



Alternative Splicing of the Lobster (*Homarus americanus*) Crustacean Hyperglycemic Hormone A and B Genes Produce 2 Protein Variants Involved in Vitellogenin Inhibition

OPEN ACCESS

Cheng Gui Wang^{1,2}, Wei Wang¹, Li Li Shi¹, Yu Chun Shen^{2*} and Siuming F. Chan^{1*}

Edited by:

Benjamin Costas,
University of Porto, Portugal

Reviewed by:

Piero Giulio Giuliani,
University of Trieste, Italy
Ting Chen,
Chinese Academy of Sciences, China

*Correspondence:

Yu Chun Shen
shenyuchun@163.com
Siuming F. Chan
siuming573@sina.com

Specialty section:

This article was submitted to
Aquatic Physiology,
a section of the journal
Frontiers in Marine Science

Received: 25 January 2022

Accepted: 11 March 2022

Published: 20 April 2022

Citation:

Wang CG, Wang W, Shi LL,
Shen YC and Chan SF (2022)
Alternative Splicing of the Lobster
(*Homarus americanus*) Crustacean
Hyperglycemic Hormone A and B
Genes Produce 2 Protein Variants
Involved in Vitellogenin Inhibition.
Front. Mar. Sci. 9:861691.
doi: 10.3389/fmars.2022.861691

¹ Laboratory of Shrimp Genetic Breeding, College of Fisheries, Guangdong Ocean University, Zhanjiang, China,

² Key Laboratory of Marine Ecology and Aquaculture Environment of Zhanjiang, College of Fisheries, Guangdong Ocean University, Zhanjiang, China

Current BLASTP search analysis results suggested that the lobster (*Homarus americanus*) *HaCHH-A* and *HaCHH-B* may be derived from two different four-exon genes. Repeated tissue expression studies have revealed much different expression patterns of these two genes from those reported in the past. With RT-PCR, rapid amplification of complementary DNA (cDNA) ends (RACE), and genomic DNA cloning, we confirmed that the *HaCHH-A* and *HaCHH-B* transcripts were derived from two different four-exon CHH genes. By an alternative splicing mechanism, each gene can produce different but larger transcript variants (i.e., *sHaCHH-A* and *sHaCHH-B*) mainly in different non-eyestalk tissues of the females. The larger and unspliced transcripts can be detected in the hepatopancreas, gill, heart, nerve cord, brain, ovary, and thoracic ganglion of the reproductive females. The expression patterns of *sHaCHH-A* and *sHaCHH-B* in other non-eyestalk tissues suggest that these transcripts have a wide spectrum of expressions during the female reproductive cycle. An *in vitro* organ explant culture system was developed to investigate the reproductive function of these cDNAs. The results showed that the recombinant proteins for *sHaCHH-A* and *sHaCHH-B* inhibited the gene expression of vitellogenin, whereas the double-stranded RNA (dsRNA) for *sHaCHH-A* and *sHaCHH-B* stimulated the expression of the vitellogenin gene *in vitro*. The results of the study may provide insights for the development of techniques to induce gonad development without using eyestalk ablation operation. This is the first in-depth report of the characterization of two four-exon CHH genes in a crustacean.

Keywords: shrimp, eyestalk, crustacean hyperglycemic hormone, alternative splicing, vitellogenin

HIGHLIGHTS

1. Multiple *CHH-A* and *CHH-B* genes exist in the lobster *Homarus americanus*, and these genes consist of four exons and are interrupted by three introns.

2. The previously reported lobster *CHH-A* and *CHH-B* cDNAs were derived from alternative splicing of the third intron, and the newly identified *sCHH-A* and *sCHH-B* transcripts were products of four-exon transcripts that exist mainly in non-eyestalk neuronal tissues.

3. The expression patterns of the newly reported *sHaCHH-A* and *sHaCHH-B* suggested that they may have a reproductive stage-related function in the biannual reproductive cycle of females.

