignalP analysis indicated that the putative protein did not contain a signal peptide. Prediction of potential functional motifs showed that the deduced peptide included two N-glycosylation sites, 10 tyrosine kinase II phosphorylation sites, three N-myristoylation sites, and six protein kinase C phosphorylation sites. In addition, the DBD of nuclear receptors was found at 124–199 amino acid residues (Figure 1).

Sequence Alignment and Phylogenetic Analysis

Sequence alignment showed that the deduced amino acid sequence shared high homology with ERR amino acid sequences of other species, as published previously, including both invertebrates and vertebrates. There was a high degree of conservation with other ERR amino acid sequences in the DBD. Based on the results of multiple sequence alignments, a phylogenetic tree was constructed (Figure 2). ERR of *M. rosenbergii* was phylogenetically closest to those of *S. paramamosain* and *P. trituberculatus*.

Tissue Distribution of *ERR* Transcripts

RT-PCR results showed that *ERR* was widely expressed in the brain ganglion, hepatopancreas, heart, ovaries, and testes, and highly expressed in the heart and ovaries (Figure 3). In gills, *ERR* expression levels of male prawns were weaker than those of female prawns (Figure 3). However, the expression levels of *ERR* in ovaries were obviously higher than those in testes (Figure 3).

Table 2. Primers used in RT-PCR and qPCR

Gene	Primer name	Sequence (5'-3')	Melt temperature (°C)	Product length (bp)
ERR	F_5	AGAACTATCCAGGGAAACATC	60	138
	R_5	ACTCCTTCCTTCAACATGCC		
18S	F_6	TGTTACGGGTGACGA	60	161
	R_6	AATTACGCAGACTCGGAAGA		

