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RESEARCH ARTICLE

# Characterization, expression and function of the pyrokinins (PKs) in the giant freshwater prawn, *Macrobrachium rosenbergii*

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## ABSTRACT

Pyrokinins (PKs) are neuropeptides that have been found to regulate a variety of physiological activities including reproduction in various insect and crustacean species. However, the reproductive roles of PKs in the giant freshwater prawn, *Macrobrachium rosenbergii*, have not yet been investigated. In this study, we identified the *MroPK* gene from next-generation sequence resources, which encodes a MroPK precursor that shares a high degree of conservation with the C-terminal sequence of FxPRLamide in other arthropods. *MroPK* is expressed within most tissues, except the hepatopancreas, stomach and gill. Within developing ovarian tissue, *MroPK* expression was found to be significantly higher during the early stages (stages 1–2) compared with the late stages (stages 3–4), and could be localized to the oogonia, previtellogenic and early vitellogenic oocytes. A role for PK in *M. rosenbergii* reproduction was supported following experimental administration of MroPK to ovarian explant cultures, which led to an increase in the production of progesterone and estradiol and upregulation of expression of steroidogenesis-related genes (*3β-HSD* and *17β-HSD*) and *vitellogenin* (*Vg*). Together, these results support a role for MroPK in regulating ovarian maturation via steroidogenesis.

**KEY WORDS: FxPRLamide, Steroidogenesis, Ovarian maturation, Reproduction**

## INTRODUCTION

The giant freshwater prawn, *Macrobrachium rosenbergii*, is highly valued as a food source in the tropical countries of Asia, for both domestic consumption and export (Hossain and Das, 2010; New and Nair, 2012). The popularity of this prawn has grown rapidly and the demand for it is getting progressively greater. Prawn aquaculture has become increasingly difficult as a result of widespread diseases and continued broodstock reproductive dysfunction in captivity leading to poor gonad maturation and spawning (Kautsky et al., 2000; Kiran et al., 2002; Brady et al., 2013). It is essential to find a new way to enhance the control of reproduction, particularly ovary maturation of the broodstock, to address the high consumption demands and the development of crustacean aquaculture. Ovarian development of *M. rosenbergii* can be characterized into four different stages based on the size, color and histology of the ovary. Oocyte (Oc) growth also gradually increases during ovarian development stages 1 to 4. Stage 1 (spent; a period after spawning) and stage 2 (proliferative; a period of regeneration of gamete cells to begin a new cycle) show mainly oogonia (Og) and previtellogenic oocytes (Oc1 and Oc2), which correspond to the early development of the ovary. Stage 3 (premature; a period of rapid gametogenesis) and stage 4 (mature; a period of fully mature gametes) display prominent vitellogenic oocytes (Oc3 and Oc4), which accumulate vitellin granules and demonstrate the mature stage of the ovary (Meeratana and Sobhon, 2007).

The practice of eyestalk ablation, which removes gonad-inhibiting hormones, has been widely used to stimulate gonad maturation, but is an impractical and unethical practice (Uawisetwathana et al., 2011). Hormones and neurotransmitters are involved in the regulation of reproduction, have been used to enhance gonadal maturation and gamete production in crustaceans (Nagaraju, 2011). For example, serotonin administration can significantly shorten the time for gonad maturation and even stimulate spawning in a variety of crustaceans, including *M. rosenbergii* (Meeratana et al., 2006; Tinikul et al., 2008), the white shrimp, *Penaeus vannamei* (Vaca and Alfaro, 2000), and the black tiger shrimp, *Penaeus monodon* (Wongprasert et al., 2006). Red pigment-concentrating hormone (RPCH) enhances ovarian maturation by increasing gonad–somatic index (GSI) and *vitellogenin* (*Vg*) expression in the ovary and hepatopancreas of the mud crab, *Scylla paramamosain* (Zeng et al., 2016). Furthermore, neuropeptide F (NPF) administration reduces ovarian maturation periods and increases oocyte diameter, GSI and hemolymph Vg concentration in *M. rosenbergii* (Tinikul et al., 2017). Octopus and lamprey gonadotropin-releasing hormone (GnRH)-like peptides can also stimulate ovarian maturation and spawning in *P. monodon* (Ngernsoungnern et al., 2008a), *M. rosenbergii* (Ngernsoungnern et al., 2008b) and the blue swimming crab, *Portunus pelagicus* (Saetan et al., 2013). Abalone egg-laying hormone (ELH) can shorten oocyte development in *M. rosenbergii* (Ngernsoungnern et al., 2009) and *P. pelagicus* (Saetan et al., 2017). Moreover, zebrafish kisspeptins induce oogonial proliferation and shorten the length of ovarian maturation in *M. rosenbergii* (Thongbuakaew et al., 2016a). Hormones, neuromodulators and other substances are speculated to control ovarian maturation by acting directly on the gonads or indirectly exerting their stimulatory effects by triggering the release of a putative gonad-stimulating factor from the nervous tissues, which may affect levels of cyclic nucleotides (cAMP and cGMP) and $Ca^{2+}$ in target tissues, leading to the regulation of vitellogenesis (Jayasankar et al., 2020). Nevertheless, there are still large gaps in our understanding of crustacean reproductive endocrinology and the role of pyrokinins (PKs).

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The PKs are a subfamily of the PRXamide family, which were found in several insect orders (Jurenka, 2015; Ahn et al., 2020). In insects, PKs have been found to regulate a variety of biophysiological processes such as myotropic activity, contraction of the oviduct, egg diapause and stimulation of sex pheromone biosynthesis (Raina et al., 1989; Raina and Kempe, 1990; Schoofs et al., 1991; Nachman et al., 1993; Suwan et al., 1994; Predel and Nachman, 2001; Choi and Vander Meer, 2012; Marciniak et al., 2012). In crustaceans, PKs were first identified in the central nervous system (CNS) of the white shrimp, *P. vannamei*, by MALDI-TOF mass spectrometry (Torfs et al., 2001). In recent years, PKs have been found in the Jonah crab, *Cancer borealis* (Saideman et al., 2007), the blue crab, *Callinectes sapidus* (Hui et al., 2012), the mud crab, *S. paramamosain* (Bao et al., 2015), the American lobster, *Homarus americanus* (Dickinson et al., 2015a, b), the freshwater crayfish, *Procambarus clarkii* (Christie and Chi, 2015), and the Australian crayfish, *Cherax quadricarinatus* (Nguyen et al., 2016), via mass spectrometry and transcriptomics. Moreover, PK is highly expressed at the early vitellogenic stage, suggesting its involvement in the initiation of vitellogenesis (Bao et al., 2015). In the Norway lobster, *Nephrops norvegicus*, PK may stimulate the maturation of immature ovaries (Nguyen et al., 2018). However, PKs have not yet been identified in all crustaceans and research on their functions is still warranted.

In this study, we aimed to identify PKs and investigate their function in *M. rosenbergii*. This study provides basic knowledge concerning the existence of PKs in the giant freshwater prawn and associates its functions with reproductive processes, specifically ovarian maturation, which may be applied to prawn culture.

## MATERIALS AND METHODS

### Ethical approval

The animal procedures used in this work were approved by the Animal Care and Use Committee of Walailak University (protocol number: 005/2019) and conformed with relevant guidelines of animal care and use under the Ethical Review Board of the Office of National Research Council of Thailand (NRCT).

### Gene mining

All relevant transcriptome data for CNS and ovarian tissues of female *M. rosenbergii* was obtained from NCBI Sequence Read Archive (SRA) database accession number SRP049917, reported by Suwansa-ard et al. (2015). Briefly, all unigenes assembled were annotated with the databases of non-redundant protein sequence (Nr), nucleotide sequence (Nt), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG) and Gene Ontology (GO), using BLAST and BLAST2GO software, with an E-value threshold of 1e−6 (Suwansa-ard et al., 2015). The list of annotated sequences was scanned for PKs. Homologs of PKs were identified via tBLASTn search against known PKs reported in previous studies (Torfs et al., 2001; Saideman et al., 2007; Hui et al., 2012), using the CLC Main Workbench version 21.0.3 (CLC Bio-Qiagen, Aarhus, Denmark). All hits were analyzed manually with their homologous proteins from various species.

### Animals and tissue collection

Live mature female prawns, *Macrobrachium rosenbergii* De Man 1879, representing different stages of reproduction [stages 1–4; stage 1 (spent), stage 2 (proliferative), stage 3 (premature), stage 4 (mature) according to the morphological and histological characteristics previously described; Meeratana and Sobhon, 2007] with 20 prawn per stage ($n$=80), each with an average mass of 50–70 g and mature male prawns ($n$=20) with an average mass of 150–200 g were obtained from a commercial farm in Surat Thani, Thailand. Prawns were acclimatized in a culture tank containing clean freshwater under natural photoperiod (12 h light:12 h dark) and a constant aeration system at 25°C, for 24 h before tissue collection. After 24 h, the animals were anesthetized by immersion in ice-cold water for 5 min and then killed. The eyestalks, brain, thoracic ganglia, abdominal ganglia, ovaries, hepatopancreas, hematopoietic tissues, gut, heart and muscles from female prawns and testes from male prawns were collected and immediately frozen in liquid nitrogen, then stored at −80°C until preparation of total RNA.

