



Characterization and expression analysis of seven putative JHBPs in the mud crab *Scylla paramamosain*: Putative relationship with methyl farnesoate

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ABSTRACT

Juvenile hormone binding proteins (JHBPs) serve as the carriers that transport juvenile hormone (JH) to target tissues to activate JH signals. In this study, seven JHBPs from mud crab *Scylla paramamosain* were obtained. All Sp-JHBPs contained one JHBP domain, and Sp-JHBP4, 5, 6 also contained one Grp7_{allergen} domain. The predicted secondary and 3D structures are conserved among the seven Sp-JHBP domains, but the complete 3D structure of Sp-JHBP4, 5, 6 were more similar to lipopolysaccharide binding protein. Additionally, the potential ligands for Sp-JHBP1, 2, 3, 7 were ubiquinone 8 (UQ8) and JH II or JH III, and the predicted binding location and sites for JHs were similar to the structured known BmJHBP, suggesting that MF is a potential ligand for these Sp-JHBPs. Furthermore, the expression analysis showed that *Sp-JHBP1* expression is relatively low in the 14 detected tissues, and *Sp-JHBP2* has the highest expression in the ovary, while the other five JHBPs are highly expressed in the hepatopancreas. During larval development, *Sp-JHBP2* is highly expressed during the Z1, M and C1 stages, which are three post-metamorphosis stages, while *Sp-JHBP3* and 4 expression levels were significantly lower during the Z5 and M stages, which are two pre-metamorphosis stages. Moreover, the *in vivo* and *in vitro* study revealed that *Sp-JHBP2* expression was reduced by MF, while the *in vitro* studies showed that *Sp-JHBP3*, 6, 7 are induced by MF in a concentration-independent manner. Taken together, we hypothesized Sp-JHBP1, 2, 3, 7 has the potential to bind MF and Sp-JHBP2, 3, 7 play a more important roles in MF signal, while Sp-JHBP4, 5, 6 were more likely to participate in immune response.

1. Introduction

The sesquiterpenoid methyl farnesoate (MF) is the non-epoxidized form or precursor of JH III, which is the most common type of juvenile hormone (JH) in insects, and MF is considered the primary juvenile hormone in crustaceans (Nagaraju, 2007). In crustaceans, MF is synthesized and secreted by the mandibular organ (MO), which is homologous to the corpora allata (CA) in insects. The biosynthetic pathway of MF has been established based on the general mevalonate pathway in vertebrates and the biosynthesis of JH in insects (Borst et al., 2001). Many genes involved in the MF biosynthesis pathway have been identified, and their putative roles in developmental regulation have been studied (Zhao et al., 2015; Li et al., 2010; Xie et al., 2016). MF has been shown to regulate gonad development (Medesani et al., 2012; Nagaraju et al., 2003), moulting (Reddy et al., 2004; Yudin et al., 1980), metamorphosis (Smith et al., 2000) and sex determination (Zhou et al.,

2018; Toyota et al., 2015). However, the molecular mechanism of MF signalling in crustaceans is poorly understood. In insects, JH is secreted by the CA, is transferred to target tissues by binding juvenile hormone binding protein (JHBP) and then binds to a bHLH-PAS family nuclear receptor methoprene-tolerant (Met) (Jindra et al., 2013) to activate gene transcription through both nuclear (Minakuchi et al., 2008) and membrane-initiated (Liu et al., 2015) signals. Recently, the molecular mechanism of JH signalling has made revolutionary progress, primarily due to the establishment of Met as the JH receptor in insects. However, knowledge regarding JHBP, especially in crustacean species, is relatively scarce.

JHs are highly hydrophobic molecules that are easily degraded by juvenile hormone esterases (JHE); thus, JHBPs perform a protective role by delivering this hormone signal (Park et al., 1993). The currently reported JHBPs included haemolymph JHBP (hJHBP), nuclear JHBP (nJHBP) and cytosolic JHBP (cJHBP). Notably, hJHBPs are involved in

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the transportation and protection of JH, and > 99% of JH molecules bind to hJHBPs (Park et al., 1993); thus, hJHBPs could be used for a quantitative assay of JH in haemolymph to some extent (Glinka et al., 1995), while nJHBP and cJHBP might participate in JH signal transduction. The nJHBP have a high affinity for JH III (Ismail and Gillott, 1996), and the mRNA expression and protein level of nJHBPs are consistent with the anti-metamorphic effects of JH, suggesting that nJHBP might transfer the JH signal in the larvae of *Manduca sexta* (Palli et al., 1994). Approximately 2500 cJHBP molecules are present per *Drosophila* Kc cell (Chang et al., 1980), and the resistant effect to JH III of *Met* mutant fruit flies might be a result of a reduced binding affinity to cJHBP (Shemshedini and Wilson, 1990). The existence of methyl farnesoate binding protein (MFBP) in some crustacean species has been confirmed (Takac et al., 1993; Tamone et al., 1997). However, knowledge regarding the sequences of these proteins is limited. To date, partial cJHBP sequences have been reported in *Neocaridina denticulata* (Sin et al., 2015), and the cJHBP sequences in *Cherax quadricarinatus* and *Lepeophtheirus salmonis* have been deposited in GenBank; Recently we had reported the cJHBP from mud crab (Qian et al., 2019), however, information regarding hJHBP and nJHBP in crustaceans is scarce.

Currently, the classification for JHBP domain containing proteins is still a problem. Except for the hJHBP, another well studied JHBP domain containing protein is Takeout. Takeout has been proved to participate in many other physiological processes, including circadian rhythm, behaviour and life span. In *Drosophila*, starvation induces Takeout expression, but mutations in the circadian rhythm genes (*clk*, *cyc*, *per* and *tim*) perturbs this inducement (Sarav-Blat et al., 2000). Takeout mRNA exhibits daily cycling expression, but this activity is delayed relative to that of the clock mRNAs, including *per* and *tim* (So et al., 2000). Additionally, Takeout participates in courtship behavior as a mutation in Takeout reduces male courtship behaviour, and this behaviour can be synergized by *fruitless* mutations (Dauwalder et al., 2002). Interestingly, the overexpression of Takeout increases longevity, and this phenotype can be reversed by treatment with the JH analog methoprene (Chamseddin et al., 2012). Thus, a comprehensive analysis of the JHBP family is highly important.

The mud crab *Scylla paramamosain* belongs to the genus *Scylla* (Family: Portunidae) and is the most common mud crab in China (Ma et al., 2006). Because this mud crab is among the most commercially important crustacean species, many research teams have explored its aquaculture and genetic breeding. The regulatory mechanism of its development, particularly larval and gonad development, deserves in-depth investigations. In this study, seven JHBP sequences were obtained from the mud crab. A comprehensive analysis of these seven JHBPs was conducted, and we hope that our results give impetus to MF functional studies and provide a direction for further studies.

2. Material and methods

2.1. Ethics statement

All animal experiments performed in this study were conducted in accordance with the relevant national and international guidelines. Our project was approved by the East China Sea Fisheries Research Institute. In China, catching mud crabs in seawater does not require specific permits. Our study did not involve endangered or protected species.

2.2. Collection of zygotes, larvae and adult tissue

Ten different stages of *S. paramamosain* zygotes and larvae were collected from the Qionghai Research Center (Hainan province), washed twice with physiological saline, fixed with RNA fixer (Bioteke, Beijing, China), and stored at -80°C until the RNA extraction. Larvae at different stages were identified under a microscope based on the morphological analysis of *Scylla serrata* (currently known as *S. paramamosain*) performed by Zeng et al. (2001).