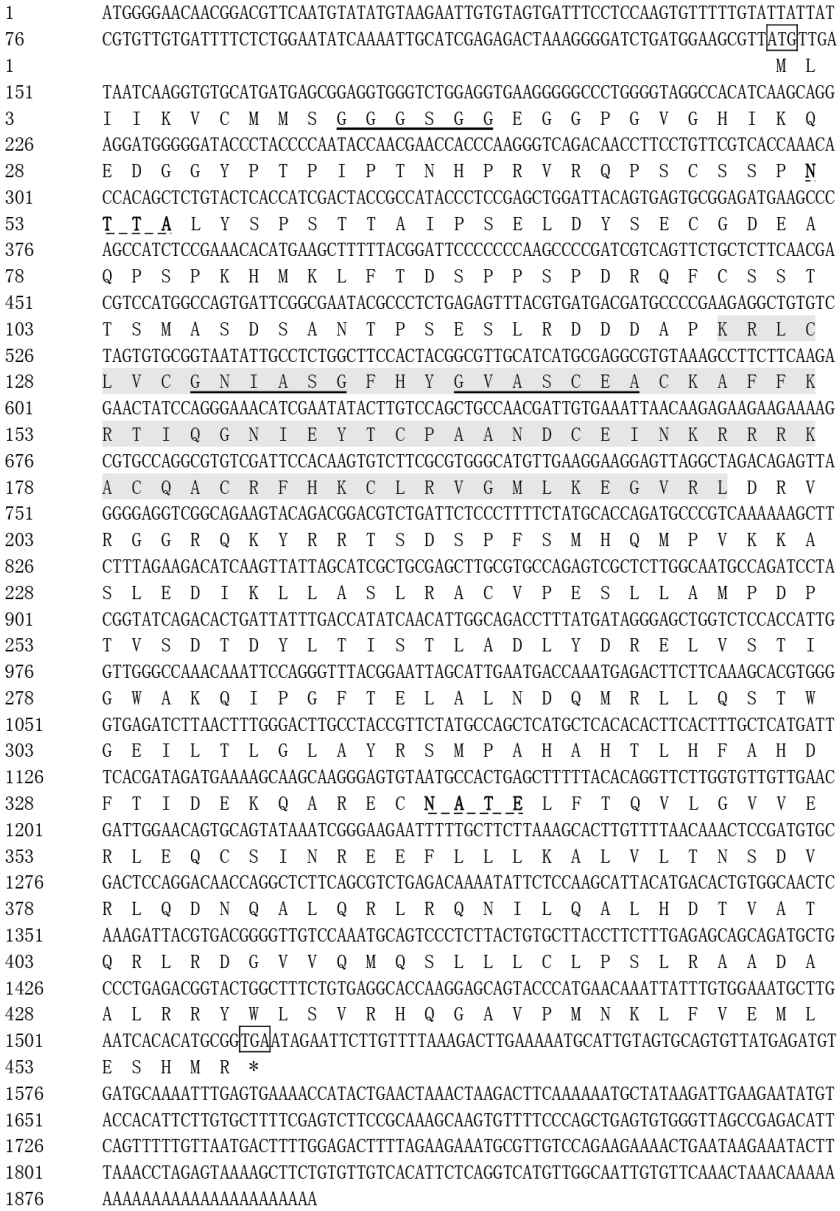

Figure 1. Nucleotide and deduced amino acid sequences of ERR in *Macrobrachium rosenbergii.*
The sequence was 1897 bp in length and encoded a protein of 457 amino acid residues. The initiating codon (ATG) and stop codon (TAA) are indicated by rectangular boxes. One nuclear hormone receptor DNA-binding domain (DBD) is highlighted by grey. Two N-glycosylation sites are indicated by a broken underline and bold. Three N-myristoylation sites are underlined.

Differentiation of Ovaries at Various Development Stages

As observed from the external shape, ovaries at stage 0 were small transparent messes with little pigmental deposition (Figure 4A). Ovaries at stage I were relatively larger than those at stage 0 and exhibited a certain appearance of milk white covered with dark pigment on the surface (Figure 4B). Ovaries at stage II increased rapidly in size and became a faint orange color (Figure 4C), whereas ovaries at stage III reached the maximum status with a bright yellow color (Figure 4D). Histological observations revealed that the ovaries at each stage contained oocytes at various developmental stages. There was a thick ovarian wall and large number of oogonia that were closely arranged in the ovaries at stage 0 (Figure 4E). In ovaries at stage I, the oocytes were increased in size and loosely arranged, and the boundaries between oocytes became clear (Figure 4F). Oocytes in the ovaries at stage II were lager than those in ovaries at stage I and contained yolk granules (Figure 4G). Ovaries at stage III were mature, and the oocyte shape was irregular to expand the oocyte volume (Figure 4H).

Expression of *ERR* in the Ovaries at Various Developmental Stages

RT-PCR results showed high expression of *ERR* at each stage (Figure 5A). qPCR results showed that *ERR*