4. Recombinant proteins for *sHaCHH-A* and *sHaCHH-B* inhibited the expression of vitellogenin, and gene knockdown of *sHaCHH-A* and/or *sHaCHH-B* caused a significant increase in the gene expression of vitellogenin in the hepatopancreas and ovary.

INTRODUCTION

Reproduction is an important physiological process and requires a large amount of energy in crustaceans. Crustacean reproduction is tightly regulated by the interactions of hormones (i.e., peptides, neuropeptides, juvenoids, and steroids) (Fingerman, 1987; Huberman, 2000; Diwan, 2005). The neuropeptides most popularly studied in crustacean endocrine research are the crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH), and gonad-inhibiting hormone (GIH). Together, these hormones belong to a large group of neuropeptides of the CHH/MIH/GIH family (Keller, 1992). These hormones are synthesized in the X-organ sinus gland complex located in the optical ganglia of the eyestalk. The eyestalk sinus gland is a neurohemal organ consisting of clustered axon endings of the neurosecretory cell somata (Chan et al., 2003; Chung et al., 2020; Keller, 1992; Montagné et al., 2008; Tensen et al., 1991; Webster et al., 2012). Many studies have shown that the removal of eyestalks could induce gonad maturation in decapods (Panouse, 1943; Primavera, 1978; Okumura and Aida, 2001; Uawisetwathana, et al., 2011; Magaña-Gallegos et al., 2021). Removal of the eyestalk could remove the source of GIH and result in rapid ovary development. However, the use of this technique has raised serious animal welfare concerns in recent years. In lobster (*Homarus americanus*), two highly similar HaCHH isoforms (i.e., *HaCHH-A* and *HaCHH-B*) have been characterized (Tensen et al., 1991; De Kleijn et al., 1994; De Kleijn and Van Herp, 1995; Chang et al., 1999). They are known to play a role in the reproduction of the female lobster. The *HaCHH-A* and *HaCHH-B* isoforms share a high overall amino acid sequence identity and differ only by eight amino acid residues for the deduced pro-hormone. Despite the high sequence homology, each CHH has been reported to regulate reproduction during the biannual reproductive cycle of adult females (De Kleijn et al.,

1995). Many CHH family neuropeptides from other crustacean species were discovered in the eyestalk from 1990 to 2000; subsequent studies indicated that the transcripts of these neuropeptides can be found in other non-eyestalk neuronal tissues (Chan et al., 2003; Diwan, 2005; Huberman, 2000; Keller, 1992; Spanings-Pierrot et al., 2000; Webster et al., 2012; Loredano-Ranjel et al., 2017) and even in many non-neuronal tissues (Chen et al., 2004; Shi et al., 2018). Although the discovery of the two lobster CHH isoforms has been reported for 25 years, little information has been acquired since their first discovery. At the time when the complementary DNAs (cDNAs) of *HaCHH-A* and *HaCHH-B* were cloned, only a few CHH family neuropeptide sequences have been reported in the GenBank. With the exponential increase in the total number of CHH family neuropeptides reported in recent years, it is important to obtain additional specific information on the gonad maturation of the lobster.

In recent years, China has imported large quantities of *H. americanus* as high-priced seafood from the US and Canada. Because of the increased need for this species as food, growing interest to develop a large-scale commercial complete aquaculture of *H. americanus* in China has arisen. One major constraint for the successful aquaculture of this species is the lack of information on the endocrine control of reproduction. Knowledge on its reproduction, larval rearing, and broodstock management is inadequate. For this reason, investigations into the involvement of CHHs in the reproductive cycle of the female lobster are urgently needed. In this study, we further characterized *HaCHH-A* and *HaCHH-B* from the study of gene structure and expression. We report new findings for two additional CHH-related peptides and confirm that they are derived from two different four-exon *CHH* genes that also gave rise to the originally discovered *HaCHH-A* and *HaCHH-B* cDNAs. We also demonstrate the potential functions of *sHaCHHs* during the reproductive cycle of the female lobster.