Healthy adult females at ovary developmental stages II and III were collected from the First Market of Wenchang City, Hainan Province. The ovarian stages were identified by visual examination according to the study by Shangguan et al. (1991). The adult tissues were collected by quick dissection, fixed in RNA fixer and stored at -80°C until the RNA extraction.

2.3. In vivo and in vitro MF treatment

Methyl farnesoate (CAS: 10485-70-8) was purchased from Echelon Biosciences (Salt Lake City, UT, USA), dissolved in DMSO (analytical grade), and serially diluted to an appropriate working concentration. Female crabs at ovary developmental stage II (early development stage, 120 ± 30 g) were used for the *in vivo* studies. The adult crabs received an injection of 50 μL MF solutions using the insulin syringes at the base of the right third pereopod. The final concentration of MF in the crab was calculated by the ratio between hemolymph volume and body mass according to Reddy (1980), in which he found that the hemolymph volume (mL) of the crab is approximately 27% of body mass. At least three crabs were sacrificed 4 h and 6 h after the injection, and the hepatopancreas and ovary were collected for RNA extraction. Crabs injected with 50 μL of 5 μM of DMSO solution were included in the control group.

An *in vitro* hepatopancreas and ovary culture was performed for six hours to study the effects of MF on JHBPs. Tissues from female crabs at the ovary developmental stage III (developing stage) were dissected and rinsed nine times with sterile saline. Then, the tissues were cut into small pieces of approximately 30 mg, and tissue fragment was placed into a well of the 12-well culture plates containing 2 mL of high Glucose DMEM medium (Thermo Fisher Scientific, Inc.) supplemented with 10% foetal bovine serum (Thermo Fisher Scientific, Inc.). MF solution were diluted to 1, 10, 100 μM by DMSO, then 20 μL MF solution were added to 2 mL media to reach the final 0.1, 1, 10 μM MF concentration. The 20 μL DMSO was added for the control group. The culture plates were incubated at room temperature (25°C) under gentle shaking on a rocker shaker. The total RNA from the hepatopancreas and ovary fragments was extracted 6 h after the addition of MF.

2.4. Identification and sequence analysis of the seven JHBPs

The seeds sequences of JHBP Pfam sequence (PF06585) was used to search for Sp-JHBP genes in a cDNA library constructed from multiple of *S. paramamosain* tissues at our laboratory using the BLAST program (E value $\leq 1e - 10$) (Li et al., 2016). The protein prediction was performed by using the open reading frame (ORF) finder tool (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The protein domains of the identified JHBP sequences were predicted with hmmscan (Finn et al., 2015) to ensure that these sequences contained the JHBP domain. The SignalP 5.0 server was used to predict the presence and location of signal peptide cleavage (Almagro Armenteros et al., 2019). The PROSITE program (<http://prosite.expasy.org/>) was used to predict the functional sites or domains in the amino acid sequence. The 3D structure of the predicted proteins was simulated by using the I-TASSER (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) (Yang et al., 2015) or Robetta (<http://robetta.bakerlab.org/>) programs. SAVES v5.0 was used to evaluated the accuracy of the predicted models (<http://servicesn.mbi.ucla.edu/SAVES/>).

2.5. Phylogenetic analysis of JHBP domains across arthropods

The JHBP sequences of sixteen species with reference genomes distributed in five classes were predicted, and 356 JHBP sequences were used in this study. The genome datasets were downloaded from Daphnia genomebase (Colbourne et al., 2011), InsectBase (Yin et al., 2016), i5k database (<https://i5k.nal.usda.gov/>) and NCBI Genome database (<https://www.ncbi.nlm.nih.gov/genome/>). First, OrthoMCL

program were used to predict the in-paralogs gene (E value $\leq 1e - 10$) (Li et al., 2003), which were filtered in the following analysis. JHBP domain sequences of the remaining JHBPs were identified using batch CD-search tool (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>), and extracted using the python script. The JHBP sequences was aligned by muscle algorithm in the default model using MEGA 7 (Kumar et al., 2016). A maximum likelihood (ML) tree was constructed using MEGA 7 with a Jones-Taylor-Thornton model, use all sites, and 1000 bootstrap replicates. The branch lengths were based on the phylogenetic distances. The phylogenetic relationships were deduced and dendrograms were produced using the MEGA 7 program (Kumar et al., 2016). The final tree was polished by using the online tool Interactive Tree of Life (<https://itol.embl.de>) (Letunic and Bork, 2016).

2.6. Expression pattern analysis

The total RNA was extracted from crabs during different larval stages, including zoeas (Z1 - Z5), megalopa (M), first and second juvenile crabs (C1-2); the haemolymph, hepatopancreas, thoracic ganglion, muscle, ovary, cerebral ganglion, mandibular organ, cuticle, antennae, antennule tissues from adult female crabs, and also antennae and antennule from the adult male crabs were extracted. Moreover, RNA from the cultured tissues, hepatopancreas and ovary, from the female crabs injected with MF were also used as a template for the quantitative real-time PCR (qRT-PCR) analysis. The total RNA was isolated by using the total RNA isolation kit (Aidlab, Beijing, China) according to the manufacturer's instructions, and the genomic DNA was removed by DNase I (Sangon, Shanghai, China) digestion. The RNA concentration was determined by spectrophotometry, and the RNA integrity was determined by the visualization of clear bands of 18S and 28S ribosomal RNA on 1.0% agarose gel electrophoresis.

Total RNA of 0.2 μ g was used for the synthesis of first strand cDNA by reverse transcription reaction using ReverTra Ace[®] qPCR RT Kit (TOYOBO, Osaka, Japan) and primer mix (oligo dT & random primers, provided by the kit) following the manufacturer's instruction. The qRT-PCR was performed on a 7900 genetic analyser system (Applied Biosystems). Specific amplification of each cDNA was verified by melting curve analysis (Table 1), and the 18S rRNA fragment was used as an internal control due to its relatively stable expression (Fang et al., 2018). The system, procedure and quantitative method of qRT-PCR have been described in our previous study (Zhao et al., 2015). A relative standard curve was developed using 10-fold serial dilutions of cDNA. The standard curves were included in all runs to calibrate the quantitative data. The concentrations of cDNA in each sample were calculated from the standard curves.

To analysis the results of the qRT-PCR, the mean and standard deviation (SD) of each sample were calculated. All data obtained from the

Table 2

Basic information of the cDNA sequences and predict amino acids sequences.

Name	The Length of ORF (predict amino acids)	Molecular weight (kDa)		Isoelectric point	Signal peptide	GenBank accession number
		Pre	Mature			
JHBP1	741 (246)	27.043	–	5.77	–	MH492027
JHBP2	753 (250)	27.75	25.81	4.94	1–20	MH492028
JHBP3	834 (277)	30.52	27.81	5.42	1–26	MH492029
JHBP4	1308 (435)	47.18	44.56	4.46	1–26	MH492030
JHBP5	1326 (441)	47.53	45.68	4.10	1–18	MH492031
JHBP6	1299 (432)	48.16	45.67	5.36	1–25	MH492032
JHBP7	663 (220)	24.14	21.91	4.58	1–22	MH492033

qRT-PCR analysis were log transformed prior to performing the data analysis with a one-way ANOVA. The *post hoc* test was carried out using a Tukey multiple comparison test. The differences were considered significant at $p < .05$. All data analyses were performed with SPSS 22.0.

3. Results

3.1. Sequence analysis of JHBP

Seven putative JHBPs, namely, Sp-JHBP1 to Sp-JHBP7, are reported in this study. The basic information of these sequences is shown in Table 2. The open reading frame (ORF) length of the seven putative JHBPs ranged from 663 bp to 1326 bp; the length of the predicted amino acids ranged from 220 to 441 amino acids; the molecular weight (M.W. values) of the pre-peptides ranged from 24.14 kDa to 47.53 kDa with the predicted mature protein ranged from 21.91 kDa to 45.68 kDa; and the isoelectric points ranged from 4.10 to 5.77.