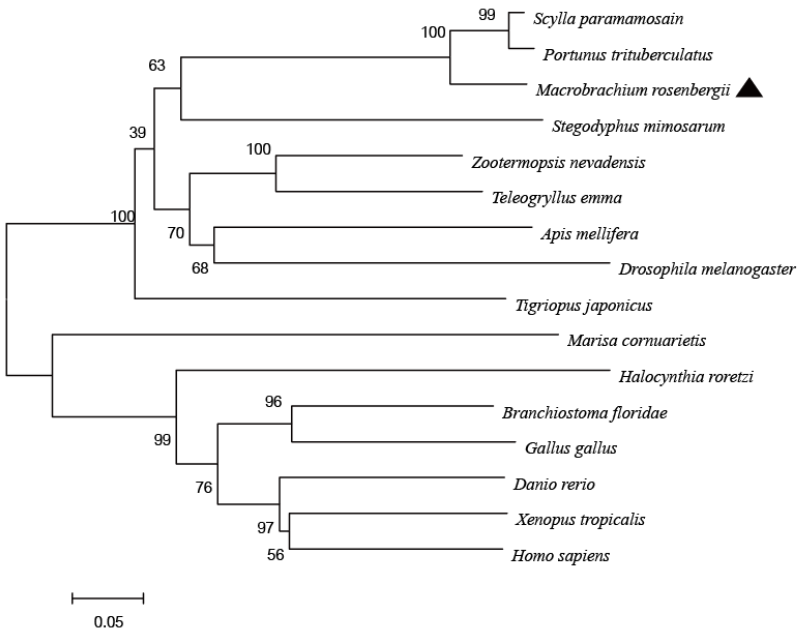

Figure 2. Phylogenetic tree constructed by the neighbor-joining method using MEGA5.0 software.
ERR amino acid sequences of other species were obtained from the GenBank database: *Scylla paramamosain ERR* (ADB43256.1), *Portunus trituberculatus ERR* (AIS76179.1), *Stegodyphus mimosarum ERR* (KFM71034.1), *Zootermopsis nevadensis ERR* (KDR06670.1), *Teleogryllus emma ERR* (FJ770332.1), *Apis mellifera ERR* (NP_001155988.1), *Drosophila melanogaster ERR* (NP_729340.1), *Tigriopus japonicus ERR* (AID52852.1), *Marisa cornuarietis ERR* (DQ923065.1), *Halocynthia roretzi ERR* (ABO42263.1), *Branchiostoma floridae ERR* (AAU88063.1), *Gallus gallus ERR* (NP_001007082.1), *Danio rerio ERR* (NP_998120.1), *Xenopus tropicalis ERR* (NP_001072756.1), and *Homo sapiens ERR* (NP_004442). Numbers at nodes indicate the bootstrap value as percentages obtained from 1000 replicates.

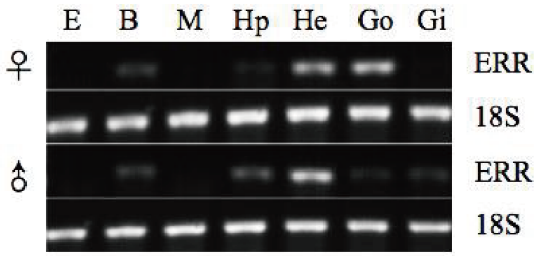

Figure 3. Tissue distribution of *ERR* transcripts in *Macrobrachium rosenbergii* determined by RT-PCR analysis.
18S rRNA was adopted as the internal reference gene. E, eyestalk; B, brain ganglion; M, muscle; Hp, hepatopancreas; He, heart; Go, gonad; Gi, gills.

was highly expressed in ovaries at stages 0 and I, and the highest expression levels appeared in ovaries at stage I (Figure 5B). However, the expression levels of *ERR* in ovaries at stages II and III, which showed no significant difference, were significantly lower than those in ovaries at stages 0 and I ($p<0.01$) (Figure 5B).

Expression Profiles of *ERR* in Ovaries After NP Exposure

The various concentrations of NP increased ovarian *ERR* expression (Figure 6). The low concentration of NP (5 µg/L) significantly increased *ERR* expression compared with the control group ($p<0.05$), and expression levels in medium (25 µg/L) and high (125 µg/L) concentration groups were significantly higher than that in the control group ($p<0.01$). However, there was no significant difference between medium and high concentration groups ($p>0.05$). To investigate correlations between *ERR* expression in ovaries and NP treatment time, the medium concentration (25 µg/L) was chosen as the optimal concentration. qPCR results showed that *ERR* was expressed in ovaries in a time-dependent manner after NP exposure for 30 days (Figure 7). The expression level of *ERR* was significantly increased day 6 ($p<0.05$), significantly increased from day 12, reached its peak at day 18, and then started to decrease with the

