**Role of Halloween genes in ecdysteroids biosynthesis of the
swimming crab (*Portunus trituberculatus*): implications from RNA
interference and eyestalk ablation**

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Abstract: Molting, including metamorphosis molting in arthropods are controlled by the ecdysteroids that are synthesized and secreted by the crustacean Y-organ (YO) or the insect prothoracic gland (PG). The Halloween genes encoding the enzymes mainly involved in the biosynthesis of ecdysteroids are well studied in insects but not in crustaceans. Given the importance of Halloween genes in ecdysteroids biosynthesis, we have previously reported the cDNA cloning of disembodied (*Dib*) in *P. trituberculatus*. Here, cDNA sequences of another two Halloween genes, Spook (*Spo*) and Shadow (*Sad*), were further identified and characterized. The predicted amino acid sequences for these two Halloween genes of *P. trituberculatus* were compared to those of several other arthropods, and several typical domains of the cytochrome P450 mono-oxygenase (CYP) were identified. Similar to the tissue distribution of *Dib*, the *Spo* and *Sad* also showed high specificity to the YO. RNA interference (RNAi) of these 3 genes indicated they all play essential role in ecdysteroids biosynthesis. To investigate the relationships of the Halloween genes to the eyestalk neuropeptides such as molt-inhibiting hormone (MIH), effects of eyestalk ablation (ESA) on the expression of *Dib*, *Spo* and *Sad* were detected. Expression of *Dib* and *Sad*, but not *Spo*, was significantly induced by ESA. The result indicated that the inhibition of MIH in ecdysteroids biosynthesis may be partly through the transcriptional regulation of certain Halloween genes, such as *Dib* and *Sad*, while the *Spo* might not be the target for MIH signal.

Keywords: Halloween genes; Ecdysteroids; molting; *Portunus trituberculatus*; expression pattern; RNAi; eyestalk ablation

1. Introduction

Molting, including metamorphosis molting in arthropods are controlled by the ecdysteroids, which function as crucial coordinators of cell proliferation, differentiation, and apoptosis (Schwedes and Carney, 2012). The ecdysteroids are polyhydroxylated C27 steriods that synthesized and secreted by a pair of molting glands: Y-organ (YO) in crustaceans and the prothoracic gland (PG) in insects. 20-hydroxyecdysone (20E) has been characterized as the primary physiologically active form of the ecdysteroids in insects, whereas the crustaceans may possess more than one active form in addition to 20E (Mykles, 2011).

Recent advances in elucidating the biosynthetic pathway for ecdysteroids in insects and crustaceans suggest the origin of this pathway is deeply rooted in the evolutionary history of arthropods (Rewitz and Gilbert, 2008; Sin et al., 2015). Among seven enzymes that are mainly involved in the biosynthesis of ecdysteroids, five of them are cytochrome P450 mono-oxygenases (CYPs). The genes for these seven enzymes, collectively known as Halloween genes, include neverland (*nvd*), non-molting glossy/shroud (*sro*), spook (*spo*, CYP307A1), phantom (*phm*, CYP306A1), disembodied (*dib*, CYP302A1), shadow (*sad*: CYP315A1) and shade (*shd*: CYP314A1). In early steps of the steroidogenic pathway, the dietary cholesterol is taken up by the molting gland, and converted to 7-dehydrocholesterol (7DC) by 7, 8-dehydrogenase, encoded by the *Nvd* gene (Yoshiyama et al., 2006).

7DC is then converted to Δ^4 -Diketol, the central precursor to the multiple derivative steroid products, by a series of reactions involving enzymes encoded by *Sro* and *Spo* or its paralogous genes (Ono et al., 2006; Niwa et al., 2010). The last four steps of this pathway are catalyzed by the hydroxylases at C25, C22, C2, and C20, which are encoded by *Phm*, *Dib*, *Sad* and *Shd*, respectively (Mykles, 2011).