Except for JHBP1, the predicted amino acid sequences of the other six putative JHBPs possessed a signal peptide in the N-terminal region. One JHBP domain was identified in all seven JHBP sequences. Additionally, Sp-JHBP4, 5 and 6 contained a Grp7_{allergen} domain (Fig. 1). The sequences of the JHBP domain were slightly conserved in all *S. paramamosain* JHBPs. Two to nine protein-binding sites were identified in six JHBPs, except for Sp-JHBP5, by using the PredictProtein server, suggesting that these proteins might interact with other proteins or among themselves to form a complex structure *in vivo*.

3.2. Predicted secondary and 3D structures of seven putative JHBPs

The secondary structure of the JHBPs is shown in Fig. 1. The strands units were slightly higher than the other components in six JHBPs, except for JHBP3, in which the highest component is the helix. A putative disulfide bridge (S–S) was observed in JHBP2 and JHBP3 by using the PredictProtein server.

The 3D structures of seven JHBPs were initially predicted using I-TASSER server, C-score (–5, 2) and the estimated TM-score were used to evaluated the credibility of the models. High C-score with a TM-score > 0.5 suggests the model to be relatively credible. Results showed that all seven models had the TM-scores > 0.5 . In addition, all models were also evaluated using SAVES v5.0, which provided five evaluation tools, namely VERIFY, ERRAT, PROVE, PROCHECK AND WHATCHECK. The model of Sp-JHBP2, Sp-JHBP4, Sp-JHBP6 and Sp-JHBP7 passed the evaluation by at least one software, suggesting the credibility of these models. The 3D structures of Sp-JHBP1, Sp-JHBP3 and Sp-JHBP5 were then predicted using Robetta server. However, only Sp-JHBP1 returned the result, and the model passed VERIFY and PROCHECK evaluation. The predicted models are shown in Fig. 2.

From the 3D structure, it is clear that Sp-JHBP1, 2, 3 and 7, which contain only one JHBP domain, had similar 3D structures, whereas Sp-JHBP4, 5 and 6 had similar 3D structures. The structurally closed target

Table 1

Oligo nucleotide primers used in this study.

Name	Sequence (5'-3')
Sp-JHBP1-RTF	CCTCCAACGACGCGATAACT
Sp-JHBP1-RTR	CTCCACCACTCACTCACTC
Sp-JHBP2-RTF	AGGGGCTGGATGGGAGTTTA
Sp-JHBP2-RTR	CGAAATGGTGGGAAGGTGA
Sp-JHBP3-RTF	ACGCAAAAAGTATGACAGGC
Sp-JHBP3-RTR	TTGATGGTAGGCAACCCGA
Sp-JHBP4-RTF	GATGTAGCCACGCCAAGAT
Sp-JHBP4-RTR	AGTCGGGAGCCATTGAGTTG
Sp-JHBP5-RTF	CCACCAACTCTCAGGTGCAT
Sp-JHBP5-RTR	CCAGTCTCAAGTAGTGGCCG
Sp-JHBP6-RTF	TGATGGCACGTTCCACTACC
Sp-JHBP6-RTR	ATCACGGGTTCCACCTTGTG
Sp-JHBP7-RTF	CGGGACGGTGTCAATGAGT
Sp-JHBP7-RTR	CCAGATTGCCGCTCTTGTG

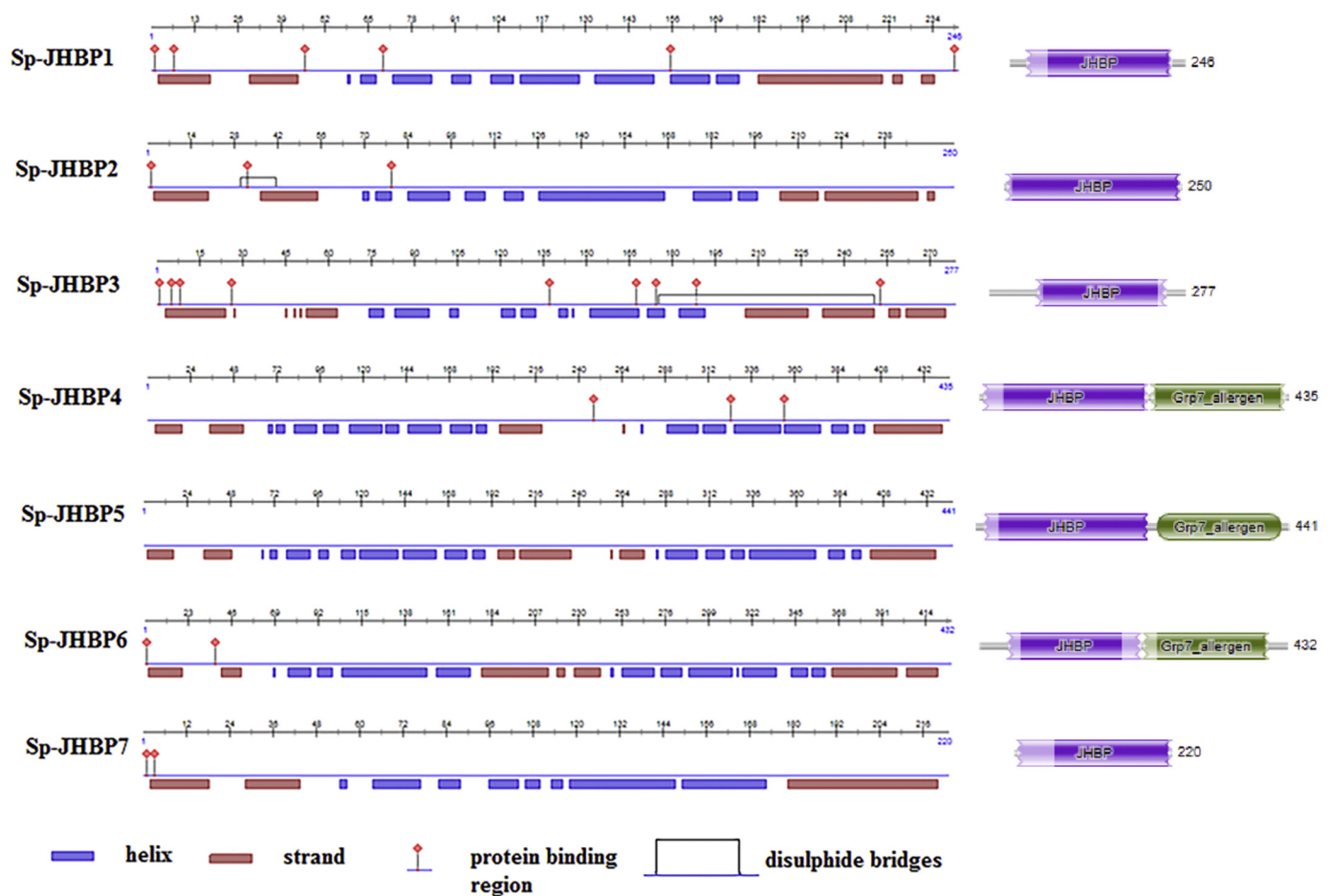


Fig. 1. The secondary structures and protein domains of the seven Sp-JHBPs. The predicted secondary structures are on the left and the protein domains are on the right. The annotations of elements in the figure are at the bottom.

identified by TM-align in the PDB for Sp-JHBP1, 2, 3 and 7 were *Epiphyas postvittana* Takeout 1 (PDB code: 3e8wA) and *Bombyx mori* JHBP (PDB code: 2rqfA), they adopted the same elongated barrel fold, and α helix wrapped in a highly curved anti-parallel β -sheet. In addition, Sp-JHBP1 had one more α helix in the N-terminal. The 3D structure of JHBP domain in Sp-JHBP4, 5 and 6 were similar to Sp-JHBP1, 2, 3 and 7, which also exhibited typical tubular structures comprising by helices and strands, whereas the predicted Grp7_allergen domain only had one long α helix and several anti-parallel β -sheet. The structurally closed target in the PDB for Sp-JHBP4, 5 and 6 were *Mus musculus* lipopolysaccharide binding protein (LBP) (PDB code: 4m4d) and human bactericidal/permeability-increasing protein (BPI) (PDB code: 1ewf).