### Total RNA extraction, molecular cloning and characterization of *MroPK*

Frozen tissues were individually homogenized and the total RNA extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol in combination with a DNaseI (Thermo Fisher Scientific) treatment to eliminate potential genomic DNA contamination. For DNaseI treatment, the reactions contained 2 µg RNA, 1 unit of DNaseI and 2 µl of 10× DNaseI reaction buffer in a final volume of 20 µl (adjusted with RNase-free $H_2O$) and were incubated at 37°C for 30 min. Then, DNaseI was heat inactivated with 2.5 µl of 25 mmol $l^{-1}$ EDTA for 5 min at 75°C, and samples were finally cooled to 4°C. The quantity and quality of RNA were measured using spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific) and gel electrophoresis. First-strand cDNA was synthesized by reverse transcription (RT) of 2 µg total RNA using RevertAid Reverse Transcriptase (Fermentas, Hanover, MD, USA) and primed using a modified oligo(dT) primer (3′-RACE CDS Primer A, Clontech, Mountain View, CA, USA) following the manufacturer's protocol. The 5′ UTR sequence of the *MroPK* gene was isolated through the published *M. rosenbergii* transcriptome as previously described. The *MroPK* gene-specific forward primer (AGCCAGTTGGGAGCTTGCAT) was designed by Primer-BLAST (Ye et al., 2012) according to the core sequence. PCR amplification of 2 µl of the first-strand cDNA was carried out with a gene-specific primer and the universal primer mix (UPM; GCCGAGGCGGCCGACATGTT; Clontech) containing the complementary sequence to the oligo(dT) primer, with PCR SuperMix (Thermo Fisher Scientific). Cycling conditions included an initial denaturation step (95°C for 5 min), 35 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s) and extension (72°C for 45 s), and a final extension (72°C for 10 min). The PCR product was analyzed by agarose gel electrophoresis. The amplicons were extracted using QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned into the pDrive vector (Qiagen). Ligated plasmids were introduced into *E. coli* strain DH5α for further gene amplification and sequencing (Duangprom et al., 2018). The recombinant plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen), and sequenced by Macrogen (Macrogen Ltd, Seoul, South Korea). The obtained sequences were analyzed using a bioinformatics tool including BLAST against the NCBI GenBank database, and the putative amino acid sequence was deduced using the Expasy bioinformatics tool (http://web.expasy.org/translate/) (Artimo et al., 2012). Signal peptide prediction was done by the online program SignalP 5.0 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) (Armenteros et al., 2019). Analysis of protein similarity was performed by protein alignment using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/)

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**Table 1. Gene-specific primers used for tissue expression and qPCR and expected amplicon sizes**

| Gene | Technique | Forward primer (5′–3′) | Reverse primer (5′–3′) | Size (bp) |
|---|---|---|---|---|
| *MroPK* | RT-PCR | GCTCCCAACTGGCTCATCTT | AGCAGAGTCCTCCTGATCGT | 474 |
| | qPCR | GGGTTTCGTGATCTCCGACT | CCCTAGTCTGGGGATGAATGC | 154 |
| *3β-HSD* | qPCR | GGGAAGCGTCTCATCGAACA | CTAGTGTCCCCCTCACCGTA | 200 |
| *17β-HSD* | qPCR | CGGCTGGAAATGCAGAAGTG | GATGTACTCGTCGCCGTAGG | 159 |
| *Vg* | qPCR | GAGTCCGATCTAGCTGCAATCC | CGCACATGGCGCGCGATAG | 108 |
| *β-actin* | RT-PCR/qPCR | GCACCCTCCACCATGAAGAT | TGCACAATTGAGGGTCCAGA | 152 |

RT-PCR, reverse transcription PCR; qPCR, quantitative PCR.

(Edgar, 2004). Prediction of conserved protein domains was performed by NCBI conserved domain database (Marchler-Bauer et al., 2017) and InterPro (https://www.ebi.ac.uk/interpro) (Finn et al., 2017). For species abbreviations and NCBI accession number, see Table S1.

### Tissue distribution of *MroPK* by RT-PCR

To determine the tissue distribution of PK gene expression, total RNA was extracted from tissues and cDNA was generated as described above. *MroPK* was amplified by gene-specific primers designed by Primer-BLAST (Ye et al., 2012) (Table 1). Primers were also prepared for the β-actin gene (GenBank accession no. AY626840.1) to be used as a positive control; a no-template negative control was also included. The PCR program consisted of an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final extension of 10 min at 72°C. PCR products were electrophoresed on an agarose gel and amplicons were confirmed by sequencing as described above.

### Ovarian expression patterns of *MroPK* by *in situ* hybridization

The distribution of *MroPK* expression in ovarian tissue sections was investigated by *in situ* hybridization (ISH). Briefly, plasmids containing the *MroPK* sequence were amplified by PCR using M13 primers (forward GTAAAACGACGGCCAGT and reverse AACAGCTATGACCATG) and the identity and orientation of the insert were checked by sequencing. PCR products generated with M13 primers covering the binding site for the T7 or SP6 RNA polymerase were used as templates for subsequent *in vitro* transcription. Furthermore, digoxigenin-labeled sense and antisense RNA probes were generated using a DIG RNA labeling kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

The developmental stages of ovaries (stage 1–4) were dissected out and fixed in fresh 4% paraformaldehyde fixative in 0.1 mol $l^{-1}$ PBS, pH 7.4, at 4°C overnight. Then, tissues were processed by a routine paraffin method (Slaoui and Fiette, 2011). Paraffin-embedded blocks were sectioned at 6 µm thickness using a Leica rotary microtome (Leica Microsystems, Wetzlar Germany). ISH was performed following a previously described protocol (Thongbuakaew et al., 2019). Briefly, the sections were deparaffinized and rehydrated through an ethanol series. Then, the sections were covered with prehybridization buffer [4× saline sodium citrate (SSC) buffer and 50% formamide] at 37°C for 10 min before being incubated with sense and antisense DIG-labeled probes in hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 10 mmol $l^{-1}$ DTT, 1 mg $ml^{-1}$ yeast t-RNA, 1 mg $ml^{-1}$ denatured and sheared salmon sperm DNA) at 55°C overnight. After hybridization, sections were washed twice in 2× SSC and twice in 0.1× SSC for 15 min at room temperature. The sections were then washed with buffer 1 [100 mmol $l^{-1}$ Tris-HCl (pH 7.5), 150 mmol $l^{-1}$ NaCl] containing 0.1% Triton X-100, and finally covered by blocking solution [buffer 1 containing 0.1% Triton X-100 and 1% normal sheep serum (Sigma)] for 2 h. Subsequently, sections were washed twice in buffer 1 and twice in buffer 2 [100 mmol $l^{-1}$ Tris-HCl (pH 9.5), 100 mmol $l^{-1}$ NaCl, 50 mmol $l^{-1}$ $MgCl_2$]. Sections were incubated with NBT/BCIP (Roche), diluted in buffer 2 in the dark overnight. Color development was terminated with stop solution [10 mmol $l^{-1}$ Tris-HCl (pH 8.1), 1 mmol $l^{-1}$ EDTA]. Sections were washed thoroughly in DEPC-treated $H_2O$ and mounted in 90% glycerol. The stained sections were observed and photographed under a Nikon E600 microscope equipped with a DXM1200F digital microscope (Nikon, Tokyo, Japan). The experiments of each stage were performed in triplicate.

### Expression of *MroPK*, steroidogenesis-related genes and *Vg* by qPCR

Quantitative real-time PCR (qPCR) was used to evaluate changes in mRNA levels of *MroPK* during the ovarian cycle (stages 1–4) of mature female prawns (7 prawns per stage; $n$=28) and the expression levels of steroidogenesis-related genes (*3β-HSD* and *17β-HSD*) and *Vg* in cultured ovarian tissue ($n$=5). Total RNA extraction and cDNA synthesis were performed as described above. All primers used for qPCR analysis are shown in Table 1. Three reference genes (encoding 16S rRNA, GAPDH and β-actin) were evaluated for their efficiency and variability through pilot tests, and *β-actin* was selected as a reference gene as it showed less variability (coefficient of variation, CV=4.82%) compared with *16S rRNA* (CV=7.12%) and *GAPDH* (CV=5.34%). Expression of *β-actin* was used as the internal control according to a previously described protocol (Thongbuakaew et al., 2019). qPCR was performed using the GeneRead qPCR SYBR Green Mastermix (Qiagen) following the manufacturer's protocol. Transcripts were quantified using a standard curve method (Larionov et al., 2005). Standard curves for *MroPK* and *β-actin* were generated by 10-fold serial dilution of known concentrations of the plasmids containing the target transcripts. The detection range, linearity and qPCR amplification efficiency of each primer pair were checked before continuing with sample analysis. qPCR reaction efficiency was calculated from the standard curve, which ranged from 95% to 100%. Expression of *β-actin* was verified before used as the internal reference to correct for differences in reverse transcription efficiency and template quantity. All standards and experimental samples were run in duplicate. The amount of target and internal reference in experimental samples was determined from the respective standard curves. Transcript levels were normalized to the level of *β-actin* and the data were expressed as relative mRNA levels.