Although the ecdysteroids biosynthesis pathways are similar in insects and crustaceans, their neurohormonal control seemed to be opposite in action. Unlike the PG ecdysteroidogenesis is under stimulatory regulation by prothoracicotropic hormone (PTTH), the primary regulation of YO ecdysteroid synthesis is inhibitory (Covi et al., 2012). The YO ecdysteroidogenesis is negatively regulated by two neuropeptides synthesized and secreted by the X-organ/sinus gland (XO-SG) complex in eyestalk, namely molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH). These neuropeptides are members of CHH family which control growth, reproduction, osmoregulation, and metabolism in crustaceans (De Kleijn and Van Herp, 1995; Lacombe et al., 1999; Nakatsuji et al., 2009; Chung et al., 2010; Webster et al., 2012). Both peptides have been proved to suppress the secretion of ecdysteroids by YO *in vitro* (Nakatsuji and Sonobe, 2004; Lee et al., 2007) and *in vivo* (Chang et al., 1990; Chung, 2010), although MIH is 10–20 times more effective than CHH (Chung and Webster, 2003). The suppression of MIH may act through the transcriptional or translational regulation (Chang and Mykles, 2011). As in the shrimp, *Marsupenaeus japonicus*, the sharply increase in *Phm* expression during premolt can be blocked by sinus gland extract and recombinant MIH (Asazuma et al., 2009), the Halloween genes may be a target of MIH signaling.

The swimming crab, *Portunus trituberculatus*, is a commercially important crustacean species and is one of the most common edible crabs in Chinese waters. As it has been extensively artificially propagated and cultivated, comprehensive understanding on the ecdysteroids-mediated molting process is essential for controlling its growth and development. Given the importance of Halloween gene in ecdysteroids biosynthesis, we have tried for several years to clone the *P. trituberculatus* Halloween gene orthologs using degenerate primers based on the *Daphnia*, *Drosophila*, and *Bombyx* genes, but only the full-length cDNA of *Dib* (GenBank accession no. **KM596851**) was obtained (Liu et al 2015). Here, we cloned the full-length cDNA of another two Halloween genes, *Pt-spo* and *Pt-sad*, based on their sequence information in our *P. trituberculatus* transcriptome database. Their tissue distribution and expression patterns during the molt cycle were detected using qPCR. In addition, the roles of these three Halloween genes in ecdysteroids biosynthesis of *P. trituberculatus* were further investigated by RNA interference (RNAi) and eyestalk ablation (ESA).

2. Materials and methods

2.1 Experimental animals

Wild swimming crabs (body weight: 35-50 g) were collected and temporarily reared at the Ninghai Deshui Aquaculture Farm. All crab investigations were carried

out according to Animal Care and Use of Science and Technology guidelines. Crabs were maintained in large tanks with through-flowing sea water and fed with live razor clams once a day. Molting stages of experimental animals were determined by observing their morphological characteristics as previously described (Shen et al., 2011). Intact crabs with no injuries or missing pereopods were selected for sampling, and YOs of each staged crab were removed, and placed in RNA preservation fluid (Sangon), and stored at -20°C. For the intermolt crabs, tissues including mandibular organ, eyestalk, thoracic ganglia, hepatopancreas, epidermis, testis, ovary, and heart were also sampled for detecting the tissue distribution of *Pt-spo* and *Pt-sad*.

2.2 RNA isolation and cDNA preparation

Total RNA of tissue samples was extracted using Trizol RNA isolation reagent (Sangon) according to the manufacturer's instructions, and genomic DNA was removed by DNase I (Takara). RNA concentrations was determined using a NanoDrop 2000 UV Spectrophotometer (Thermo Fisher), and RNA integrity was determined by visualization of clear bands of the 18S and 28S ribosomal RNA on agarose gel. cDNA was then prepared with 1 µg RNA using Perfect Real Time PrimeScript® RT reagent Kit (Takara), and stored in -80°C.