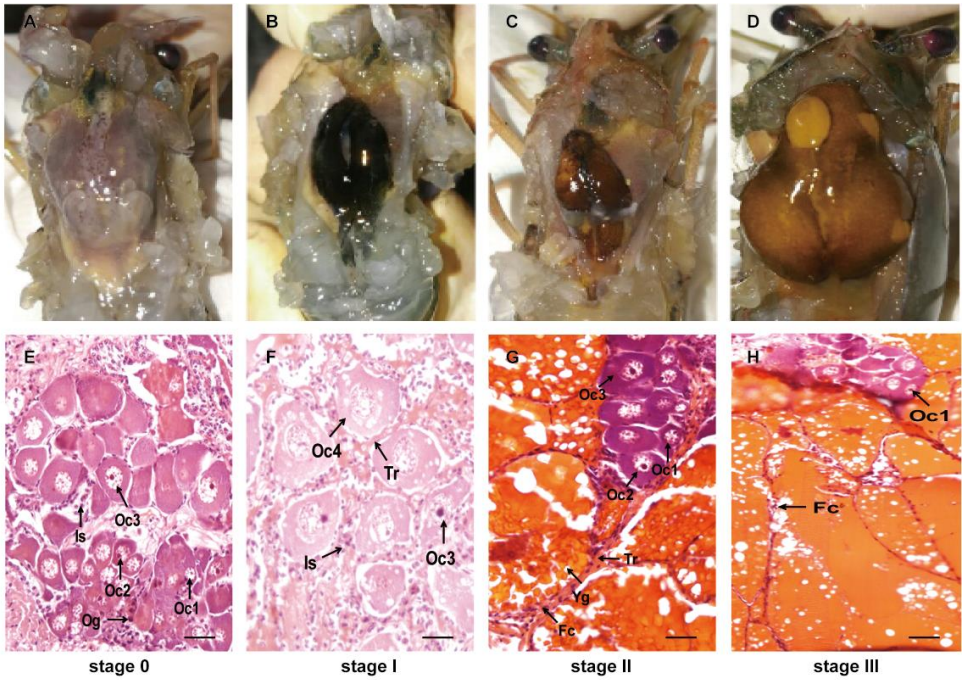

Figure 4. Outer profile and histological observation of ovaries at various developmental stages.
(A, E) Outer profile and histological observation of the ovary at stage 0. (B, F) Outer profile and histological observation of the ovary at stage I. (C, G) Outer profile and histological observation of the ovary at stage II. (D, H) Outer profile and histological observation of the ovary at stage III. Og: oogonia; Oc1: early oocyte of previtellogenesis; Oc2: later oocyte of previtellogenesis; Oc3: early oocyte of vitellogenesis; Oc4: later oocyte of vitellogenesis; Is: interlobular septum. Yg: yolk granule; Fc: follicular cell. Scale bar = 20 µm.

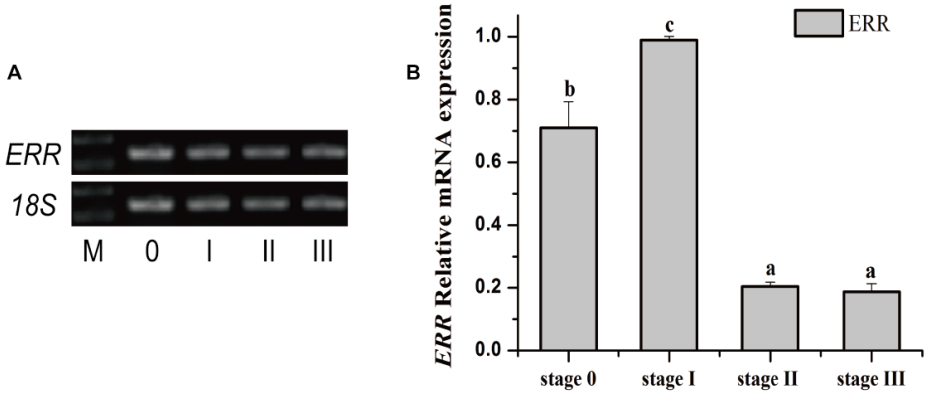

Figure 5. *ERR* expression in the ovaries at various developmental stages.
(A) Expression patterns detected by RT-PCR. *18S rRNA* was adopted as the internal reference gene. M: Marker, 0: stage 0; I: stage I, II: stage II; III: stage III. (B) Relative expression profiles verified by qPCR. Data are shown as the mean ± SEM. Values with different letters (a–c) are significantly different ($p<0.05$) (n= 6).

extension of treatment time. However, the expression levels at days 24 and 30 were still significantly higher than those of the control groups.

Discussion

Although a unique ERR has been respectively isolated from two Decapod crustaceans, *S. paramamosain* and *P. trituberculatus*, detailed studies with respect to its role have not been reported so far. As in the many species of invertebrates (Dehal *et al.*, 2002; Park & Kwak, 2010; Misra *et al.*, 2017), in the current study, we obtained a unique full length ERR cDNA of 1897 bp from *M. rosenbergii*, which contained a 1374 bp ORF encoding 457 amino acid residues. The blast search of the deduced amino acid sequence in the GenBank database showed that ERR of *M. rosenbergii* included two conserved regions of nuclear receptors, DBD and LBD, and had a high degree of similarity with that of *P. trituberculatus* and *S. paramamosain.* Signal peptides was not found by searching for signal sequences, indicating that the ERR was a non-secreted