MATERIALS AND METHODS

Animals

Female lobsters (average carapace length = 70 ± 3 cm, weight = 500 ± 50 g) were purchased from the local seafood market. These animals were imported from the US or Canada to China. They were brought back to the Marine Research Station of the Guangdong Ocean University, Zhanjiang, China. The lobsters were acclimatized (>1 week) in 1-mt circular fiberglass tanks equipped with running seawater (salinity = $32 \pm 5\%$), temperature of $20 \pm 3^\circ\text{C}$, and an aeration system under conditions of natural photoperiod. They were fed twice daily with commercial pellet diets and fresh oysters. The molt stages of the lobsters were determined by setogenesis specifically for lobster (Helluy and Beltz, 1991). Except for the tissue extraction studies, females were used in all cloning and expression experiments. Before dissection, the lobsters were anesthetized in ice for 30 min. All experiments were conducted

in compliance with the guidelines of the Animal Care and Use Committee of Guangdong Ocean University.

Cloning of *HaCHH-A* and *HaCHH-B* Genes

To avoid cloning of multiple highly homologous CHH isoforms and highly polymorphic gene transcripts from different individuals, genomic DNA from a single animal was used as the template. For genomic DNA extraction, tissues from the hepatopancreas (Hp) or testis were dissected from the animal and extracted in an extraction buffer. Purified high-quality genomic DNA was used as the template for PCR. Gene-specific primers were designed to specifically amplify either the *HaCHH-A* or the *HaCHH-B* gene, and these primers were located in the exon of the published CHH gene from other decapods (Table 1).

Cloning of the *sHaCHH-A* and *sHaCHH-B* cDNA

Total RNA was extracted from various tissues using TransZol Up Plus RNA spin column-based kit (TransGen, Beijing, China). The RNA concentration was determined using a NanoDrop spectrophotometer, and 1 µg of total RNA was reverse transcribed using the SMART RACE cDNA Amplification Kit (TaKaRa, Shiga, Japan). PCR amplification of RACE (rapid amplification of cDNA ends) products was performed using a 5'- and 3'-RACE Kit (TaKaRa, Shiga, Japan). The forward primers CHH-F1A: 5'-CGTCATGTTTCGCTGC AGAACTCTG-3' and CHH-F1B: TCATGATGGCCTGCAGAGCGCTGT and the common reverse primer CHH-RP: 5'-TTACTTG CCGACCATCTGGACG-3' were used. The internal primers used to generate *HaCHH-A* and *HaCHH-B* isoform-specific RT-PCR products were as follows: full-length *HaCHH-A* and *HaCHH-B* were generated using the forward primer 5'-CCCATTTGCAAAGCGATGAGTTCG-3' and the reverse primer 5'-TGTGCCAA ATGGCTCACAGAA-3'; the amplified

fragment was cloned into the pMD19 TA cloning vector (Sangon Biotech, Shanghai, China). All the clones were confirmed by nucleotide sequencing analyses.

To determine the messenger RNA (mRNA) expression profiles of *sHaCHH-A* and *sHaCHH-B* in the biannual ovarian development cycle, females at different reproductive stages were sacrificed for total RNA extraction. The reproductive stages of these animals were determined based on the coloration of the ovary and the Gonadosomatic Index (GSI) as previously reported (Tiu et al., 2009).

The amino acid sequences of the positive clones were analyzed using ORF Finder (NCBI; <http://www.ncbi.nlm.nih.gov/projects/gorf/orf.cgi>). Sequence identities were verified using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignment was performed using the amino acid sequence, and the online Multiple Sequence Alignment software ClustalW (genome.jp) was employed for the various CHH sequences chosen to represent the major subgroups of the type I CHH genes, using only the most highly conserved orthologous amino acids within the CHH/MIH/GIH from representative decapods. The amino acid sequences used in our phylogenetic analysis are listed in Supplemental Data File 1. The amino acid sequences of *HaCHH-A* and *HaCHH-B* were compared to those of other decapods to confirm the identification of each family member.