2.3 Cloning and sequence analysis of *Pt-spo* and *Pt-sad*

All Gene-specific primers in cDNA cloning were designed from the sequence information in our *P. trituberculatus* transcriptome database (Table 1). The cDNA of YO was used as templates for PCR validation of the sequence information. To obtain the full-length cDNA, 5' RACE and 3' RACE were performed according to the manufacturer's instructions of SMARTer™ RACE cDNA Amplification kit (Clontech, USA). The following program was used for each PCR amplification: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C (58°C in 3' RACE) for 30 s, and 72°C for 90 s, with a final elongation at 72°C for 10 min. All PCR products were analyzed by 1% agarose gel electrophoresis, and the PCR products were gel-purified using the PCR purification kit (Sangon), ligated with the pMD19-T vector (Takara), and transformed into competent *Escherichia coli* cells. After transformation, three positive clones were picked for sequencing (Invitrogen).

Sequence splicing and open reading frame (ORF) identification were performed using Vector NTI 10.0 software. The deduced protein sequences were aligned with representative Spo and Sad sequences selected in the NCBI database using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). The phylogenetic tree was generated based on the representative protein sequences of Halloween genes using MEGA 5.0.

2.4 RNA interference

Amplicons of *Pt-spo*, *Pt-dib*, *Pt-sad*, and green fluorescent protein (GFP) were cloned into pMD19-T vector (Takara) and amplified by PCR using a T7 promoter linked primers (Table 1). The resultant DNA was used as templates to synthesize

dsRNA. The dsRNA was synthesized and purified according to the manufacturer's instructions of the MEGAscript RNAi Kit (Ambion). Integrity of the dsRNA was checked on agarose gel, and concentration of the dsRNA were checked with a NanoDrop 2000 UV Spectrophotometer (Thermo Fisher).

The RNAi experiment was conducted in a stage-dependent manner. Pt-spo dsRNA was injected into the intermolt crabs, while Pt-dib and Pt-sad dsRNA were respectively injected into the premolt (D₁ stage) crabs. For each stage, crabs receiving crab saline (Duan and Cooke, 1999) and GFP dsRNA were set as control. 150 µL crab saline or 300 µg (dissolved in 150 µL crab saline) dsRNA was injected via the base of the last walking leg with 1 mL syringe. 5 crabs in each injection group were sacrificed at 0, 1, 2, and 4 d post injection. YO and hemolymph sample of each crab were then obtained for further analysis.

2.5 Eyestalk ablation (ESA)

The intermolt crabs were divided into two groups, the eyestalk ablation (ESA) group and the non-surgery group. For the ESA group, the eyestalks of individual crabs were clipped bilaterally using sharp sterile scissors and the wounds were cauterized with flame-heated sharp spatula to minimize hemolymph loss and infection. 5 crabs in each group were sacrificed at 0, 1, 2, 4 and 8 d post ESA. YO and hemolymph sample of each crab were then obtained for further analysis.

2.6 Analysis of gene expression

Gene expression in this study were all determined using quantitative real time PCR (qPCR). The cDNAs prepared above were amplified with gene specific primers and normalized relative to the beta-actin gene of *P. trituberculatus* (Table 1). PCR was carried out using the SYBR[®] Premix Ex TaqTM II kit (Takara) according to the manufacturer's instructions. PCR conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, and 58°C for 20 s. To confirm product specificity, a melting curve analysis of the amplified DNA was performed following amplification, at temperatures between 55 and 95°C, with the temperature increasing at a rate of 0.2°C /s. Each cDNA sample was assayed three times and data expressed as mean $\pm$ SEM. Results were analyzed by the $2^{-\Delta\Delta C_t}$ method. Transcript abundance in a representative replicate was set as calibrator. All other quantities were expressed as an n-fold difference relative to the calibrator.

2.7 Quantification of hemolymph 20E titer

The 20E levels in above hemolymph samples were detected using HPLC-QqQ MS as previously described (Zhao et al., 2011). In briefly, 0.3 ml hemolymph was collected through the arthrodial membrane between the coxa and the base of the cheliped using a 1-mL syringe with a 23-gauge needle. The hemolymph was then homogenized in 1.2 ml acetonitrile with 30 ng homobrassinolide added as internal standard and centrifuged for 15 min at 12000 rpm. The supernatant was evaporated and re-dissolved in a solvent mixture of 0.25 mL methanol and 0.05 mL 0.5% acetic acid. Samples were eluted through a Hypersil Gold C18 column (100 mm $\times$ 2.1 mm

× 1.7 μm, Thermo Fisher) and analyzed by TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer (Thermo Fisher). The mass spectrometry was performed in positive mode using ions at m/z 445.2 and 459.4 as quantitative ions for 20E and homobrassinolide, respectively. The amount of 20E in each sample was determined by comparing the peak area of 20E to that of homobrassinolide.