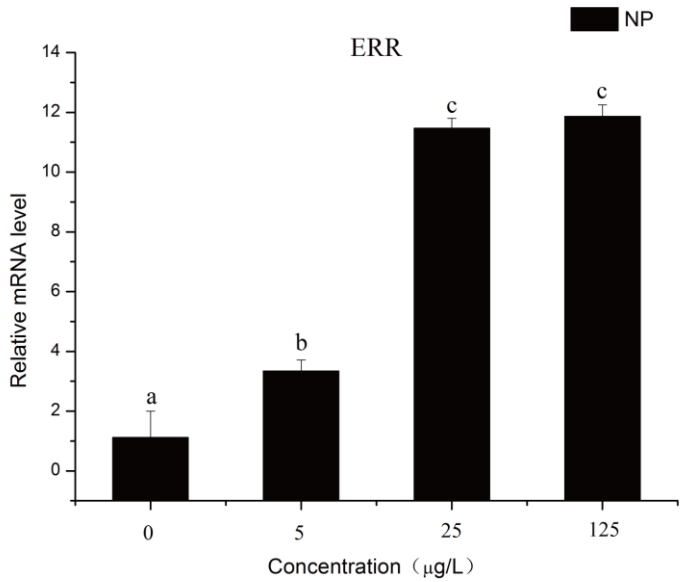

Figure 6. Effects of various concentrations of NP on ERR expression levels in female *M. rosenbergii.*
Data are shown as the mean±SEM. Values with different letters (a–c) are significantly different (p<0.05) (n= 6).

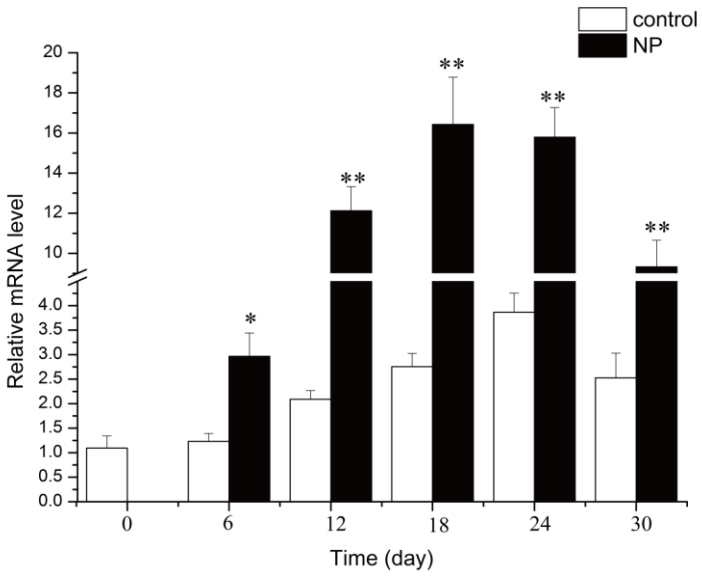

Figure 7. Temporal effects of NP on ERR expression levels in female *M. rosenbergii.*
Values are means±SEM. *p<0.05, **p<0.01 (n= 6).

protein, which is consistent with the characteristic of nuclear receptors. Analysis of potential functional motifs showed that the putative protein contained many protein kinase C and tyrosine kinase phosphorylation sites, revealing that the ERR likely plays a role by phosphorylation and dephosphorylation.

Alignments of deduced ERR amino acid sequences from invertebrates and vertebrates showed a high degree of sequence identity in the typical DBD with cysteine residues comprising the Zinc finger, revealing that ERR might share identical target genes in vertebrates and invertebrates. However, low conservation was observed in the LBD, implying that ERR might recognize different ligands in different species. It is generally accepted that there are three ERR isoforms in mammals. However, at least three ERR isoforms have been found in fish such as zebrafish (*Danio rerio*) (Bardet *et al.*, 2004). A unique *ERR* has been cloned from invertebrates so far, which is consistent with our results. Analysis of the phylogenetic

tree confirmed that ERRs of crustaceans were clustered and had close phylogenetic relationships with the ERRs of other invertebrates, indicating that all isoforms of ERR might originate from an ancestral ERR. These results also support the hypothesis: a single *ERR* gene underwent at least two waves of duplication during the invertebrate to vertebrate transition (Bardet *et al.*, 2004).