Real-Time Quantitative PCR

The transcript levels of *HaCHH-A*, *sHaCHH-A*, *HaCHH-B*, and *sHaCHH-B* were determined by both semi-quantitative PCR and real-time quantitative PCR (RT-qPCR). For the semi-quantitative PCR, each PCR was carried out in a final volume of 10 µl containing 5 µl of 2× PCR Master Mix (ABI, Richmond Hill, Canada), 0.3 µl of forward and reverse primers, 2.4 µl of nuclease-free water, and 2 µl of cDNA template. For the internal control, we

TABLE 1 | Primer sequences used in the RT-PCR, RACE, gDNA PCR and RNAi for the lobster CHH.

Name	Sequence	Description:genomic/RT-PCR/RNAi
HaCHH-F4	TCAGGTGTTGACCAGGCGTGT	Common primer for genomic,RT-PCR
HaCHHRP	TTACTTGCCGACCATCTGGACG	
HaCHH-F1A	CGTCATGTTTCGCTGCAGAACTCTG	Specific primer for HaCHH-A
HaCHH-F1B:	TCATGATGGCCTGCAGAGCGCTGT	Specific primer for HaCHH-A
HaCHH-R2:	GTTGTAACAGTCCTCACACACCGG	Common primer for genomic,RT-PCR
T7HaCHHAex3F:	TAATACGACTCACTATAGGGAAACCTTTCGTCGCCACCACCTG	dsRNA
T7HaCHHAex3R:	TAATACGACTCACTATAGGGTATCATATCCCTGATCTCGAGGT	
T7HaCHHBex3F:	TAATACGACTCACTATAGGGAAACCTTTCATCGTCACCACCTG	dsRNA
T7HaCHHBex3R:	TAATACGACTCACTATAGGGTATCATATCCCTGAACCTCAAGGA	
sHaCHH-A_F:	CTCGTCCAATTCGCCGTCGT	qT-PCR
sHaCHH-A_R:	TCCTTCCTCCTCACCAACCACITAAAGT	
HaCHH-A_F:	CTCGTCCAATTCGCCGTCGT	qT-PCR
HaCHH-A_R:	CGAGACATTGACGGAACCCCAATTAC	
sHaCHH-B_F:	CTCGTCCAATTCGCCGTCGT	qT-PCR
sHaCHH-B_R:	CATCACACACCTCGGGAATGTGT	
HaCHH-B_F	CTCGTCCAATTCGCCGTCGT	qT-PCR
HaCHH-B_R:	CGTTGGAGACGTACTCGTCGAT	
HaEF1aF	AATACCGCGGCCCTTTAGTTTG	qT-PCR
HaEF1aR:	TTGGTGTGGGTTAAGGAACCTG	
Ha1VgF:	AGACGGACGTGGTAGGGAAGTG	qT-PCR
Ha1VgR:	CAGACTACCTTGAGGGATGGCAG	

have used the elongation factor 1 (*Ef1 α*) and the β -actin gene of the lobster. The PCR conditions were as follows: 94°C for 5 min, 34 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and 72°C for 3 min. For RT-qPCR, the CFX96 Real Time System (Bio-Rad, Hercules, CA, USA) and qPCR Master Mix (TaKaRa) were used. Total RNA was extracted from the different tissues of mature lobsters. Total RNA from each tissue (1 μ g) was treated with gDNA Eraser at 37°C for 15 min to avoid contamination with genomic DNA. Total RNAs were used for cDNA synthesis using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). The cDNAs from females with vitellogenic oocytes were used for tissue distribution. The relative mRNA abundance of each gene was calculated using the $\Delta\Delta C_t$ method, then normalized to that of the *Ef1 α* and β -actin for each sample. Measurements of the expression levels were performed in triplicate or duplicate.