Additionally, I-TASSER also predicted the possible ligands for seven Sp-JHBPs using the COACH meta-server approach (Yang et al., 2013a; Yang et al., 2013b). For Sp-JHBP1, 2, 3 and 7, the rank 1st predicted ligands were all ubiquinone 8 (UQ8), and the rank 2nd were JH II or JH III. And also, the predicted JHs binding location were close to the N- and C-termini (W cavity) (Fig. 3). However, for Sp-JHBP4, 5 and 6, the rank 1st predicted ligands were phosphatidylcholine, and no JHs were predicted.

To examine the difference of ligands binding sites among different JHBPs, multi-alignment was conducted between Sp-JHBPs and other JHBP domain containing proteins, which has been functionally characterized (Fig. 4). The alignment results revealed that these sequences have relatively low identity with each other. The first S–S, which was conserved in JHBP and Takeout insects, were only found in Sp-JHBP2. The second S–S was conserved in four insect JHBPs but not conserved in two insect Takeouts or Sp-JHBPs, only Sp-JHBP3 had a

predicted S–S at the near location (boxed). In addition, the location of predicted binding sites for JHs in Sp-JHBP1, 2, 3 and 7 (indicated by red background), were similar to the residues that are essential for JH binding (indicated by asterisks) (Suzuki et al., 2011). Most of those are not conserved but some has the similar properties with BmJHBP. It should be mentioned that Tyr128 (Y128) in BmJHBP (indicated by blue background), which interacted with epoxy of JHs through hydrogen bond (Suzuki et al., 2011), are not converted in two Takeout proteins in insects and seven JHBPs. However, Ser137 (S137) (marked by blue background) in Sp-JHBP2, has the similar properties with Tyr, both of them are the polar and neutral amino acids.

3.3. Phylogenetic analysis of the JHBPs

Using the e-value of $1e - 10$ as the cutoff, several in-paralogs pairs were identified. One inparalogs was found in *Tigriopus californicus*, three in *Apis mellifera*, six in *Bombyx mori*, 58 in *Drosophila melanogaster*, eight in *Danaus plexippus*, 52 in *Limulus Polyphemus*, 4 in *Nilaparvata lugens*, 10 in *Plutella xylostella*, 47 in *Tribolium castaneum* and six in *Zootermopsis nevadensis*, suggesting that JHBPs had undergone an expansion in those species. In the further studies, only one sequence was leaved for the phylogenetic analysis. A phylogenetic tree was constructed using JHBP domain sequences from sixteen species, including two crustacean (seven *S. paramamosain* and five *Daphnia pulex*), two copepoda (two *Eurytemora affinis* and two *T. californicus*), two Arachnida (one *Ixodes scapularis* and two *Tetranychus urticae*), one Merostomata (two *L. polyphemus*) and 10 insects (25 *N. lugens*, 19 *Pediculus humanus*, 33 *P. xylostella*, 25 *T. castaneum*, 16 *A. mellifera*, 36 *B. mori*, 18 *D. melanogaster*, 34 *D. plexippus*, 27 *Heliconius melpomene* and

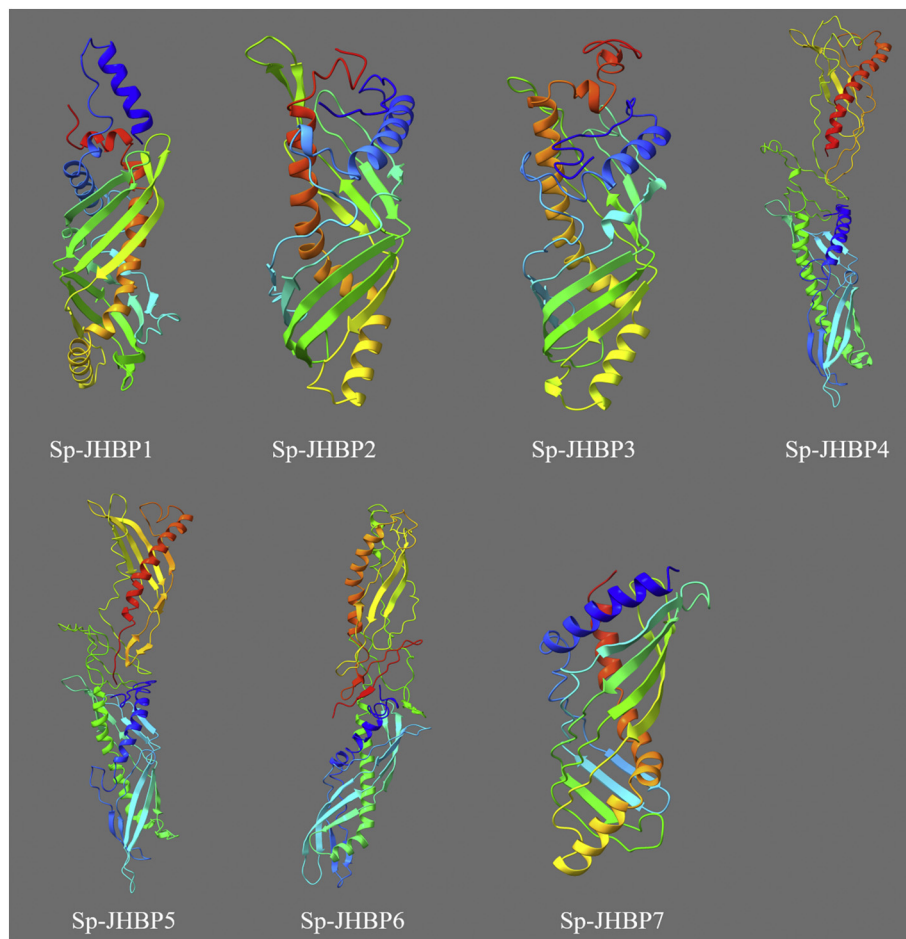


Fig. 2. Predicted 3D structures of the seven Sp-JHBPs. The 3D structures are shown in rainbow. The N-terminal and C-terminal are in blue and red color, respectively.

18 *Z. nevadensis*) (Supplementary File 1. and Supplementary Fig. 1).

The tree was divided into three major branches. However, only branch I had a low bootstrap support. All seven Sp-JHBPs belonged to branch II, but Sp-JHBP2 was not clustered with other Sp-JHBPs. The functionally characterized BmJHBPd18 also belonged to branch II, but was not clustered with any Sp-JHBPs. *D. melanogaster* Takeout belonged to the branch III, and was not clustered with any other crustacean

JHBPs. Nearly all JHBPs in Lepidoptera were first clustered with Lepidoptera JHBPs then with other sequences, and a lepidoptera specific cluster was found in branch III, in which only one sequence from *Z. nevadensis* was included.