RT-PCR results showed a wide distribution of *ERR* transcripts in various tissues of male and female prawns, such as the brain ganglion, hepatopancreas, heart, and gonads, but it was specifically expressed in the gills of male prawns, which suggested that ERR might have multifunctionality in *M. rosenbergii*. In mammals, ERRs are involved in the actions of the estrogen signaling pathway and related to energy metabolism (Giguére, 2002; Schreiber *et al.*, 2003). In present study, a high expression level was observed in the hearts of both male and female prawns, implying that ERR of *M. rosenbergii* likely plays role in energy metabolism processes similar to the function of ERRα in mammals. In addition, we observed that expression of *ERR* in the male hepatopancreas was slightly higher than that in the female hepatopancreas, which also supports the aforementioned hypothesis, because the hepatopancreas is mainly a digestive organ and the growth of male *M. rosenbergii* is predominantly faster than that of females. Interestingly, *ERR* expression in the ovaries of *M. rosenbergii* was obviously higher than that in the testes. This result was similar to the expression patterns of *ERRα* in Japanese Medaka (Zhang *et al.*, 2008), indicating that ERR might play an important role in ovary development of *M. rosenbergii*.

According to the previous description (Damrongphol *et al.*, 1991), the Ovaries at different development stages were classified by observing the the external shape and histological structure. RT-PCR and qPCR were performed to investigate expression patterns of *ERR* in ovaries at various developmental stages. The results showed that the expression of *ERR* in ovaries at each stage was different, with the highest expression in ovaries at stage I. Prawns whose ovaries were at stage I were chosen to detect the expression of *ERR* under NP exposure. In the current study, all exposure concentrations of NP significantly increased the expression levels of *ERR* in ovaries of *M. rosenbergii*. This result was similar to the expression patterns of *ERR* in *Chironomus riparius* (Park & Kwak, 2010). In vertebrates, NP exerts estrogenic effects by mimicking the action of endogenous estrogen (Ashley *et al.*, 2003). However, ERs that are present in vertebrates appear to be absent in arthropods (Thomson *et al.*, 2009). In addition, studies suggest that ERs and ERRs share overlapping recognizable sequences (Lu *et al.*, 2001), and transcriptional activation of ERRs is inhibited by the synthetic estrogen diethylstilbestrol in vertebrates (Coward, Lee, Hull, & Lehmann, 2001; Tremblay *et al.*, 2001). These results suggest that ERR may be directly responsible for NP effects in arthropods. *ERR* was expressed in a time-dependent manner under NP exposure, and its highest expression occurred at day 18, inferring that there are other responsible pathways that lagged behind the response of ERR to NP in *M. rosenbergii*. VTG of oviparous organisms is sensitive to environmental estrogens and regarded as an environmental indicator to detect environmental pollution by estrogens or estrogenic compounds (Wang *et al.*, 2015). VTG of oviparous organisms is sensitive to environmental estrogens and regarded as an environmental indicator to detect environmental pollution by estrogens or estrogenic compounds (Wang *et al.*, 2015). In the study of Ara and Damrongphol (Ara & Damrongphol, 2014), the relative expression level of *VTG* approximately increased 3-fold after 50 ug/L NP exposure for 4 days. In our study, however, the relative expression level of *ERR* increased 3 fold after 25 ug/L NP exposure for 6 days, suggesting that the sensitivity of ERR to NP might be better than that of VTG and ERR might be an more excellent environmental indicator.

Acknowledgements

We thank the Guangdong Province Marine Fishery Science and Technology Promotion 305 Project (Nos. B201500B02, A201608B01 and 2017A0012), Key research and development 306 plan of Hainan Province (ZDYF2018225), and Laboratory for Marine Fisheries Science and 307 Food Production Processes, Qingdao National Laboratory for Marine Science and Technology 308 (No. 2016LMFS-B12).