***sHaCHH-A* and *sHaCHH-B* Functional Study by *In vitro* Bioassay**

Recombinant protein and RNAi technologies were used to study the function of *sHaCHH-A* and *sHaCHH-B* in lobster gonad vitellogenesis. For the detailed protocol of recombinant protein production, see Liang et al. (2019). Briefly, the restriction enzyme site sequence-linked primers for *sHaCHH-A* (expHaCHH-AF: GGATCCCGGTCTGTAGAAGGAGCATC; expHaCHH-AR: GAGCTCCTACCCCTTGATCATATCC) and *sHaCHH-B* (*sHaCHH-B*; rHaCHH-B: GGATCCCGGTCAGTAGAAGGAGCGGATATGATCAAGGGGTAGGAGCTC) (Table 1) were used to amplify the mature peptide of *sHaCHH-A* or *HaCHH-B*. The cDNA template was derived from the cDNA clones from the above cDNA cloning procedure. The PCR-amplified cDNA fragments were digested with the same enzyme and ligated to the pET32A vector plasmid digested previously with the same restriction enzymes (*Bam*HI and *Sac*I). After bacterial transformation, positive clones were screened by PCR using the T7 promoter and T-terminator primers. The positive clones were verified by PCR with a gene-specific primer for either the *HaCHH-A* or *HaCHH-B* gene and DNA sequence determination. The recombinant proteins were produced using *Escherichia coli* DE21 strain and subsequently purified. The purified and re-natured recombinant proteins for *sHaCHH-A* and *sHaCHH-B* were used for *in vitro* bioassay to study their potential functions.

RNA interference approaches were used to knock down the functions of *sHaCHH-A* or *sHaCHH-B*. For double-stranded RNA (dsRNA) synthesis, primers amplifying exon 3 of *sHaCHH-A* and *sHaCHH-B* were designed (Table 1). The templates were the cDNAs for *sHaCHH-A* and *sHaCHH-B* from the cDNA cloning described above. DsRNAs were synthesized using the T7 RNA Transcription Kit (Vazyme, Nanjing, China). For control, the dsRNA for the tiger frog virus ATPase gene was used (Tiu et al., 2008). The dsRNA template for each gene was amplified (PCR amplification procedure: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, then 72°C for 10 min) with the above primers and purified using the FastPure Gel DNA Extraction Mini Kit (Vazyme). After detection of the

concentrations of the DNA templates (NanoDrop 2000; Thermo Fisher Scientific, Inc., Waltham, MA, USA), dsRNA was produced with DNA templates using the T7 RNA Transcription Kit (Vazyme). The final dsRNA was diluted to an appropriate concentration (1 μ g/ μ l) with phosphate-buffered saline (PBS). *In vitro* RNA interference experiments ($n = 8$) were then performed.

The lobsters were dissected and the ovary developmental stage was determined. Individuals with ovary at the early (yellow) and middle (light green) stages were dissected for the Hp and ovary (i.e., 50–80 mm³). The tissues were first rinsed with ice-cold PBS and then placed into the wells of a sterile culture plate containing 1.5 ml nutrient medium (M199; Sigma, St. Louis, MO, USA). Individual wells were treated with either the tested recombinant proteins, dsRNAs, or the control (Tiu et al., 2008). The culture plate was then shaken on the platform of an orbital shaker at 24–26°C for 3 h. At the end of the culture period, the tissues were extracted for total RNA and for cDNA synthesis. The expression of the lobster vitellogenin gene was monitored by RT-PCR, as previously described. The *HaVg1* primers were derived from the lobster vitellogenin cDNA (GenBank no. EF422415.1).