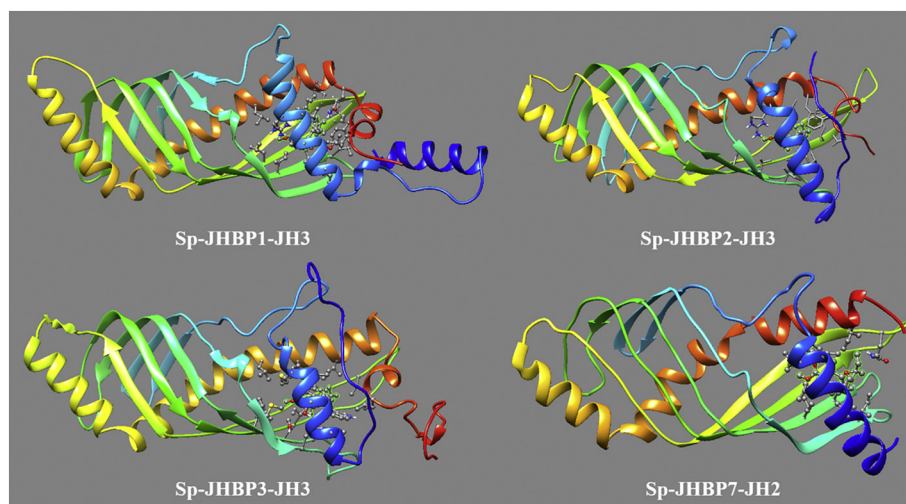


Fig. 3. Predicted binding model between JH III or JH II and Sp-JHBPs. The N-terminal and C-terminal are in blue and red color, respectively. The ligands and interacted residues are in ball & stick format and ligands are marked with green fluorescent edge.

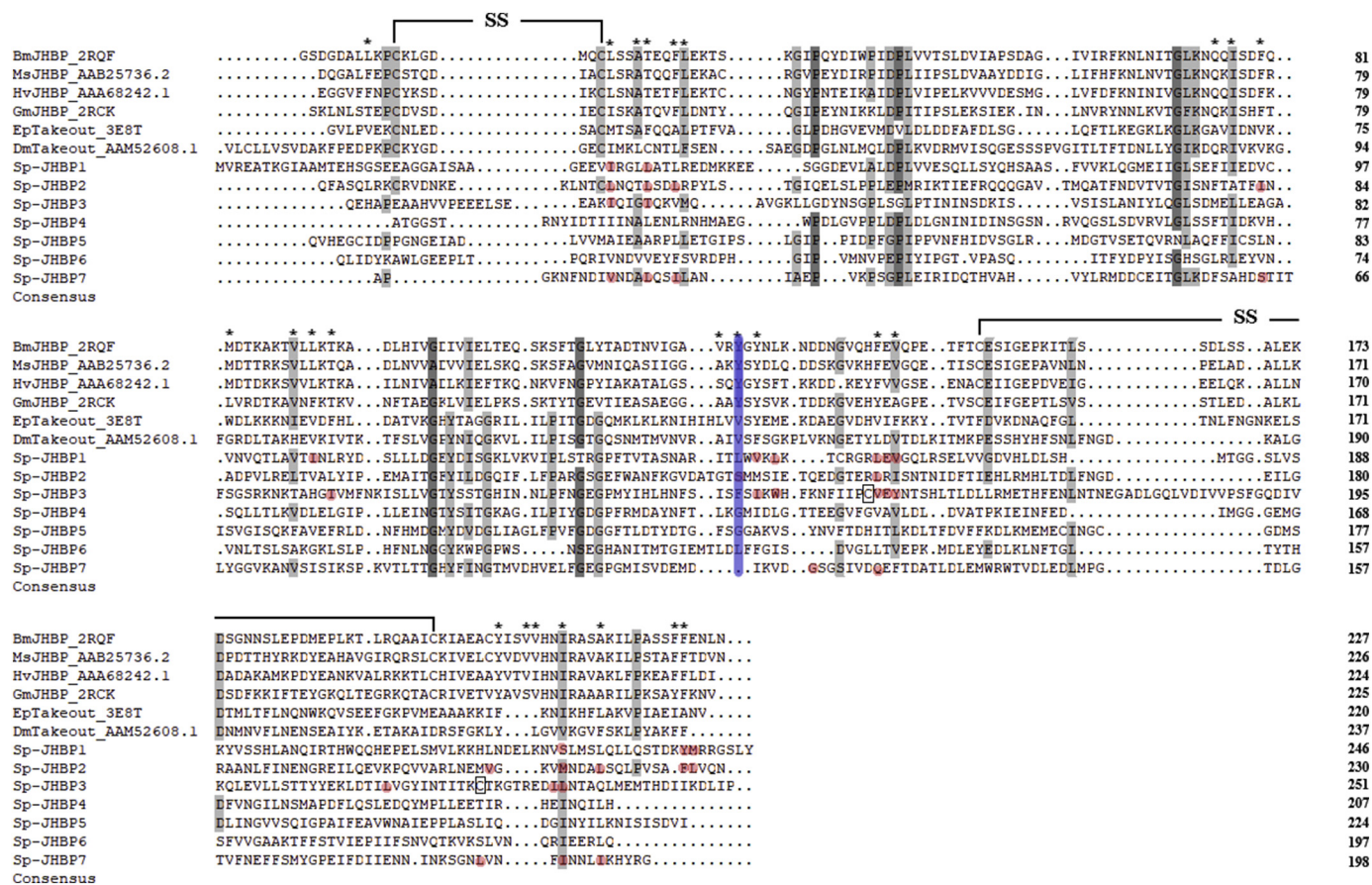


Fig. 4. Multi-alignment between four insect JHBPs, Takeout and Sp-JHBPs. The * indicates residues essential for JH binding in BmJHBP⁴². SS represents the disulfide bond. Residues in the red background indicate the predicted binding sites for JHs binding. Residues in the blue background indicate the residues interacted with epoxy of JH II. The putative disulfide bond in Sp-JHBP3 is underlined.

3.4. Tissues distribution of Sp-JHBPs mRNA

The mRNA distributions of all seven JHBPs was examined in different tissues (Fig. 5). Among all seven JHBPs, Sp-JHBP1 showed a relatively low expression (with a Ct value larger than 28.5) in the 12 detected tissues; with relatively higher expression in the thoracic ganglions, followed by the haemolymph and ovary tissues. Sp-JHBP2 showed the highest expression in the ovary, followed by the antenna, cuticle, and the expression of Sp-JHBP2 in the antenna of female crabs was slightly higher than that in the antenna of male crabs. Sp-JHBP3 and Sp-JHBP4 showed the highest expression in the hepatopancreas, with low expression in the cuticle. Sp-JHBP5, 6 and 7 were nearly specifically expressed in the hepatopancreas.

3.5. Expression profile of seven Sp-JHBPs during larval development

From Zoea stage 1 (Z1) to the second juvenile crab (C2), the expression of Sp-JHBP1 was low, with a comparatively lower expression during the Z3, Z5, M and C2 stage. Sp-JHBP2 exhibited the highest expression during the Z1 stage, followed by megalopa (M) and the first juvenile crab stage (C1); the expression during the Z5 stage was very low and undetectable the other four stages. Sp-JHBP3 and Sp-JHBP4 showed similar variations during larval development, and the highest expression of both genes was observed during the Z2 stage; with a significant reduction at Z5 and M stages, followed by an increased expression at C1 stage and a significant decrease at C2 stage. Sp-JHBP5 was rarely expressed from Z1 to C2. The variations in Sp-JHBP6 and Sp-JHBP7 expression were similar to some extent, as both genes showed increased expression from Z1 to Z3, reduced expression at Z4, a

subsequent increase until Z5, and then a gradual decrease until the C2 stage. However, Sp-JHBP7 showed much higher expression than Sp-JHBP6 (Fig. 6).