2.8 Statistical analysis

All results represent mean ± 1 SEM (n), in which “n” is the number of replicates. SPSS 19.0 was used to evaluate the statistical significance of the results. The data were subjected to the normality test using the Kolmogorov–Smirnov and Cochran tests prior to all statistical tests. The data that did not show a normal distribution were analyzed using nonparametric ANOVA (Mann-Whitney Rank Sum test). In all cases, statistical significance was accepted at P < 0.05 and noted with letters or asterisks.

3. Results

3.1 Cloning and characterization of *Pt-spo* and *Pt-sad*

Sequences of the full length cDNA for *Spo* (*Pt-spo*) and *Sad* (*Pt-sad*) were submitted to GenBank with the accession number **KM030021** and **KM880023** respectively (Supplementary File 1). Analysis of these sequences revealed the open reading frame (ORF) encoding putative protein of 520 amino acids for *Pt-spo* and 533 amino acids for *Pt-sad* (Supplementary File 2 and 3). The predicted amino acid sequences for *Pt-spo* and *Pt-sad* in *P. trituberculatus* shared the highest identities (62% for *Pt-spo* and 54% for *Pt-sad*) with those in the shrimp *Neocaridina denticulate*, although the sequences in *N. denticulate* are incomplete. *Pt-sad* have five conserved cytochrome P450 motifs, such as WxxxR (Helix-C), GxE/DTT/S (Helix-I), ExxR (Helix-K), PxxFxPE/DRF (PERF motif) and PFxxGxRxCxG/A (heme-binding domain), where ‘x’ means any amino acid sequence (Feyereisen, 1999). While in *Pt-spo*, Helix-C and Helix-I were not well conserved. A phylogenetic tree was constructed based on the amino acid sequences of representative CYP Halloween genes selected in the NCBI database. The phylogenetic tree clearly separated into two clusters of CYP2 clan (*Spo* and *Phm*) and mitochondrial clan (*Dib*, *Sad* and *Shd*) (Nelson, 2006), and showed individual clusters for each gene (Supplementary File 4).

3.2 Tissue- and molting stage-specific expression of *Pt-spo* and *Pt-sad*

By using qPCR, the transcript level of *Pt-spo* and *Pt-sad* were detected in several tissues including YO, mandibular organ (MO), eyestalk (ES), thoracic ganglia (TG), hepatopancreas (HP), epidermis (EP), testis (TE), ovary (OV), and heart (HT). Similar to the tissue distribution of *Pt-dib* in previous study (Liu et al., 2015), *Pt-spo* and *Pt-sad* in this study were highly expressed in the YO, while their transcripts were barely detected in other tissues (Figure 1). During the molt cycle, the expression of *Pt-spo* increased from postmolt (stage A and B), to the maximum

at intermolt (stage C), then slowly declined during premolt. As for the expression pattern of *Pt-sad*, it showed a significantly increase at D₁ substage of premolt, and the level was maintained at D₂ substage, then sharply dropped at D₃ and D₄ substage (Figure 2).

3.3 Effect of RNAi on gene expression and hemolymph 20E titers

Role of *Pt-spo*, *Pt-dib*, and *Pt-sad* in the ecdysteroids synthesis was examined using the RNAi-mediated gene knockdown. As shown in Figure 3, expression of *Pt-spo*, *Pt-sad*, and *Pt-dib* were all significantly down-regulated by injection of their corresponding dsRNA, indicating a high RNAi efficiency in this study. In addition, RNAi of *Pt-spo* decreased the mRNA level of *Pt-dib* and *Pt-sad*, but *Pt-spo* expression was not affected by *Pt-dib* or *Pt-sad* dsRNA treatment. By contrast, the mRNA level of *Pt-sad* was significantly down-regulated by RNAi of *Pt-spo* and *Pt-dib*, but *Pt-sad* dsRNA treatment had no effect on expression of *Pt-spo* and *Pt-dib* (Figure 3).