### Ovarian tissue culture with PK peptide

The protocols for ovarian tissue culture used in this study were based on Thongbuakaew et al. (2019) in *M. rosenbergii*. Briefly, fragments of early-stage ovaries (stage 1–2; 0.2–0.3 g) from mature female prawns ($n$=5) were dissected and washed in crustacean physiological saline (CPS: NaCl 29 g, KCl 0.71 g, $CaCl_2{\cdot}2H_2O$

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2.38 g, $MgSO_4 \cdot 7H_2O$ 3.16 g, $NaHCO_3$ 0.5 g, $MgCl_2 \cdot 6H_2O$ 0.17 g, Hepes 4.76 g in 1 liter of distilled water) (Tinikul et al., 2008) containing antibiotic-buffered mixture (1000 IU $ml^{-1}$ penicillin and 1000 µg $ml^{-1}$ streptomycin). These samples were then cultured in Leibovitz's L-15 medium (Gibco, Grand Island, NY, USA) containing 100 µg $ml^{-1}$ of streptomycin and 100 IU $ml^{-1}$ of penicillin. The culture was performed in 24-well culture plates (Nunc, Wiesbaden, Germany) with each sample in 2 ml of L-15 medium and 10 µl of 1, 10, 100 and 1000 nmol $l^{-1}$ PK peptide was added into the media (final concentration: 0.005, 0.05, 0.5 and 5 nmol $l^{-1}$). PK peptide (FSPRL-NH2) used in this experiment was commercially synthesized by GenScript (Piscataway, NJ, USA). CPS was added to the control groups instead of PK peptide. All plates were incubated with gentle shaking in the dark at 25°C for 1 h, and the experiments were performed in duplicate. At the end of the incubation period, medium from each well was transferred to a microcentrifuge tube and frozen at −20°C. After removal of the medium, the ovarian fragments were collected and immediately frozen in liquid nitrogen and stored at −80°C.

### Steroid hormone measurements

Frozen medium samples from ovarian tissue culture ($n$=5) were concentrated using a Freeze Dryer (ScanVac CoolSafe freeze dryer, Labogene, Lillerød, Denmark) and analyzed using 17b-estradiol (E2) and progesterone (P4) EIA kits, based on a competitive ELISA technique following the manufacturer's recommendations (Cayman Chemical Company, Ann Arbor, MI, USA; E2 cat. no. 582251, P4 cat. no. 582601). Briefly, the media samples and E2 or P4 standard diluted in EIA buffer were added to a 96-well plate, with AChE tracer and the corresponding EIA antiserum. The plates were covered with plastic film and incubated for 1 h at room temperature with gentle shaking. The plates were then emptied and rinsed 5 times with EIA wash buffer and developed with Ellman's reagent provided in the EIA kits (Cayman Chemical Company) for 1 h in the dark. The plates were read at a wavelength of 420 nm. Duplicate E2 or P4 standard curves were prepared consisting of eight concentrations ranging from 0.0066 to 4 ng $ml^{-1}$ E2 and 0.0078 to 1 ng $ml^{-1}$ P4. Parallelism of serial dilution curves for samples was assessed and demonstrated that these dilution curves were parallel with the standard curve. Moreover, the cross-reactivity of various steroids with the antisera of the P4 and E2 EIA kits is shown in Table 2. Each EIA kit exhibited high specificity.

### Statistical analysis

Experimental data are expressed as a means±s.d., and were analyzed and compared using a one-way analysis of variance (ANOVA), followed by a Tukey's *post hoc* multiple comparison. A probability value less than 0.05 ($P$<0.05) indicated a significant difference.

**Table 2. Cross-reactivity of steroids with the antisera of the P4 and E2 EIA kits**

| | Cross-reactivity (%) | |
|---|---|---|
| Steroid | P4 | E2 |
| Pregnenolone | 27 | 0 |
| Progesterone | 100 | <1 |
| Estriol | <1 | <1 |
| Estrone | <1 | 5 |
| Estradiol | <1 | 100 |
| Testosterone | 0 | <1 |
| Dihydrotestosterone | 0 | 0 |
| 20-Hydroxyecdysone | <1 | <1 |

## RESULTS

### Identification of PKs in *M. rosenbergii* and peptide characterization

In the female *M. rosenbergii* transcriptome (combined CNS and ovarian tissue), we found a transcript that encoded a MroPK precursor. The MroPK precursor is 275 amino acids in length, with a 21 amino acid signal peptide (Fig. 1A). It also contains multiple

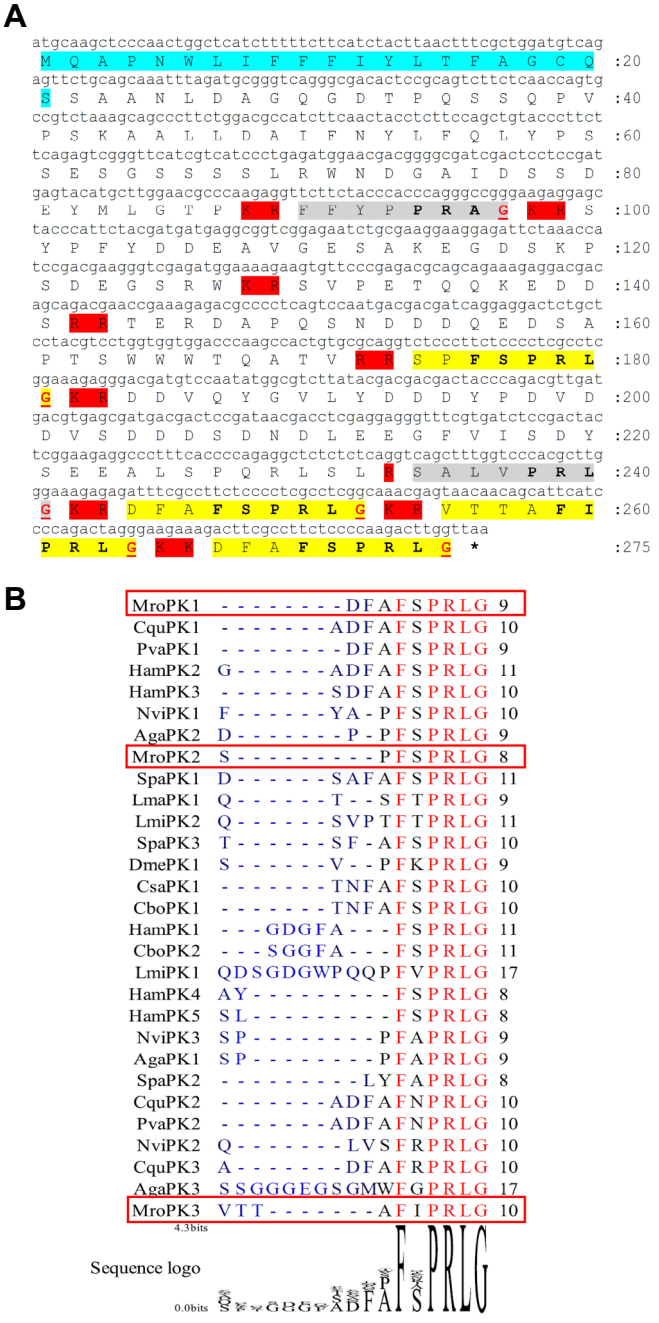


**Fig. 1. Characterization of *Macrobrachium rosenbergii* pyrokinin (*MroPK*).** (A) Nucleotide and amino acid sequence of the *MroPK* gene, showing signal peptide (blue), putative cleavage sites (red), PK peptides (FxPRL; yellow), PRXamide (gray) and glycine for amidation (red underline). Asterisk represents stop codon. (B) Amino acid sequence alignment of mature MroPK with PKs of other species. Conserved amino acids are shown in red font, characterized as the C-terminal pentapeptide sequence FxPRLamides. Species names and NCBI accession numbers are shown in Table S1. The list of amino acid sequences of mature PKs is shown in Table S2.

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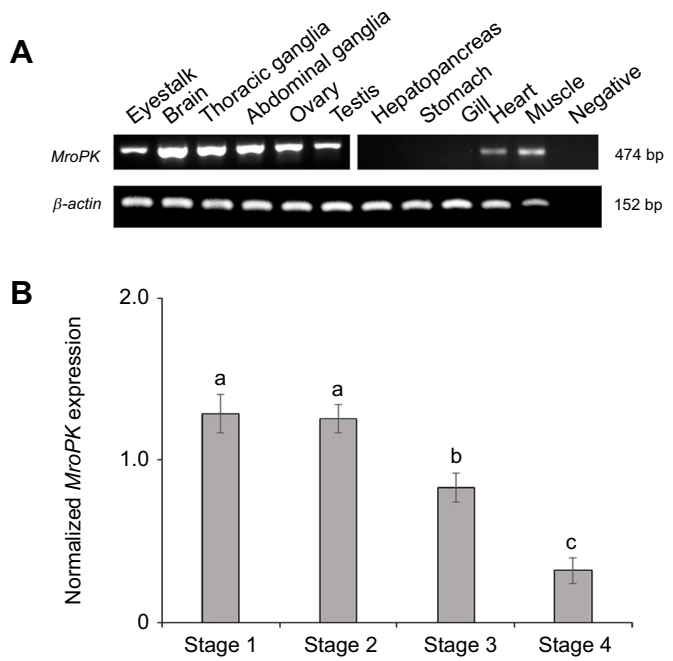


**Fig. 2. Tissue-specific and temporal expression of *M. rosenbergii PK*.** (A) Agarose gel showing tissue-specific expression of *MroPK* following RT-PCR with gene-specific primers. The *β-actin* gene was used as a positive control; the negative control was obtained by PCR without cDNA template. The expected amplicon size is shown in base pairs (bp, right). (B) Quantitative expression profile of *MroPK* in ovarian tissues during ovarian maturation (stages 1–4). Data were normalized against *β-actin* and the relative expression levels are means±s.d. (*n*=7 at each stage). Different letters indicate a statistically significant difference ($P<0.05$).

predicted cleavage sites (K88R, K98R, K128R, R142R, R172R, K182R, R233, K242R, K253R and K265R), which may produce three mature PK peptides (DFAFSPRL-$NH_2$, SPFSPRL-$NH_2$ and VTTAFIPRL-$NH_2$) and two mature PRXamide peptides (FFYPPRA-$NH_2$ and SALVPRL-$NH_2$) (Fig. 1A). All three mature PK peptides are conserved and predicted to be functional peptides with C-terminal amidation (Fig. 1A). Amino acid alignment of mature MroPKs with PK peptide sequences of other crustaceans and insects supports high conservation of the C-terminal sequence FxPRLamide (Fig. 1B). For the list of amino acid sequences of mature PKs, see Table S2.