3.6. Role of MF in the regulation of JHBP transcription

The regulatory influence of MF on six JHBPs, including Sp-JHBP2–7, was analyzed by *in vivo* and *in vitro* studies. The *in vitro* studies showed that the expression of Sp-JHBP2 was reduced by the MF treatment and that Sp-JHBP3, 6 and 7 were induced by the MF treatment in a concentration-independent manner, while Sp-JHBP4 and 5 showed no obvious regulation by MF (Fig. 7). The *in vivo* studies suggested that the expression of these six JHBPs greatly varied among individuals as revealed by the large standard error in the histogram, and Sp-JHBP5 was the most variable. Nevertheless, the average of Sp-JHBP2 was reduced to approximately half of the control group at both time points but not significant ($P > .05$), especially 6 h after the MF injection, while MF had a non-constant effect on the expression levels of the other JHBPs, suggesting that MF might have a non-direct regulatory effect on these JHBPs (Fig. 8).

4. Discussion

MF functions as a crustacean juvenile hormone that participates in the regulation of many physiological processes. After its synthesis in the MO, MF is transported to target tissues through MFBP (Takac et al., 1993; Tamone et al., 1997). However, knowledge regarding MFBP is limited. In this study, a preliminary analysis of seven JHBPs from mud crab was conducted to provide impetus to the MF functional pathway in

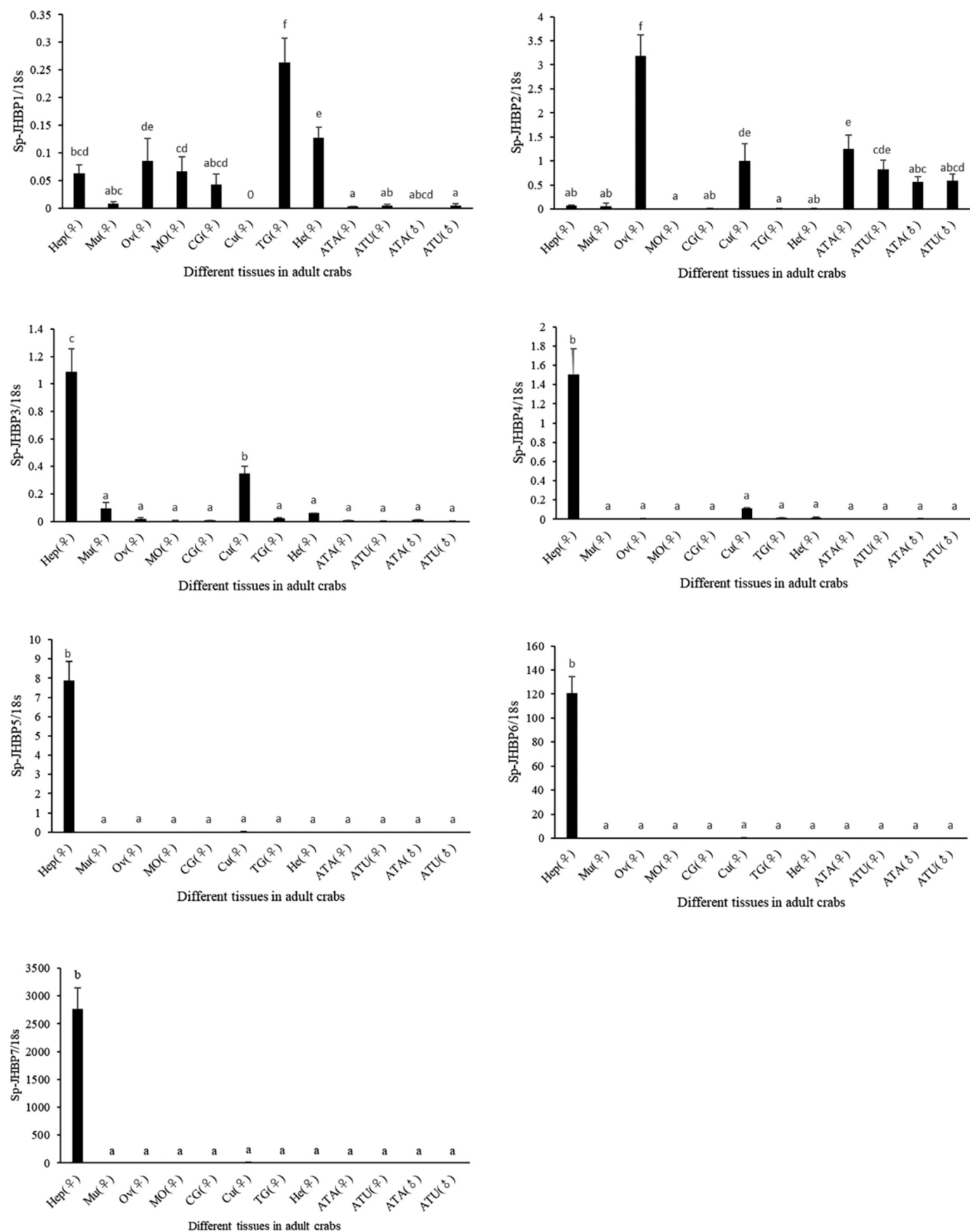


Fig. 5. The expression profiles of *Sp-JHBPs* in different tissues. Ten tissues of female crabs: Hepatopancreas: Hep(♀), muscle: Mu(♀), ovary: Ov(♀), mandibular organ: MO(♀), cerebral ganglion: CG(♀), cuticle: Cu(♀), thoracic ganglion: TG(♀), Haemolymph: He(♀), antennae: ATA(♀), antennule: ATU(♀); Two tissues of male crabs: antennae: ATA(♂), antennule: ATU(♂). The “Y” axis represents the relative ratio of the *Sp-JHBPs*/18S rRNA mRNA expression levels. The data are shown as the means $\pm$ SD ($n \geq 3$), different letters indicate significant differences ($p < .05$).