References

Ara, F., & Damrongphol, P. (2014). Vitellogenin gene expression at different ovarian stages in the giant freshwater prawn, *Macrobrachium rosenbergii*, and stimulation by 4-nonylphenol. *Aquaculture Research*, 45, 320-326. https://dx.doi.org/10.1111/j.1365-2109.2012.03229.x

Ashley, J., Moore, A., Stapleton, H., Velinsky, D., & Wilhelm, M. (2003). Sedimentary nonylphenol contamination in an urbanized, industrialized segment of the Delaware River estuary, USA. *Bulletin of Environmental contamination and Toxicology*, 70, 0978-0984. https://dx.doi.org/10.1007/s00128-003-0078-1

Bardet, P.L., Obrecht-Pflumio, S., Thisse, C., Laudet, V., Thisse, B., & Vanacker, J.M. (2004). Cloning and developmental expression of five *estrogen-receptor related genes* in the zebrafish. *Development genes and evolution*, 214, 240-249. https://dx.doi.org/10.1007/s00427-004-0404-6

Bonami, J.R., & Widada, J.S. (2011). Viral diseases of the giant freshwater prawn *Macrobrachium rosenbergii*: a review. *Journal of invertebrate pathology*, 106, 131-142. https://dx.doi.org/10.1016/j.jip.2010.09.007

Bonnelye, E., Vanacker, J.M., Dittmar, T., Begue, A., Desbiens, X., Denhardt, D.T., … Fournier, B. (1997). The ERR-1 orphan receptor is a transcriptional activator expressed during bone development. *Molecular Endocrinology*,

11, 905-916. https://dx.doi.org/10.1210/mend.11.7.9948

Bonnelye, E., Merdad, L., Kung, V., & Aubin, J. (2001). The orphan nuclear estrogen receptor–related receptor α (Errα) is expressed throughout osteoblast differentiation and regulates bone formation in vitro. *The Journal of cell biology*, 153, 971-984. https://dx.doi.org/10.1083/jcb.153.5.971

Carrier, J.C., Deblois, G., Champigny, C., Levy, E., & Giguère, V. (2004). Estrogen-related receptor α (ERRα) is a transcriptional regulator of apolipoprotein A-IV and controls lipid handling in the intestine. *Journal of Biological Chemistry*, 279, 52052-52058. https://dx.doi.org/10.1074/jbc.M410337200

Chen, H.P., Li, S.S., Xiao, L., Zhang, Y., Li, G.L., Liu, X.C., & Lin, H.R. (2015). Wnt4 in protogynous hermaphroditic orange-spotted grouper (Epinephelus coioides): Identification and expression. *Comparative Biochemistry and Physiology, Part B,* 183, 67-74. https://dx.doi.org/10.1016/j.cbpb.2015.01.002

Coward, P., Lee, D., Hull, M.V., & Lehmann, J.M. (2001). 4-Hydroxytamoxifen binds to and deactivates the estrogen-related receptor γ. *Proceedings of the National Academy of Sciences*, 98, 8880-8884. https://dx.doi.org/10.1073/pnas.151244398

Damrongphol, P., Eangchuan, N., & Poolsanguan, B. (1991). Spawning cycle and oocyte maturation in laboratory-maintained giant freshwater prawns (*Macrobrachium rosenbergii*). *Aquaculture*, 95, 347-357. https://dx.doi.org/10.1016/0044-8486(91)90099-S

Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., Tomaso, A.D., ... Rokhsar D.s. (2002). The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science*, 298, 2157-2167. https://dx.doi.org/10.1126/science.1080049

Ghekiere, A., Verslycke, T., & Janssen, C. (2006). Effects of methoprene, nonylphenol, and estrone on the vitellogenesis of the mysid *Neomysis integer*. *General and comparative endocrinology*, 147, 190-195. https://dx.doi.org/10.1016/j.ygcen.2005.12.021

Giguère, V., Yang, N., Segui, P., & Evans, R.M. (1988). Identification of a new class of steroid hormone receptors. *Nature*, 331, 91-94. https://dx.doi.org/10.1038/331091a0

Giguère, V. (2002). To ERR in the estrogen pathway. *Trends in Endocrinology & Metabolism*, 13, 220-225. https://dx.doi.org/10.1016/S1043-2760(02)00592-1

Hannas, B.R., Wang, Y.H., Thomson, S., Kwon, G., Li, H., & LeBlanc, G.A. (2011). Regulation and dysregulation of vitellogenin mRNA accumulation in daphnids (*Daphnia magna*). *Aquatic toxicology*, 101, 351-357. https://dx.doi.org/10.1016/j.aquatox.2010.11.006