### Tissue-specific and temporal expression of *MroPK*

RT-PCR was performed in order to determine the tissue-specific expression of *MroPK* in *M. rosenbergii* tissues (Fig. 2A). The results showed that *MroPK* was expressed within the testes and all (female) tissues, except the hepatopancreas, stomach and gill. Consistent low-intensity amplicons were observed within the eyestalk and muscle. We further analyzed the relative gene expression in the ovarian tissues during the ovarian cycle by qPCR (Fig. 2B). The results revealed that the *MroPK* gene was expressed at significantly higher levels during the early stages (stage 1, spent; and stage 2, proliferative) and gradually decreased toward the late stages (stage 3, premature; and stage 4, mature) of the ovarian cycle.

### Distribution of *MroPK* in ovary

The tissue expression of *MroPK* was investigated by ISH in the developmental stages of ovaries (stage 1–4). The experiments of each stage were performed in triplicate. *MroPK* staining was identified in the Og, and previtellogenic (including Oc1 and Oc2) and early vitellogenic (Oc3) oocytes (Fig. 3A–C). Intense positive staining was observed within the Oc1 and Oc2, whereas the Og and Oc3 showed staining at lower intensity (Fig. 3B,C). No expression was observed within the late vitellogenic oocyte (Oc4) and follicular cells. Likewise, no positive staining was observed in the negative control, in which sense riboprobes were used (Fig. 3D).

### Effect of PK on *in vitro* ovarian steroid secretion

This experiment was performed based on the hypothesis that PKs may stimulate the secretion of female sex steroids in early-stage ovaries (stage 1–2). Our results showed that PK can increase both P4 and E2 levels, as measured by EIA (Fig. 4A,B). For P4, all doses of PK tested (0.005–5 nmol $l^{-1}$) stimulated a significant increase for stage 1 and 2 ovaries, when compared with the control group (Fig. 4A). This was also observed for E2 levels following PK incubation (Fig. 4B). Higher concentrations of P4 were found in the stage 1 ovary, whereas the

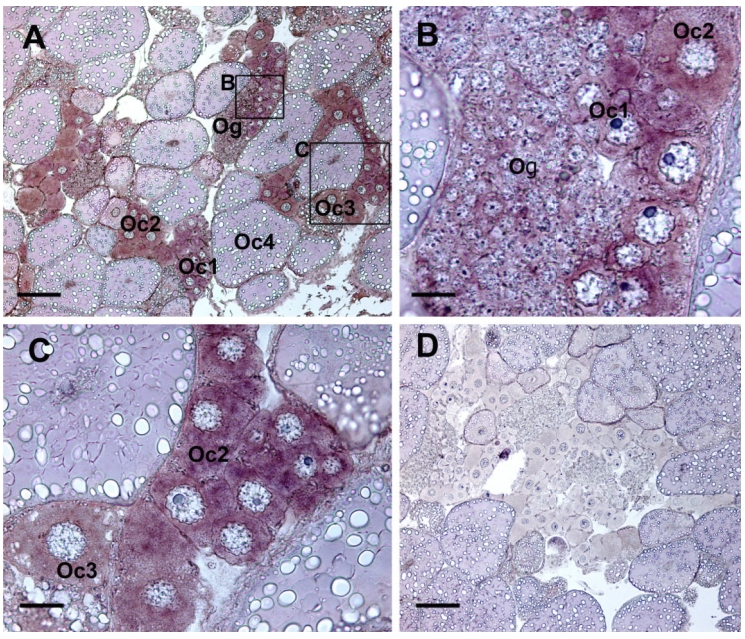


**Fig. 3. Localization of *MroPK* mRNA in the ovary at stage 2–3.** (A) *In situ* hybridization of *MroPK* mRNA in the cytoplasm of oogonia (Og), previtellogenic oocytes (Oc1 and Oc2) and early vitellogenic oocytes (Oc3). (B) High-magnification micrograph showing positive staining of Og, Oc1 and Oc2 in the cytoplasm. (C) High-magnification micrograph showing *MroPK* expression of Oc2 and Oc3 within the cytoplasm. (D) Negative control micrograph using a sense riboprobe, showing no positive signal in the ovary. Scale bars: 100 µm in A and D; 25 µm in B and C. The experiments at each stage were performed in triplicate.

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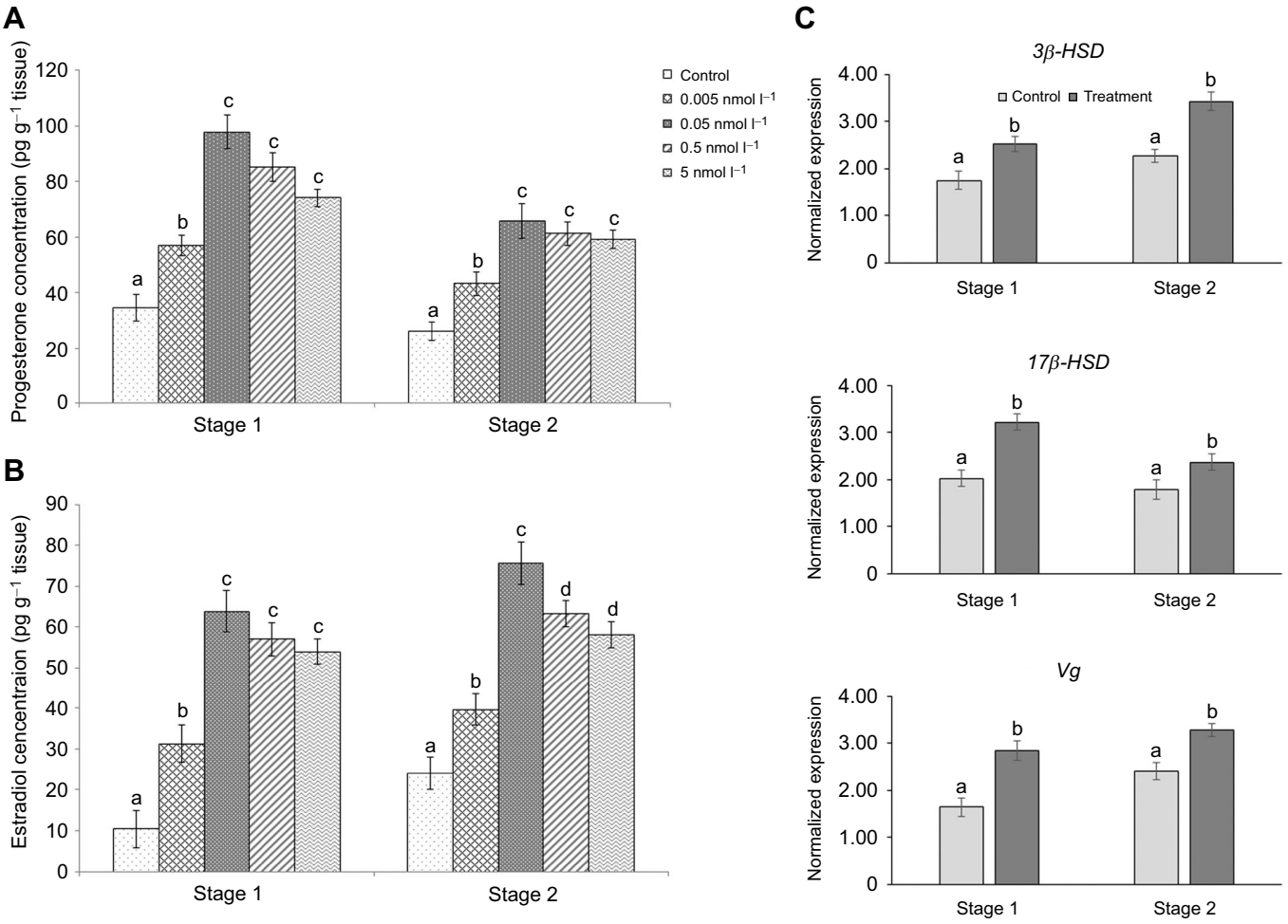


**Fig. 4. Effect of PK peptide on sex steroid production and expression of steroidogenesis-related genes and *vitellogenin* (*Vg*) in ovarian explant cultures of *M. rosenbergii*.** (A) Histograms showing a significant increase in levels of progesterone in treated groups with all doses of PK, compared with the control group at 1 h. (B) Histograms showing a significant increase in levels of estradiol in treated groups with all doses of PK, compared with the control group at 1 h. (C) Effect of PK (0.05 nmol $l^{-1}$) on gene expression levels of *3β-HSD*, *17β-HSD* and *Vg*, shown by qPCR. Data were normalized against *β-actin*. Values are means±s.d. ($n$=5) and the different letters indicate a statistically significant difference ($P$<0.05).

highest E2 concentration was observed from the stage 2 ovary (Fig. 4A, B). Maximally stimulation of P4 and E2 levels was achieved with PK concentrations of 0.05 nmol $l^{-1}$. In addition, P4 and E2 levels tended to decrease as the concentration of PK is increased (0.5–5 nmol $l^{-1}$) (Fig. 4A,B). There was no difference between control and PK-treated groups at concentrations of 0.00005–0.0005 nmol $l^{-1}$ (data not shown).