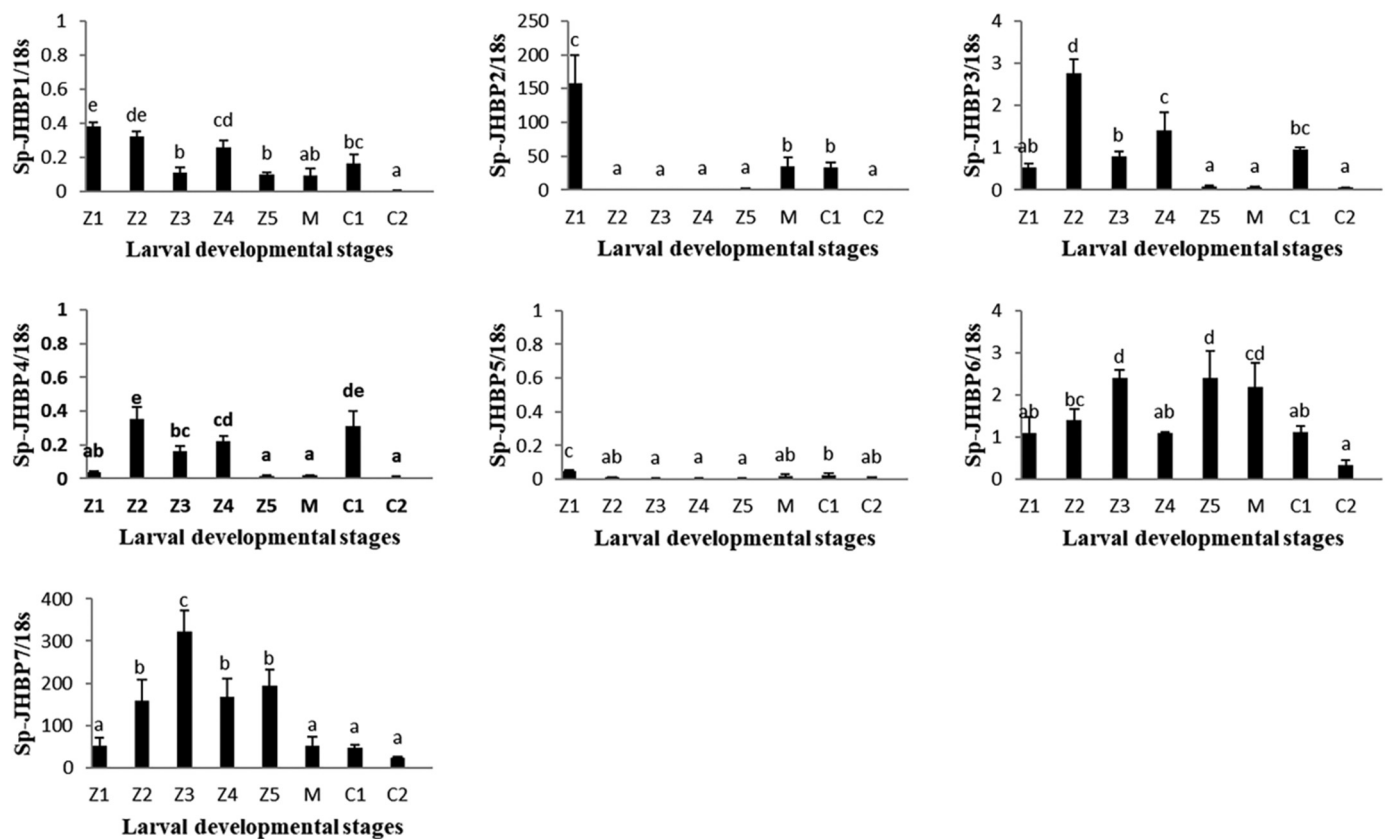


Fig. 6. The expression profiles of *Sp-JHBPs* in different larval stages by quantitative real-time PCR. The mRNA levels of the *Sp-JHBPs* genes were normalized to the 18S rRNA transcript level. The data are shown as the means $\pm$ SD ($n \geq 6$). Different letters indicate significant differences during these stages ($p < .05$). The "Y" axis represents the relative ratio of the *Sp-JHBPs*/18S rRNA mRNA expression levels. Z1-Z5 represent the five developmental phases of the zoeae stage, M represents the megalopa stage, and C + number represents the numberth juvenile crab.

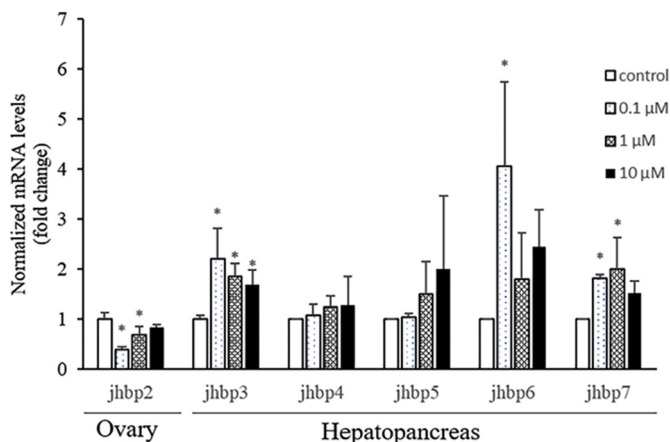


Fig. 7. *In vitro* effect of MF on the *Sp-JHBPs* expression in the ovary or hepatopancreas. The data are shown as the means $\pm$ SD of three independently repeated experiments. The "Y" axis represents the relative ratio of the *Sp-JHBPs*/18S rRNA mRNA expression levels and all data were shown as fold change compared with the control group. The * indicate significant differences between control and treated groups ($p < .05$).

crustaceans.

The currently reported JHBPs included hJHBP, nJHBP and cJHBP. Notably, the cJHBP gene is homologous to human Glod-4, which contains Glo_EDI_BRP_like_21 and Glo_EDI_BRP_like domains and is highly conserved across different species (Li et al., 2015). However, differentiating hJHBP and nJHBP based on sequence information is difficult as both proteins contain at least one JHBP domain. Seven JHBP domain

containing proteins were identified in the mud crab, three of them contained another Grp7_allergen domain. The Grp7_allergen domain was also identified in JHBPs in silkworm (Li et al., 2016), and currently, the function of this domain in JHBPs remains unknown.

The 3D structure for all seven *Sp-JHBPs* were also predicted and evaluated. Interestingly, these seven JHBPs possessed low identity with each other but showed the similar 3D structures. *Sp-JHBP1*, *Sp-JHBP2*, *Sp-JHBP3* and *Sp-JHBP7* adopts the unusual α/β -wrap fold, which similar to the known crystal structure of JHBPs (Suzuki et al., 2009; Hamiaux et al., 2009; Kolodziejczyk et al., 2008), suggesting that they might perform similar functions. The predicted 3D structures of *Sp-JHBP4*, *Sp-JHBP5* and *Sp-JHBP6* were similar to *M. musculus* LBP and human BPI. LBP and BPI specifically binds bacterial LPS to initiate an immune response (Eckert et al., 2013; Kleiger et al., 2000), suggesting a possible immune response role for *Sp-JHBP4*, *Sp-JHBP5* and *Sp-JHBP6*.

The predicted ligands for *Sp-JHBP1*, *Sp-JHBP2*, *Sp-JHBP3* and *Sp-JHBP7* included ubiquinone 8 (UQ8) and JH II or JH III, suggesting a possible binding ability for JHs. The predicted binding location were in the cavity close to the N- and C-termini (W cavity), which were similar to the *B. mori* JHBP (Suzuki et al., 2011). The N-terminal S-S plays a critical role in JH binding, as indicated by mutational studies of JHBP from *Heliothis virescens* (Wojtaszek and Prestwich, 1995). However, this S-S was only conserved in *Sp-JHBP2* but not in other *Sp-JHBPs*. The second S-S is conserved in insect JHBPs but not found in Takeout proteins and *Sp-JHBPs*, only *Sp-JHBP3* had a predicted S-S at the near location. This S-S bond anchors $\alpha 3$ to the β -sheet in the central part of the barrel and triggers the formation of two separate cavities (Kolodziejczyk et al., 2008), the loss of this bond creating more space in the core of the protein and a long continuous tunnel instead of two

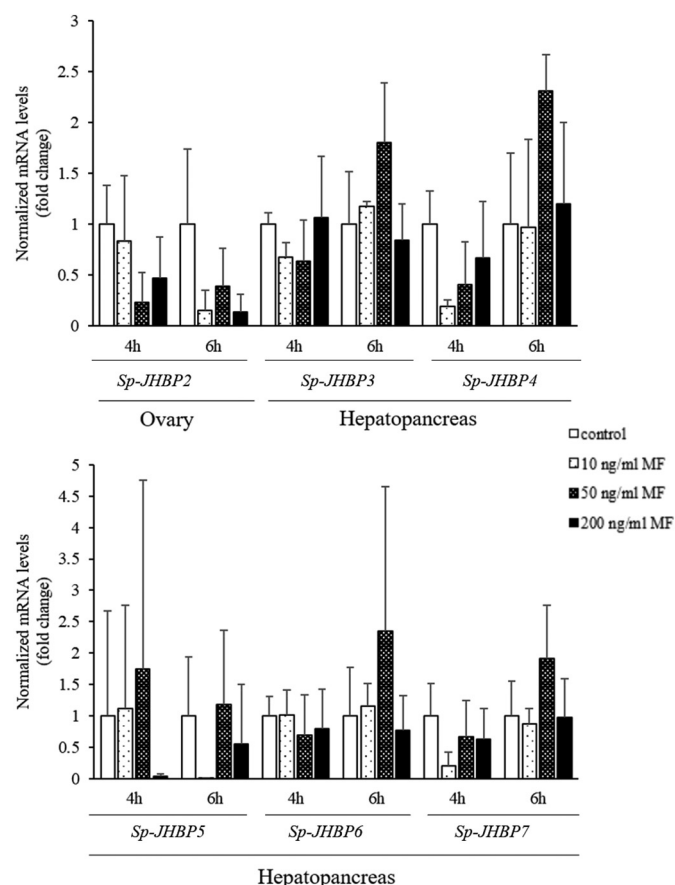


Fig. 8. *In vivo* effect of MF on the *Sp-JHBPs* expression in the ovary or hepatopancreas. The data are shown as the means $\pm$ SD of three independently repeated experiments. The “Y” axis represents the relative ratio of the *Sp-JHBPs*/18S rRNA mRNA expression levels and all data were shown as fold change compared with the control group. The * indicate significant differences between control and treated groups ($p < .05$).