The effect of RNAi on hemolymph 20E titer was estimated by comparing the 20E titer of dsRNA injection group to that of crab saline injection group. In the *Pt-spo* dsRNA injection group, hemolymph 20E titer was lower than that in the crab saline injection group at 2 d post injection, but not statistically significant (Figure 4A). Treatment with *Pt-dib* dsRNA caused a significant decrease in 20E titer at 2d post injection (Figure 4B), while *Pt-sad* dsRNA caused a significant decrease in 20E titer at 4d post injection (Figure 4C).

3.4 Effect of ESA on gene expression and hemolymph 20E titers

To further investigate the relationships of *P. trituberculatus* Halloween genes to the eyestalk neuropeptides such as MIH, the effects of the eyestalk removal on gene expression of *Pt-spo*, *Pt-dib*, and *Pt-sad* were examined. Expression of *Pt-dib* and *Pt-sad* was induced by ESA, and the significantly induction of *Pt-dib* occurred at 2 d, 4 d, and 8 d post ESA, while of *Pt-sad* the induction occurred at only 4 d post ESA (Figure 5). By contrast, there was no significant difference in *Pt-spo* expression at any time point post ESA (Figure 5).

ESA caused a rapid and strong increase in hemolymph 20E titer. As showed in Figure 6, the 20E titer was significantly elevated from about 1.78 ng/mL to about 2.5-fold higher in 2 days after ESA. Much higher 20E level was found at 4 d and 8 d post ESA, and the corresponding 20E titer was about 12.57 ng/mL and 14.92 ng/mL, respectively (Figure 6). We also observed a slowly climb in 20E titer in the non-surgery group, and the 20E titer increased to 2.77 ng/ml at 8 d post ESA. As most intact crabs at this time had progressed into the premolt stage (Data not shown), this result would be reflecting the variation of hemolymph 20E titer in part of the normal molting process.

4. Discussion

We have now obtained the full length cDNA of three Halloween genes in the

swimming crab, *P. trituberculatus*, *Pt-dib* in our previous study (Liu et al., 2015), and *Pt-spo* and *Pt-sad* in the present study. All three genes belong to the cytochrome P450 mono-oxygenases (CYPs) gene family and contain the typical P450 motifs, such as Helix-C, Helix-I, Helix-K, PERF motif and heme-binding domain (Feyereisen, 1999). These motifs are well conserved in *Pt-dib* and *Pt-sad*, while in *Pt-spo*, the Helix-C and Helix-I are not conserved. In insect *Spos*, the Helix-C and Helix-I are not conserved either, but the putative sequences of these region are very similar, making them a common characteristic of insect *Spos* (Iga and Smagghe, 2010; Marchal et al., 2011). In crustacean *Spos*, this is also the case for the sequence of Helix-I, but not for that of Helix C. The putative sequence of Helix C in *Pt-spo* was not even similar to that in the shrimp *N. denticulate*. Whether the difference in Helix C means a varied functional role of *Spo* remains to be seen.

The essential role of Halloween genes on ecdysteroids biosynthesis has been largely described in insects, and mutations in the Halloween genes result in cuticle deformities and embryonic lethality attributable to ecdysteroids deficiencies (Chavez et al., 2000; Gilbert, 2004; Rewitz et al., 2006; Niwa and Niwa, 2011). Similarly, deficiencies of *Pt-spo*, *Pt-dib* and *Pt-sad* expression all led to a reduction in hemolymph 20E titer, suggesting the essential role of Halloween genes in the crustacean ecdysteroidogenesis. In addition, the transcripts of *Pt-spo* and *Pt-sad* were mainly located in YO, and this is also the distribution property for *Pt-dib* transcripts (Liu et al., 2015). As YO is the primary site of ecdysteroids biosynthesis in crustaceans (Mykles, 2011), the tissue distribution of Halloween genes indicated they may be functional specific to ecdysteroid biosynthesis in crustaceans.