### Effect of PK on steroidogenesis-related and *Vg* expression

Next, we investigated whether 0.05 nmol $l^{-1}$ PK could change *3β-HSD*, *17β-HSD* and *Vg* expression within stage 1–2 ovary explants. The results showed that PK could significantly upregulate expression of *3β-HSD* and *17β-HSD* in both stage 1 and 2 ovaries, compared with the control (Fig. 4C). Moreover, ovarian explants incubated with PK exhibited a gradual increase of *Vg* gene expression compared with the control (Fig. 4C).

## DISCUSSION

The PK subfamily is a major group of insect and crustacean neuropeptides that has now been found in several crustacean species including the white shrimp, *P. vannamei* (Torfs et al., 2001), the Jonah crab, *C. borealis* (Saideman et al., 2007; Christie and Pascual, 2016), the blue crab, *C. sapidus* (Hui et al., 2012), the mud crab, *S. paramamosain* (Bao et al., 2015), the American lobster, *H. americanus* (Dickinson et al., 2015a,b), the Australian crayfish, *C. quadricarinatus* (Nguyen et al., 2016), and the Norway lobster, *N. norvegicus* (Nguyen et al., 2018). In this study, we successfully identified and characterized a *MroPK* precursor in the giant freshwater prawn, *M. rosenbergii*. We additionally describe tissue and ovarian gene expression of *MroPK*, as well as the changes in its expression during ovarian maturation. Moreover, a link with ovarian steroid secretion was established through *in vitro* oocyte culture bioassays using the PK peptide.

The MroPK precursor comprises a predicted signal peptide, cleavage sites and various mature peptides. The presence of a signal peptide is required for the endoplasmic reticulum-to-Golgi pathway of secretion and membrane translocation of newly synthesized proteins. Three mature MroPKs share a highly conserved C-terminal sequence FxPRLamide that is observed in other arthropods. This pentapeptide sequence appears to be the primary biologically active region required for reception activation leading to biological functions (Torfs et al., 2001; Saideman et al., 2007; Choi et al., 2015). It is possible that PK peptides derived from a common precursor may be co-released and may interact with their distinctive receptors localized to specific target tissues to modulate

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physiological functions (Paluzzi and O'Donnell, 2012). Moreover, the MroPK precursor also produced two other PRXamide peptides, which are not members of the PK subfamily (FxPRLamide). PKs are a subfamily of the PRXamide family that are differentiated by variations in the C-terminal motif and the type of receptor (Hull et al., 2021). In arthropods, a single gene was found to encode a precursor with multiple PRXamide, but generally members from different families of peptides are not encoded within a single gene (Jurenka, 2015; Choi et al., 2015; Ahn et al., 2020). However, a single gene that produces peptides from two different peptide families was reported in mollusks (Ahn et al., 2020). Interestingly, C-terminal PRXamide comprises a core motif for PRXamide–PRXamide receptor binding, but when C-terminal PR(X)amide was replaced by the amino acid alanine (A), the receptor responses to the ligands almost completely disappeared (Ahn et al., 2020). This suggests that FFYPPRA-NH2 may possess less potent activity at the receptors in controlling physiological functions compared with SALVPRL-NH2 (Ahn et al., 2020). It will be interesting for future studies to examine the structure and activity of PRXamide peptides in crustaceans.

In crustaceans and insects, PKs are broadly distributed within the nervous system, suggesting neurohormonal control of biological processes (Torfs et al., 2001, 2002; Choi et al., 2001; Saideman et al., 2007; Christie et al., 2010; Erica et al., 2014; Bao et al., 2015). Furthermore, *PK* genes are expressed in the ovaries of the Australian crayfish, *C. quadricarinatus*, and the Norway lobster, *N. norvegicus*, suggesting they help regulate reproductive processes (Nguyen et al., 2016, 2018). PK receptor is also expressed in both male and female reproductive tissues of the insect *Rhodnius prolixus*, indicating that PK may be involved in regulating the growth and maturation of reproductive tissue (Paluzzi and O'Donnell, 2012). In addition to nervous and gonadal tissues, our study also detected the expression of *MroPK* in other tissues including the heart and muscle. Similarly, PKs showed bioactivity in the cardiac neuromuscular system in the lobster *H. americanus* (Dickinson et al., 2015a). The synthetic PKs revealed myotropic activity in the crayfish *Astacus leptodactylus* hindgut (Torfs et al., 2001). *MroPK* was not present within the stomach, hepatopancreas and gill. We propose that MroPK may be involved in the movement of food in the digestive tract and does not regulate the activity of digestion and osmoregulation in *M. rosenbergii* or may be modulated by PKs produced in the nervous system (Morris, 2001; Paluzzi and O'Donnell, 2012; Dickinson et al., 2015b). Thus, this further implicates PKs in a variety of physiological activities in *M. rosenbergii*, including reproductive functions.

The mRNA expression pattern and quantitative expression can provide insight into gene function and biological importance. We found *MroPK* transcript expression specifically in the Og, and previtellogenic (Oc1 and Oc2) and early vitellogenic (Oc3) oocytes. Moreover, qPCR demonstrated that expression levels of *MroPK* during the ovarian maturation cycle were significantly higher in the early stages (stages 1–2). This finding was similarly observed in the mud crab, *S. paramamosain*, where highest expression of *PK* was found at the early vitellogenic stages, leading us to speculate that PK may be involved in the beginning of vitellogenesis (Bao et al., 2015). In addition, PK is expressed in the ovaries of immature female Norway lobster, *N. norvegicus*, suggesting that PK may play a role in stimulating the maturation of ovaries (Nguyen et al., 2018). Hence, MroPK may be involved in ovarian development by stimulating primary vitellogenesis, which is characterized by the differentiation of endoplasmic reticulum and the formation of endogenous yolk stored in vesicles (Subramoniam, 2011).

Moreover, the role of PKs in reproduction was supported by *in vitro* ovary explant culture experiments showing MroPK peptide induced sex steroid secretion (P4 and E2), as well as upregulation of steroidogenesis-related (*3β-HSD* and *17β-HSD*) and *Vg* gene expression. These results are consistent with previous studies on PK receptors in insects, where expression was prominent in female reproductive tissues (Paluzzi and O'Donnell, 2012; Gondalia et al., 2016), suggesting its involvement in reproduction. Recent work has demonstrated that both *3β-HSD* and *17β-HSD* genes are likely involved in the biosynthesis of steroids in invertebrates (Wen and Pan, 2015; Thitiphuree et al., 2019). Also, the presence of P4, E2 and enzymes involved in the steroidogenic pathway was reported in the ovarian tissue of *M. rosenbergii* using LC-MS/MS and transcriptomic analysis (Thongbuakaew et al., 2016b). In addition to steroid metabolism, *3β-HSD* and *17β-HSD* have been shown to possess other functions, such as a role in ecdysteroid biosynthesis and fatty acid metabolism (Dauphin-Villemant et al., 1997; Sakurai et al., 2006). Notably, CYP19 is absent from crustaceans, which suggests that estrogens are synthesized via an alternative metabolic pathway (Thongbuakaew et al., 2016b; Knigge et al., 2021). Interestingly, the coral performed A-ring aromatization to produce a steroid with an aromatic A-ring, that is usually catalyzed by vertebrate CYP19, suggesting the possibility that invertebrates could synthesize aromatized steroids for transcriptional activation of the ancestral estrogen receptor (Markov et al., 2017). Apart from the genes involved in steroidogenesis, the existence of sex steroid receptors is still controversial in crustaceans. Progesterone and estrogen receptors have been identified in various crustacean species using immunohistochemistry (Paolucci et al., 2002; Ye et al., 2010; Yang et al., 2012). However, the identification of high-affinity sex steroid binding proteins and sex steroid receptor genes has not yet been reported in crustaceans (Knigge et al., 2021). Moreover, Swetha et al. (2016) reported the interaction of the estrogenic hormone with the ecdysteroid receptor in the edible crab *Oziothelphusa senex senex*, suggesting that its reproductive functions may occur through the ecdysteroid pathway (Swetha et al., 2016). However, a complete understanding of steroidogenesis pathways and sex steroid receptors in crustaceans remains to be elucidated (Janer and Porte, 2007; Knigge et al., 2021). Furthermore, P4 and E2 have been implicated in the stimulation of vitellogenesis, which is an important final step in oocyte maturation in crustaceans and supports a role for these hormones in crustacean reproduction (Coccia et al., 2010; Subramoniam, 2011, 2017). Taken together, these findings suggest that mature MroPKs may be produced from the precursor and released by the oocytes to stimulate sex steroid secretion, which possibly interacts with the ecdysteroid receptor in order to enhance the vitellogenic process, resulting in oocyte development and ovarian maturation in *M. rosenbergii*.