smaller cavities in Takeout proteins (Hamiaux et al., 2009). Although the low sequence identity was found between these four insect JHBP, two Takeout and seven *Sp-JHBPs*, many of the predicted binding sites in *Sp-JHBPs* were at the same or near location of BmJHBP, also suggesting a potential binding ability with JHs for *Sp-JHBP1*, *Sp-JHBP2*, *Sp-JHBP4* and *Sp-JHBP7*. However, due to the lack of S–S, it would be interesting to investigate the binding model between MF and *Sp-JHBPs*. It should be mentioned that Y128 in BmJHBP, which interacts with epoxy of JH II (Suzuki et al., 2011), were not conserved in all *Sp-JHBPs*, this was consistent with the JH type in crustacean, in which only MF was found and MF lack the epoxy. The interesting thing is that this residue was not conserved in the two Takeout proteins, but *Sp-JHBP2* has a S137 residue which has the similar properties to Tyr, was found at this location, a possible explanation is that these proteins might have different suitable ligands for binding, and this is worth of deep exploration.

To investigate whether JHBPs could be divided into groups based on their sequence information, a phylogenetic tree, including 273 JHBP domain sequence from 16 species representing four clades in arthropods, was constructed. The tree did not exhibit four clades as in the six insects (Li et al., 2016). The tree was divided into three major branches. However, only branch I had a low bootstrap support, perhaps more data could help to reach a better result. *Sp-JHBP2* was not clustered with other *Sp-JHBPs*, suggesting that there had a differentiation between *Sp-JHBP2* and other *Sp-JHBPs*.

The tissue location of genes can reflect their functions. However, the expression of *Sp-JHBP1* was relatively low in the 10 tissues of female

crabs and two tissues of male crabs, suggesting that *Sp-JHBP1* might function in other undetected tissues or have a stage specific expression in adult crabs. *Sp-JHBP2* is most highly expressed in the ovary, followed by the antenna, cuticle and antennule, suggesting that *Sp-JHBP2* might be involved in reproduction. In addition, expression in antennule suggesting that *Sp-JHBP2* might also be involved in sensory functions or some behavior processes, but *Sp-JHBP2* has no sequence homology to the newly found mosquito juvenile hormone-binding protein (mJHBP), which contains two odorant-binding protein domains and has the potential to influence the effects of JH on mating behavior, feeding, or other processes (Kim et al., 2017). The other five *Sp-JHBPs* showed the highest expression in the hepatopancreas, which was consistent with the phylogenetic tree, and *Sp-JHBP3* and *Sp-JHBP4* also showed low expression in the cuticle. The hepatopancreas is a homologous organ of the fat body in insects, and this organ is considered a major organ for the synthesis of JHBP (Orth et al., 1999; Lee et al., 1995; Rittachyeng et al., 2012). In total, 41 JHBPs in silkworm are generally highly expressed in the head, integument, midgut, fat body, testis, and ovary on day 3 of the fifth instar (Li et al., 2016). A comparative analysis of the tissues distribution and the phylogenetic tree revealed that only a slight correlation exists between gene expression and classification, suggesting that the function of JHBPs were highly differentiated. In addition, the high expression in hepatopancreas for *Sp-JHBP4*, *Sp-JHBP5* and *Sp-JHBP6* might also be correlated with the immune response process, as the hepatopancreas is a very important immune defense tissue for crabs, as indicated by the high expression of many immune related genes (Wei et al., 2018; Fang et al., 2013).

Sp-JHBP1 had a slightly higher expression from the Z1 to C2 stages during larval development than in the adults, suggesting *Sp-JHBP1* might play a more important role during larval development. *Sp-JHBP2* and *Sp-JHBP7* were highly expressed during larval development, suggesting that these genes may play important roles in larval development. *Sp-JHBP2* showed the highest expression during the Z1 stage, followed by the M and C1 stages, with rare expression during the other stages. In *Drosophila*, Takeout is induced by starvation (Sarav-Blat et al., 2000), and the expression pattern of *Sp-JHBP2* might be correlated with starvation because the newborn Z1 stage is the first stage requiring exogenous nutrition, while the M and C1 stages are the first stages after metamorphosis. Interestingly, *Sp-JHBP3* and *Sp-JHBP4* showed similar variation trends, and their expression patterns seem to correlate with metamorphosis process, as both genes showed apparently decreased expression during the Z5 and M stages. The expression patterns were similar to those of *Sp-HMGR*, which is a key enzyme involved in MF biosynthesis (Zhao et al., 2015). The expression of the other *Sp-JHBPs* was also analyzed. However, due to the lack of related behavioural, biological and physiological information during the larval development, we cannot draw any further conclusions.

The expression of *Sp-JHBP2* was reduced by MF both *in vivo* and *in vitro*. The down regulation role of JHs has been observed in *GB19811* in honeybee (Hagai et al., 2007) and *molting* in *Manduca* (Orth et al., 2003), but *Sp-JHBP2* did not clustered with *GB19811* (ame-1A0A087ZUN6) as revealed by the phylogenetic tree. *Sp-JHBP3*, *Sp-JHBP6* and *Sp-JHBP7* were induced by the MF treatment in a concentration-independent manner *in vitro*, and *Sp-JHBP4* and *Sp-JHBP5*, showed no obvious regulation by MF. These findings were inconsistent with the *in vivo* study primarily due to the obvious inconsistency among individuals in the same group, particularly for *Sp-JHBP5*. These findings suggest that the regulation of *Sp-JHBPs in vivo* is more complicated. In insects, some JHBPs can also be induced by JHs (Orth et al., 1999; Orth et al., 2003; Rittachyeng et al., 2013), suggesting the presence of some conserved function between crustaceans and insects.

In conclusion, we report seven JHBP sequences in the mud crab *S. paramamosain*. A preliminary analysis, including the bioinformatics analyses of the sequences, expression profile in tissues, at different larval development stages, and under MF challenge, was conducted. Based on these results we hypothesize that *Sp-JHBP1*, 2, 3 and 7 show

the potential to bind MF, but *Sp-JHBP2,3* and 7 might play a more important role in MF signalling, mainly because the potential binding to JHs, close correlation with metamorphosis and under MF challenge. To extend this study, several issues need further investigation, including the following four main aspects: (1) the binding properties of the seven JHBPs to MF and other JHs; (2) the tissue distribution and subcellular location of the seven JHBPs proteins; (3) the mechanism of the regulatory impact of MF on Sp-JHBPs and the significance of these regulations, and (4) the correlation between behaviour and the gene.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbpb.2019.110390>.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgments

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