As the major active form of ecdysteroids, 20E initiates and coordinates the crustacean molting process. Hemolymph 20E concentration fluctuates dramatically during the molt cycle of *P. trituberculatus*, i.e. from < 2 ng/mL in postmolt and intermolt crabs to >20 ng/mL in the late premolt (D₃ substage) crabs (Wang et al., 2013). When comparing the expression of the Halloween genes and the hemolymph 20E titer during the molt cycle of *P. trituberculatus*, it was striking that the expression of *Pt-sad* showed a steep increase and the level was kept at the maximum level between D₁ and D₂ substage of premolt. The expression pattern of *Pt-sad* is consistent with that of *Pt-dib* previously reported (Liu et al., 2015), and concerts with the hemolymph fluctuation in 20E titer. As for *Pt-spo*, it was most expressed in the intermolt stage, and the level was not significantly reduced until D₂ substage. The earlier peak of *Pt-spo* expression during the molt cycle suggested a putative regulatory role of *Pt-spo* on the expression of genes located downstream of the ecdysteroids biosynthesis pathway. More direct evidence for this conclusion is provided by RNAi experiment which indicated that the *Pt-spo* dsRNA treatment caused significantly decreases in expression of *Pt-dib* and *Pt-sad*, while the RNAi of the latter two genes had no effect on *Pt-spo* expression. In fact, similar relationship was also found between the expression of *Pt-dib* and *Pt-sad*, as deficiency of *Pt-dib* expression led to the reduction of *Pt-sad* but not vice versa. Thus, the control of ecdysteroids biosynthesis by Halloween genes might be cascaded.

In the shrimp, *Marsupenaeus japonicus*, the sharply increase in *Phm* expression

during premolt can be blocked by sinus gland extract and recombinant MIH (Asazuma et al., 2009). Therefore, the negative control of MIH on YO ecdysteroidogenesis may act through the transcriptional regulation of the Halloween genes. We applied the eyestalk ablation surgery to test this hypothesis. ESA caused a rapidly and sharply increase in the hemolymph 20E titer, similar to the results reported in some other species (Chang et al., 1976; McDonald et al., 2011; Abuhagr et al., 2014). The rise in hemolymph 20E titer may be partly due to the induced expression of *Pt-dib* and *Pt-sad* by ESA, suggesting an inhibitory role of MIH on expression of these two genes. Our result also indicated that *Pt-spo* might not be the transcriptional target for MIH signal, as the mRNA level of *Pt-spo* was not affected by ESA. Studies on illustrating the inhibition of MIH in YO ecdysteroidogenesis have revealed that, during a natural molt cycle, the reduction in amount of MIH during early premolt triggers YO activation, which results in increased ecdysteroids biosynthetic capacity of this organ (Chang and Mykles, 2011). Therefore, the MIH target genes should be elevated during the premolt and after the amount of MIH is reduced. By checking the expression level of *Pt-spo* at different molting stages, which was not elevated during premolt, we also concluded that *Pt-spo* is not involved in the transcriptional inhibition by MIH.

In conclusion, we cloned the full-length cDNA sequences of another two Halloween genes in *P. trituberculatus*, namely *Pt-spo* and *Pt-sad*, in addition to the previously reported *Pt-dib*. All three genes are predominantly expressed in YO and play essential role in ecdysteroids biosynthesis. Relationships of their gene expression to MIH was further investigated by eyestalk ablation. We found that the inhibition of MIH in ecdysteroids biosynthesis may be partly through the transcriptional regulation of certain Halloween genes. The *Dib* and *Sad* were the putative MIH targets, while the *Spo* might not be regulated by MIH signal.

Acknowledgments

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Legends

Figure 1. Tissues distribution of Halloween genes in *P. trituberculatus*. Tissue abbreviations are as follows: YO, Y-organs; MO, mandibular organ; ES, eyestalk; TG, thoracic ganglia; HP, hepatopancreas; EP, epidermis; TE, testis; OV, ovary; HT, heart. Analysis was done by qPCR using tissues from five different animals.