The ovary development of crustaceans is controlled by a complex neuroendocrine regulatory network (Jayasankar et al., 2020; Liu et al., 2021). In the giant freshwater prawn, *M. rosenbergii*, several exogenous hormones and crustacean peptides have been found that play a role in ovarian maturation, such as octopus and lamprey GnRH-like peptide (Ngernsoungnern et al., 2008b), abalone ELH (Ngernsoungnern et al., 2009), zebrafish kisspeptins (Thongbuakaew et al., 2016a), NPF (Tinikul et al., 2017), crustacean female sex hormone (CFSH) (Thongbuakaew et al., 2019), and also PKs. Moreover, neurotransmitters apparently trigger the release of the gonad-stimulating hormone (GSH), which is involved in gonad maturation in crustaceans (Fingerman, 1997; Nagaraju, 2011; Jayasankar et al., 2020). Serotonin also

enhances vitellogenesis in *M. rosenbergii*, which may indicate its involvement in ovarian maturation (Kuo et al., 2009). Furthermore, serotonin induces the expression of CFSH in the ovary of *M. rosenbergii*, resulting in ovarian development via the regulation of Vg synthesis (Thongbuakaew et al., 2019). It has been postulated that the crosstalk between neurotransmitters and hormones may play a role in controlling ovarian maturation possibly through vitellogenesis (Jayasankar et al., 2020). However, identifying the functions of PKs and the crosstalk between other neurotransmitters and hormones in controlling vitellogenesis and ovarian maturation of crustaceans will be necessary for further studies.

## Conclusions

*MroPK* gene was identified in *M. rosenbergii*, and encodes a precursor containing PK peptides with a conserved C-terminal motif (i.e. FxPRLamide) found with other arthropods. *MroPK* is expressed in the ovary, and its expression levels change during ovarian development, which implies a function in reproductive maturation. Moreover, MroPK may control ovarian maturation by stimulating the secretion of sex steroids, thereby resulting in Vg synthesis. However, the exact biological activities of MroPKs in reproduction need to be verified and this is the subject of our future investigation using advance knowledge and methodology. If a gonadal stimulatory role for PK is proven, it is possible that this hormone may assist in aquaculture technology development for commercially important crustaceans.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: T.T., S.F.C., P.S.; Methodology: T.T., S.M., A.C., K.K., T.K.; Formal analysis: T.T.; Investigation: T.T., S.M., A.C., K.K., T.K.; Resources: T.T.; Writing - original draft: T.T.; Writing - review & editing: T.T., S.F.C., P.S.; Funding acquisition: T.T.

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### References

**Ahn, S. J., Mc Donnell, R. J., Corcoran, J. A., Martin, R. C. and Choi, M. Y.** (2020). Identification and functional characterization of the first molluscan neuromedin U receptor in the slug, *Deroceras reticulatum*. *Sci. Rep.* **10**, 1-13. doi:10.1038/s41598-019-56847-4

**Armenteros, J. J. A., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., Heijne, G. V. and Nielsen, H.** (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat. Biotechnol.* **37**, 420-423. doi:10.1038/s41587-019-0036-z

**Artimo, P., Jonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., De Castro, E., Duvaud, S., Flegel, V., Fortier, A., Gasteiger, E. et al.** (2012). ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Res.* **40**, 597-603. doi:10.1093/nar/gks400

**Bao, C., Yang, Y., Huang, H., Ye, H.** (2015). Neuropeptides in the cerebral ganglia of the mud crab, *Scylla paramamosain*: transcriptomic analysis and expression profiles during vitellogenesis. *Sci. Rep.* **5**, 1-15. doi:10.1038/srep17055

**Brady, P., Elizur, A., Cummins, S. F., Ngyuen, N. H., Williams, R. and Knibb, W.** (2013). Differential expression microarrays reveal candidate genes potentially associated with reproductive dysfunction of captive-reared prawn *Penaeus monodon*. *Aquaculture* **400**, 14-28. doi:10.1016/j.aquaculture.2013.02.038

**Choi, M. Y. and Vander Meer, R. K.** (2012). Molecular structure and diversity of PBAN/pyrokinin family peptides in ants. *Front. Endocrinol.* **3**, 32. doi:10.3389/fendo.2012.00032

**Choi, M. Y., Rafaeli, A. and Jurenka, R. A.** (2001). Pyrokinin/PBAN-like peptides in the central nervous system of *Drosophila melanogaster*. *Cell Tissue Res.* **306**, 459-465. doi:10.1007/s00441-001-0467-x

**Choi, M. Y., Sanscrainte, N. D., Estep, A. S., Vander Meer, R. K. and Becnel, J. J.** (2015). Identification and expression of a new member of the pyrokinin/pban gene family in the sand fly *Phlebotomus papatasi*. *J. Insect Physiol.* **79**, 55-62. doi:10.1016/j.jinsphys.2015.06.001

**Christie, A. E. and Chi, M.** (2015). Prediction of the neuropeptidomes of members of the Astacidea (Crustacea, Decapoda) using publicly accessible transcriptome shotgun assembly (TSA) sequence data. *Gen. Comp. Endocrinol.* **224**, 38-60. doi:10.1016/j.ygcen.2015.06.001

**Christie, A. E. and Pascual, M. G.** (2016). Peptidergic signaling in the crab *Cancer borealis*: tapping the power of transcriptomics for neuropeptidome expansion. *Gen. Comp. Endocrinol.* **237**, 53-67. doi:10.1016/j.ygcen.2016.08.002

**Christie, A. E., Stemmler, E. A. and Dickinson, P. S.** (2010). Crustacean neuropeptides. *Cell Mol. Life Sci.* **67**, 4135-4169. doi:10.1007/s00018-010-0482-8

**Coccia, E., Lisa, E. D., Cristo, C. D., Cosmo, A. D. and Paolucci, M.** (2010). Effects of estradiol and progesterone on the reproduction of the freshwater crayfish *Cherax albidus*. *Biol. Bull.* **218**, 36-47. doi:10.1086/BBLv218n1p36

**Dauphin-Villemant, C., Böcking, D., Blais, C., Toullec, J. Y. and Lafont, R.** (1997). Involvement of a 3β-hydroxysteroid dehydrogenase activity in ecdysteroid biosynthesis. *Mol. Cell. Endocrinol.* **128**, 139-149. doi:10.1016/S0303-7207(97)04031-8

**Dickinson, P. S., Sreekrishnan, A., Kwiatkowski, M. A. and Christie, A. E.** (2015a). Distinct or shared actions of peptide family isoforms: I. Peptide-specific actions of pyrokinins in the lobster cardiac neuromuscular system. *J. Exp. Biol.* **218**, 2892-2904.

**Dickinson, P. S., Kurland, S. C., Qu, X., Parker, B. O., Sreekrishnan, A., Kwiatkowski, M. A., Williams, A. H., Ysasi, A. B. and Christie, A. E.** (2015b). Distinct or shared actions of peptide family isoforms: II. Multiple pyrokinins exert similar effects in the lobster stomatogastric nervous system. *J. Exp. Biol.* **218**, 2905-2917.

**Duangprom, S., Ampansri, W., Suwansa-Ard, S., Chotwiwatthanakun, C., Sobhon, P. and Kornthong, N.** (2018). Identification and expression of prostaglandin E synthase (PGES) gene in the central nervous system and ovary during ovarian maturation of the female mud crab, *Scylla olivacea*. *Anim. Reprod. Sci.* **198**, 220-232. doi:10.1016/j.anireprosci.2018.09.022

**Edgar, R. C.** (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinform.* **5**, 113. doi:10.1186/1471-2105-5-113

**Erica, H., Tyasning, N., Lyric, B. and Russell, J.** (2014). Pyrokinin/PBAN-like peptides in the central nervous system of mosquitoes. *Cell Tissue Res.* **356**, 39-47. doi:10.1007/s00441-013-1782-8

**Fingerman, M.** (1997). Roles of neurotransmitters in regulating reproductive hormone release and gonadal maturation in decapod crustaceans. *Invertebr. Reprod. Dev.* **31**, 47-54. doi:10.1080/07924259.1997.9672562

**Finn, R. D., Attwood, T. K., Babbitt, P. C., Bateman, A., Bork, P., Bridge, A. J., Chang, H. Y., Dosztányi, Z., El-Gebali, S., Fraser, M. et al.** (2017). InterPro in 2017—beyond protein family and domain annotations. *Nucleic Acids Res.* **45**, 190-199. doi:10.1093/nar/gkw1107