Hong, H., Yang, L., & Stallcup, M.R. (1999). Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. *Journal of Biological Chemistry*, 274, 22618-22626. https://dx.doi.org/10.1074/jbc.274.32.22618

Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., …Tilson, H.A. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the US EPA-sponsored workshop. *Environmental health perspectives*, 104, 715-740. https://dx.doi.org/10.1289/ehp.96104s4715

Lu, D., Kiriyama, Y., Lee, K.Y., & Giguere, V. (2001). Transcriptional regulation of the estrogen-inducible pS2 breast cancer marker gene by the ERR family of orphan nuclear receptors. *Cancer research*, 61, 6755-6761. http: // cancerres.aacrjournals.org

Luo, J., Sladek, R., Carrier, J., Bader, J.A., Richard, D., & Giguère, V. (2003). Reduced fat mass in mice lacking orphan nuclear receptor estrogen-related receptor α. *Molecular and cellular biology*, 23, 7947-7956. https://dx.doi.org/10.1128/MCB.23.22.7947-7956.2003

Misra, S., Pandey, A.K., Gupta, S., Kumar, A., Khanna, P., & Ram, K.R. (2017). Estrogen related receptor is required for the testicular development and for the normal sperm axoneme/mitochondrial derivatives in *Drosophila* males. *Scientific reports*, 7, 40372. https://dx.doi.org/10.1038/srep40372

Park, K., & Kwak, I.S. (2010). Molecular effects of endocrine-disrupting chemicals on the *Chironomus riparius* estrogen-related receptor gene. *Chemosphere*, 79, 934-941. https://dx.doi.org/10.1016/j.chemosphere.2010.03.002

Schreiber, S.N., Knutti, D., Brogli, K., Uhlmann, T., & Kralli, A. (2003). The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor α (ERRα). *Journal of Biological Chemistry*, 278, 9013-9018. https://dx.doi.org/10.1074/jbc.M212923200

Sluder, A.E., Mathews, S.W., Hough, D., Yin, V.P., & Maina, C.V. (1999). The nuclear receptor superfamily has undergone extensive proliferation and diversification in nematodes. *Genome Research*, 9, 103-120. https://dx.doi.org/10.1101/gr.9.2.103

Sullivan, A.A., & Thummel, C.S. (2003). Temporal profiles of nuclear receptor gene expression reveal coordinate transcriptional responses during *Drosophila* development. *Molecular Endocrinology*, 17, 2125-2137. https://dx.doi.org/10.1210/me.2002-0430

Thomson, S.A., Baldwin, W.S., Wang, Y.H., Kwon, G., & LeBlanc, G.A. (2009). Annotation, phylogenetics, and expression of the nuclear receptors in *Daphnia pulex*. *BMC genomics*, 10, 500-514. https://dx.doi.org/10.1186/1471-2164-10-500

Tremblay, G.B., Kunath, T., Bergeron, D., Lapointe, L., Champigny, C., Bader, J.A., … Giguère, V. (2001). Diethylstilbestrol regulates trophoblast stem cell differentiation as a ligand of orphan nuclear receptor ERRβ. *Genes & development*, 15, 833-838. https://dx.doi.org/10.1101/gad.873401

Turner, R.T., Riggs, B.L., & Spelsberg, T.C. (1994). Skeletal effects of estrogen. *Endocrine reviews*, 15, 275-300. https://dx.doi.org/10.1210/edrv-15-3-275

Wang, J., Shan, R., Zhang, X., Tian, H., Wang, W., & Ru, S. (2015). Development of a lipovitellin-based sandwich ELISA for quantification of vitellogenin in surface mucus and plasma of goldfish (*Carassius auratus*). *Ecotoxicology & Environmental Safety*, 120, 80-87. https://dx.doi.org/10.1016/j.ecoenv.2015.05.010

Zhang, Z., Hu, J., Sai, S., Zhao, Y., Huang, C., & Tian, X. (2008). Gene cloning, sequence analysis and tissue expression of estrogen-related receptor alpha (Erralpha) in Japanese medaka and its transcriptional responses after differential EDCs exposure. *Huan jing ke xue*, 29, 3153-3158. https://dx.doi.org/10.13227/j.hjkx.2008.11.006