Figure 2. Relative expression of *Pt-spo* and *Pt-sad* in YO during the molt cycle of *P. trituberculatus*. Molt stages are postmolt (A and B), intermolt (C) and premolt (D0–D4). A replicate from stage A was used as calibrator for each gene. Bars represent mean + SEM (n=5). Different letters indicates statistically significant differences ($P < 0.05$).

Figure 3. RNAi efficiency of *Pt-spo* (A), *Pt-dib* (B), and *Pt-sad* (C) dsRNA. The mRNA level of *Pt-spo*, *Pt-dib*, and *Pt-sad* in YO was detected at 1 d post injection. A replicate from the crab saline injection group was used as calibrator for each gene. Bars represent mean + SEM (n=5). Asterisks (*) represent values statistically ($P < 0.05$) different from the crab saline injection group.

Figure 4. Time-coursed effect of *Pt-spo* (A), *Pt-dib* (B), and *Pt-sad* (C) dsRNA treatments on hemolymph 20E titer. Bars represent mean + SEM (n=5). Asterisks (*) represent values statistically different ($P < 0.05$) from the crab saline injection group at same time-point.

Figure 5. Effect of ESA on YO expression of *Pt-spo*, *Pt-dib*, and *Pt-sad*. A replicate from the intact animals (0 d) was used as calibrator for each gene. Bars represent mean + SEM (n=5). Asterisks (*) represent values statistically ($P < 0.05$) different from the intact animals.

Figure 6. Effect of ESA on hemolymph 20E titer. Bars represent mean + SEM (n=5). Different letters indicates statistically significant differences within each group ($P < 0.05$), and asterisks (*) represent values statistically ($P < 0.05$) different from the intact group at same time-point.

Table 1. Primers used in this study

Primer	Sequence(5' - 3')	Purpose
Spo-F	CGCACGGAGACGAAGAAA	RT-PCR
Spo-R	CAAAGCAGGCGTCCACTAAG	RT-PCR
Sad-F	TAACCGCTTTGGGTCGCT	RT-PCR
Sad-R	GGATAACCATCGTTCCACCAG	RT-PCR
Spo-GSPF	GTGGAGGACTTAGTGGACGC	3'-RACE
Spo-GSPR	GCGGTTTCCTCCGAAGTAG	5'-RACE
Sad-GSPF	ACGCCTTCATCCCCTTTG	3'-RACE
Sad-GSPR	TGATAGTGCGGTGTTTTCTCC	5'-RACE
Spo-QF	GGGACGAGCCCAATAAGTT	qPCR
Spo-QR	CTGGTGCTGAAAGGGATGA	qPCR
Dib-QF	TGCGAGTCTGCTTGAGGTG	qPCR
Dib-QR	AGCCATTGTCAGTGGGGAG	qPCR
Sad-QF	CACGGCATTTCCTCAAGGAGA	qPCR
Sad-QR	AAGGCGTCATCCAGGCACT	qPCR
Actin-QF	CGAAACCTTCAACACTCCCG	qPCR
Actin-QR	GATAGCGTGAGGAAGGGCATA	qPCR
Spo-IF	<u>TAATACGACTCACTATAGGG</u> ACTTCCAACAAGCCTCACCG	RNAi
Spo-IR	<u>TAATACGACTCACTATAGGGG</u> GTTGAGGGAGCAGTATGACA	RNAi
Dib-IF	<u>TAATACGACTCACTATAGGG</u> AAGATGAGGCGTGAAGGG	RNAi
Dib-IR	<u>TAATACGACTCACTATAGGGG</u> CAATGAGGTAAAGCCAAGA	RNAi
Sad-IF	<u>TAATACGACTCACTATAGGG</u> GCGAAGTGCCTGGATGACG	RNAi
Sad-IR	<u>TAATACGACTCACTATAGGGT</u> GTATTTCGGAGCCAACG	RNAi
GFPIF	<u>TAATACGACTCACTATAGGG</u> GCGACGTAAACGGCCACAAGT	RNAi
GFPIR	<u>TAATACGACTCACTATAGGG</u> GCTTGACAGCTCGTCCATGC	RNAi

T7 promoter sequence was underlined.