**Gondalia, K., Qudrat, A., Bruno, B., Medina, J. F. and Paluzzi, J. P. V.** (2016). Identification and functional characterization of a pyrokinin neuropeptide receptor in the Lyme disease vector, *Ixodes scapularis*. *Peptides* **86**, 42-54. doi:10.1016/j.peptides.2016.09.011

**Hossain, M. S. and Das, N. G.** (2010). GIS-based multi-criteria evaluation to land suitability modelling for giant prawn (*Macrobrachium rosenbergii*) farming in Companigonj Upazila of Noakhali, Bangladesh. *Comput. Electron. Agr.* **70**, 172-186. doi:10.1016/j.compag.2009.10.003

**Hui, L., Xiang, F., Zhang, Y. and Li, L.** (2012). Mass spectrometric elucidation of the neuropeptidome of a crustacean neuroendocrine organ. *Peptides* **36**, 230-239. doi:10.1016/j.peptides.2012.05.007

**Hull, J. J., Brent, C. S., Choi, M. Y., Mikó, Z., Fodor, J. and Fónagy, A.** (2021). Molecular and functional characterization of pyrokinin-like peptides in the western tarnished plant bug *Lygus hesperus* (Hemiptera: Miridae). *Insects* **12**, 914. doi:10.3390/insects12100914

**Janer, G. and Porte, C.** (2007). Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. *Ecotoxicology* **16**, 145-160. doi:10.1007/s10646-006-0110-4

**Jayasankar, V., Tomy, S. and Wilder, M. N.** (2020). Insights on molecular mechanisms of ovarian development in decapod crustacea: Focus on vitellogenesis-stimulating factors and pathways. *Front. Endocrinol.* **11**, 674. doi:10.3389/fendo.2020.577925

**Jurenka, R.** (2015). The PRXamide neuropeptide signalling system: conserved in animals. *Adv. In Insect Phys.* **49**, 123-170. doi:10.1016/bs.aiip.2015.07.001

**Kautsky, N., Rönnbäck, P., Tedengren, M. and Troell, M.** (2000). Ecosystem perspectives on management of disease in shrimp pond farming. *Aquaculture* **191**, 145-161. doi:10.1016/S0044-8486(00)00424-5

**Kiran, R. B., Rajendran, K. V., Jung, S. J. and Oh, M. J.** (2002). Experimental susceptibility of different life stages of the giant freshwater prawn, *Macrobrachium*

*rosenbergii* (de Man), to white spot syndrome virus (WSSV). *J. Fish Dis.* **25**, 201-207. doi:10.1046/j.1365-2761.2002.00357.x

**Knigge, T., LeBlanc, G. A. and Ford, A. T.** (2021). A crab is not a fish: unique aspects of the crustacean endocrine system and considerations for endocrine toxicology. *Front. Endocrinol.* **12**, 98. doi:10.3389/fendo.2021.587608

**Kuo, C. M., Chen, Y. N., Fan, H. F., Chuang, H. C. and Hsieh, S. L.** (2009). Enhancement of vitellogenin synthesis by serotonin in the giant freshwater prawn *Macrobrachium rosenbergii* (de Man). *Zool. Stud.* **48**, 597-606.

**Larionov, A., Krause, A. and Miller, W. A.** (2005). Standard curve based method for relative real time PCR data processing. *BMC Bioinform.* **6**, 62. doi:10.1186/1471-2105-6-62

**Liu, J., Zhou, T., Wang, C., Chan, S. and Wang, W.** (2021). Deciphering the molecular regulatory mechanism orchestrating ovary development of the Pacific whiteleg shrimp *Litopenaeus vannamei* through integrated transcriptomic analysis of reproduction-related organs. *Aquaculture* **533**, 736160. doi:10.1016/j.aquaculture.2020.736160

**Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C. J., Lu, S., Chitsaz, F., Derbyshire, M. K., Geer, R. C., Gonzales, N. R. et al.** (2017). CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res.* **45**, 200-203. doi:10.1093/nar/gkw1129

**Marciniak, P., Szymczak, M., Pacholska-Bogalska, J., Audsley, N., Kuczer, M. and Rosiński, G.** (2012). New myotropic and metabotropic actions of pyrokinins in tenebrionid beetles. *Gen. Comp. Endocrinol.* **177**, 263-269. doi:10.1016/j.ygcen.2012.04.008

**Markov, G. V., Gutierrez-Mazariegos, J., Pitrat, D., Billas, I. M. L., Bonneton, F., Moras, D., Hasserodt, J., Lecointre, G. and Laudet, V.** (2017). Origin of an ancient hormone/receptor couple revealed by resurrection of an ancestral estrogen. *Sci. Adv.* **3**, e1601778. doi:10.1126/sciadv.1601778

**Meeratana, P. and Sobhon, P.** (2007). Classification of differentiating oocytes during ovarian cycle in the giant freshwater prawn, *Macrobrachium rosenbergii* de man. *Aquaculture* **270**, 249-258. doi:10.1016/j.aquaculture.2007.03.009

**Meeratana, P., Withyachumnarnkul, B., Damrongphol, P., Wongprasert, K., Suseangtham, A. and Sobhon, P.** (2006). Serotonin induces ovarian maturation in giant freshwater prawn broodstock, *Macrobrachium rosenbergii* de Man. *Aquaculture* **260**, 315-325. doi:10.1016/j.aquaculture.2006.06.010

**Morris, S.** (2001). Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. *J. Exp. Biol.* **204**, 979-989. doi:10.1242/jeb.204.5.979

**Nachman, R. J., Holman, G. M., Schoofs, L. and Yamashita, O.** (1993). Silkworm diapause induction activity of myotropic pyrokinin (FXPRLamide) insect neuropeptides. *Peptides* **14**, 1043-1048. doi:10.1016/0196-9781(93)90084-T

**Nagaraju, G. P. C.** (2011). Review Reproductive regulators in decapod crustaceans: an overview. *J. Exp. Biol.* **214**, 3-16. doi:10.1242/jeb.047183

**New, M. B. and Nair, C. M.** (2012). Global scale of freshwater prawn farming. *Aquac. Res.* **43**, 960-969. doi:10.1111/j.1365-2109.2011.03008.x

**Ngernsoungnern, A., Ngernsoungnern, P., Kavanaugh, S., Sower, S. A., Sobhon, P. and Sretarugsa, P.** (2008b). The identification and distribution of gonadotropin-releasing hormone-like peptides in the central nervous system and ovary of the giant freshwater prawn, *Macrobrachium rosenbergii*. *Invertebr. Neurosci.* **8**, 49-57. doi:10.1007/s10158-008-0067-5

**Ngernsoungnern, A., Ngernsoungnern, P., Weerachatyanukul, W., Chavadej, J., Sobhon, P. and Sretarugsa, P.** (2008a). The existence of gonadotropin-releasing hormone (GnRH) immunoreactivity in the ovary and the effects of GnRHs on the ovarian maturation in the black tiger shrimp *Penaeus monodon*. *Aquaculture* **279**, 197-203. doi:10.1016/j.aquaculture.2008.04.018

**Ngernsoungnern, P., Ngernsoungnern, A., Weerachatyanukul, W., Meeratana, P., Hanna, P. J., Sobhon, P. and Sretarugsa, P.** (2009). Abalone egg-laying hormone induces rapid ovarian maturation and early spawning of the giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture* **296**, 143-149. doi:10.1016/j.aquaculture.2009.08.011

**Nguyen, T. V., Cummins, S. F., Elizur, A. and Ventura, T.** (2016). Transcriptomic characterization and curation of candidate neuropeptides regulating reproduction in the eyestalk ganglia of the Australian crayfish, *Cherax quadricarinatus*. *Sci. Rep.* **6**, 1-19. doi:10.1038/s41598-016-0001-8

**Nguyen, T. V., Rotllant, G. E., Cummins, S. F., Elizur, A. and Ventura, T.** (2018). Insights into sexual maturation and reproduction in the Norway lobster (*Nephrops norvegicus*) via *in silico* prediction and characterization of neuropeptides and G protein-coupled receptors. *Front. Endocrinol.* **9**, 430. doi:10.3389/fendo.2018.00430

**Paluzzi, J. P. and O'Donnell, M. J.** (2012). Identification, spatial expression analysis and functional characterization of a pyrokinin-1 receptor in the Chagas' disease vector, *Rhodnius prolixus*. *Mol. Cell Endocrinol.* **363**, 36-45. doi:10.1016/j.mce.2012.07.007

**Paolucci, M., Cristo, C. D. and Cosmo, A. D.** (2002). Immunological evidence for progesterone and estradiol receptors in the freshwater crayfish *Austropotamobius pallipes*. *Mol. Reprod. Dev.* **63**, 55-62. doi:10.1002/mrd.10158

**Predel, R. and Nachman, R. J.** (2001). Efficacy of native FXPRLamides (pyrokinins) and synthetic analogs on visceral muscles of the American cockroach. *J. Insect Physiol.* **47**, 287-293. doi:10.1016/S0022-1910(00)00113-X

**Raina, A. K. and Kempe, T. G.** (1990). A pentapeptide of the C-terminal sequence of PBAN with pheromonotropic activity. *Insect Biochem.* **20**, 849-851. doi:10.1016/0020-1790(90)90103-2

**Raina, A. K., Jaffe, H., Kempe, T. G., Keim, P., Blacher, R. W., Fales, H. M., Riley, C. T., Klun, J. A., Ridgway, R. L. and Hayes, D. K.** (1989). Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science* **244**, 796-798. doi:10.1126/science.244.4906.796

**Saetan, J., Senarai, T., Tamtin, M., Weerachatyanukul, W., Chavadej, J., Hanna, P. J., Parhar, I., Prasert, S. and Prapee, S.** (2013). Histological organization of the central nervous system and distribution of a gonadotropin-releasing hormone-like peptide in the blue crab, *Portunus pelagicus*. *Cell Tissue Res.* **353**, 493-510. doi:10.1007/s00441-013-1650-6

**Saetan, J., Senarai, T., Thongbuakaew, T., Kruangkum, T., Chansela, P., Khornchatri, K., Tamtin, M., Sobhon, P. and Sretarugsa, P.** (2017). The presence of abalone egg-laying hormone-like peptide in the central nervous system and ovary of the blue swimming crab, *Portunus pelagicus*, and its effect on ovarian maturation. *Aquaculture* **479**, 412-422. doi:10.1016/j.aquaculture.2017.06.007

**Saideman, S. R., Ma, M., Kutz-Naber, K. K., Cook, A., Torfs, P., Schoofs, L., Li, L. and Nusbaum, M. P.** (2007). Modulation of rhythmic motor activity by pyrokinin peptides. *J. Neurophysiol.* **97**, 579-595. doi:10.1152/jn.00772.2006

**Sakurai, N., Miki, Y., Suzuki, T., Watanabe, K., Narita, T., Ando, K., Yung, T. M. C., Aoki, D., Sasano, H. and Handa, H.** (2006). Systemic distribution and tissue localizations of human 17beta-hydroxysteroid dehydrogenase type 12. *J. Steroid Biochem. Mol. Biol.* **99**, 174-181. doi:10.1016/j.jsbmb.2006.01.010

**Schoofs, L., Holman, G. M., Hayes, T. K., Nachman, R. J. and De Loof, A.** (1991). Isolation, primary structure, and synthesis of locustapyrokinin: a myotropic peptide of *Locusta migratoria*. *Gen. Comp. Endocrinol.* **81**, 97-104. doi:10.1016/0016-6480(91)90129-T

**Slaoui, M. and Fiette, L.** (2011). Histopathology procedures: from tissue sampling to histopathological evaluation. In *Drug Safety Evaluation. Methods in Molecular Biology (Methods and Protocols)*, Vol. 691 (ed. J. C. Gautier), pp. 69-82. Humana Press.

**Subramoniam, T.** (2011). Mechanisms and control of vitellogenesis in crustaceans. *Fish Sci.* **77**, 1-21. doi:10.1007/s12562-010-0301-z

**Subramoniam, T.** (2017). Steroidal control of vitellogenesis in Crustacea: a new understanding for improving shrimp hatchery production. *Proc. Indian Natl. Sci. Acad.* **83**, 595-610.

**Suwan, S., Isobe, M., Yamashita, O., Minakata, H. and Imai, K.** (1994). Silkworm diapause hormone, structure-activity relationships indispensable role of C-terminal amide. *Insect Biochem. Mol. Biol.* **24**, 1001-1007. doi:10.1016/0965-1748(94)90137-6

**Suwansa-ard, S., Thongbuakaew, T., Wang, T., Zhao, M., Elizur, A., Hanna, P. J., Sretarugsa, P., Cummins, S. F. and Sobhon, P.** (2015). In silico neuropeptidome of female *Macrobrachium rosenbergii* based on transcriptome and peptide mining of eyestalk, central nervous system and ovary. *PLoS One* **10**, e0123848. doi:10.1371/journal.pone.0123848

**Swetha, C. H., Girish, B. P. and Reddy, P. S.** (2016). Elucidation of the role of estradiol and progesterone in regulating reproduction in the edible crab, *Oziothelphusa senex senex*. *RSC Adv.* **6**, 24959-24967. doi:10.1039/C5RA23637A

**Thitiphuree, T., Nagasawa, K. and Osada, M.** (2019). Molecular identification of steroidogenesis-related genes in scallops and their potential roles in gametogenesis. *J. Steroid Biochem. Mol. Biol.* **186**, 22-33. doi:10.1016/j.jsbmb.2018.09.004

**Thongbuakaew, T., Saetan, J., Suwansa-ard, S., Kankoun, W., Sumpownon, C., Parhar, I., Meeratana, P., Sobhon, P. and Sretarugsa, P.** (2016a). The existence of kisspeptin-like peptides and effects on ovarian development and maturation in the giant freshwater prawn *Macrobrachium rosenbergii*. *Aquaculture* **455**, 50-62. doi:10.1016/j.aquaculture.2016.01.006

**Thongbuakaew, T., Siangcham, T., Suwansa-Ard, S., Elizur, A., Cummins, S. F., Sobhon, P. and Sretarugsa, P.** (2016b). Steroids and genes related to steroid biosynthesis in the female giant freshwater prawn, *Macrobrachium rosenbergii*. *Steroids* **107**, 149-160. doi:10.1016/j.steroids.2016.01.006

**Thongbuakaew, T., Suwansa-ard, S., Sretarugsa, P., Sobhon, P. and Cummins, S. F.** (2019). Identification and characterization of a crustacean female sex hormone in the giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture* **507**, 56-68. doi:10.1016/j.aquaculture.2019.04.002

**Tinikul, Y., Engsusophon, A., Kruangkum, T., Thongrod, S., Tinikul, R. and Sobhon, P.** (2017). Neuropeptide F stimulates ovarian development and spawning in the female giant freshwater prawn, *Macrobrachium rosenbergii*, and its expression in the ovary during ovarian maturation cycle. *Aquaculture* **469**, 128-136. doi:10.1016/j.aquaculture.2016.11.026

**Tinikul, Y., Mercier, A. J., Soonklang, N. and Sobhon, P.** (2008). Changes in the levels of serotonin and dopamine in the central nervous system and ovary, and their possible roles in the ovarian development in the giant freshwater prawn, *Macrobrachium rosenbergii*. *Gen. Comp. Endocrinol.* **158**, 250-258. doi:10.1016/j.ygcen.2008.07.009

**Torfs, P., Nieto, J., Cerstiaens, A., Boon, D., Baggerman, G., Poulos, C., Waelkens, E., Derua, R., Calderón, J., Loof, A. D. et al.** (2001). Pyrokinin neuropeptides in a crustacean: isolation and identification in the white shrimp *Penaeus vannamei*. *Eur. J. Biochem.* **268**, 149-154. doi:10.1046/j.1432-1327.2001.01858.x

**Torfs, P., Nachman, R. J., Poulos, C., De Loof, A. and Schoofs, L.** (2002). Activity of crustacean myotropic neuropeptides on the oviduct and hindgut of the crayfish *Astacus leptodactylus*. *Invertebr. Reprod. Dev.* **41**, 137-142. doi:10.1080/07924259.2002.9652744

**Uawisetwathana, U., Leelatanawit, R., Klanchui, A., Prommoon, J., Klinbunga, S. and Karoonuthaisiri, N.** (2011). Insights into eyestalk ablation mechanism to induce ovarian maturation in the black tiger shrimp. *PLoS One* **6**, e24427. doi:10.1371/journal.pone.0024427

**Vaca, A. A. and Alfaro, J.** (2000). Ovarian maturation and spawning in the white shrimp, *Penaeus vannamei*, by serotonin injection. *Aquaculture* **182**, 373-385. doi:10.1016/S0044-8486(99)00267-7

**Wen, J. and Pan, L.** (2015). Short-term exposure to benzo [a] pyrene disrupts reproductive endocrine status in the swimming crab *Portunus trituberculatus*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **174**, 13-20. doi:10.1016/j.cbpc.2015.06.001

**Wongprasert, K., Asuvapongpatana, S., Poltana, P., Tiensuwan, M. and Withyachumnarnkul, B.** (2006). Serotonin stimulates ovarian maturation and spawning in the black tiger shrimp *Penaeus monodon*. *Aquaculture* **261**, 1447-1454. doi:10.1016/j.aquaculture.2006.08.044

**Yang, X., Zhao, L., Zhao, Z., Hu, B., Wang, C., Yang, Z. and Cheng, Y.** (2012). Immunolocalization of estrogen receptor α in *Neomysis japonica* oocytes and follicle cells during ovarian development. *Tissue Cell.* **44**, 95-100. doi:10.1016/j.tice.2011.12.001

**Ye, H., Huang, H., Song, P. and Wang, G.** (2010). The identification and distribution of progesterone receptors in the brain and thoracic ganglion in the mud crab *Scylla paramamosain* (Crustacea: Decapoda: Brachyura). *Invertebr. Neurosci.* **10**, 11-16. doi:10.1007/s10158-010-0100-3

**Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T. L.** (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC bioinform.* **13**, 134. doi:10.1186/1471-2105-13-134

**Zeng, H., Bao, C., Huang, H., Ye, H. and Li, S.** (2016). The mechanism of regulation of ovarian maturation by red pigment concentrating hormone in the mud crab *Scylla paramamosain*. *Anim. Reprod. Sci.* **164**, 152-161. doi:10.1016/j.anireprosci.2015.11.025