RESULTS

Characterization of *sHaCHH-A* and *sHaCHH-B*

Current GenBank BLASTP sequence homology search results for *HaCHH-A* (GenBank no. P19806.3) and *HaCHH-B* (GenBank no. 2105187B) indicated that they are most similar to the prepro-CHH isoform A of the lobster *Nephrops norvegicus* (GenBank no. AAQ22391.1) and the CHH isoform B (GenBank no. AAQ22392.1). Compared to the crayfish, *HaCHH-A* and *HaCHH-B* are most similar to the CHH of *Procambarus clarkii* (GenBank no. Q25683.3). Compared to the CHH of shrimps, *HaCHH-A* and *HaCHH-B* are most similar to the CHH-like of *Litopenaeus vannamei* (GenBank no. AAN86055.1) and *Penaeus monodon* (GenBank no. XP037787977), sharing 68%–69% sequence identity in the mature peptide region. Moreover, the *HaCHH-A* and *HaCHH-B* sequences are more similar to the CHH of the freshwater shrimp *Macrobrachium rosenbergii* (GenBank no. AAF29534.1). Compared to the crab CHH, the lobster sequences shared the highest sequence identity with the CmCHH (GenBank no. P14944) of *Carcinus maenas*. Further analysis of these decapod CHH sequences revealed that they were derived from four-exon genes. Alternative splicing of the pre-mRNA for these CHHs gave rise to the transcripts being analyzed. Therefore, the results suggested that the lobster *HaCHH-A* and *HaCHH-B* may also be derived from different four-exon genes. Unlike a previous report (DeKleijn et al., 1995), the current RT-PCR results showed that two transcripts can be detected in the eyestalk cDNA (Figure 1A). The small cDNA fragment was about 150 bp, and a larger and more abundant DNA fragment of 310 bp was also amplified. Similar results were observed for the *HaCHH-B* gene when primers *HaCHH1b* and

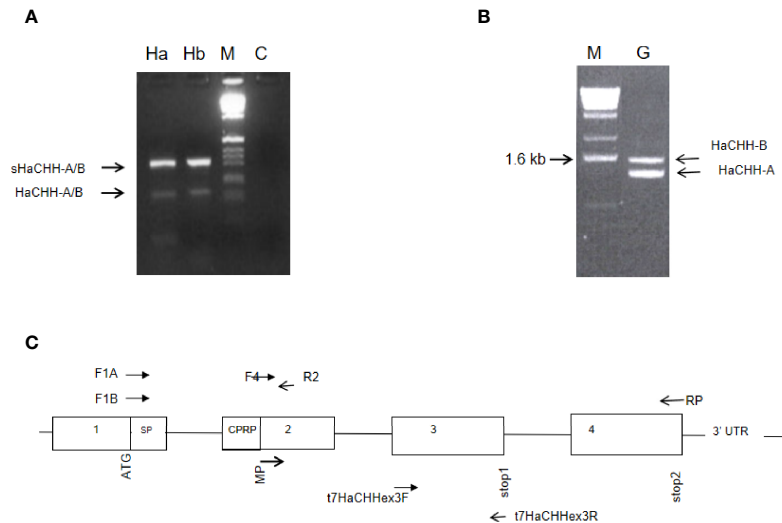


FIGURE 1 | (A) Identification of transcript variants for the *HaCHH-A* and *HaCHH-B* genes. Gene-specific forward primers for *HaCHH-A* (F1A) and *HaCHH-B* (F1B) were paired with common reverse primer (R2) in the RT-PCR amplification of cDNA from eyestalk (Es). Two major fragments were amplified by each pair of primers. The larger fragment (upper arrow) is the *sHaCHH-A* or the *sHaCHH-B* transcript variant; the smaller band (lower arrow) is for transcripts *HaCHH-A* and *HaCHH-B*. **(B)** Gene from eyestalk cDNA of female lobster. Lane Ha: PCR results using primer pair F1A and RP; lane Hb: results for primer F1B/RP; M: DNA size marker; and C: negative, no template control. **(B)** Genomic PCR detection of multiple *HaCHH-A* and *HaCHH-B* genes. The primer pair F4/RP (Figure 2 and Table 1) was used and two distinctive DNA fragments (i.e., 1.4 and 1.6 kb) were amplified. **(C)** Schematic diagram showing the locations of the primers and the proposed gene structure of the *HaCHH* genes used in this study.

HaCHH-RP were used in PCR (Figure 1A). These two DNA fragments were subcloned into pMD19 in *E. coli* for DNA sequencing. The results revealed that the smaller cDNAs carried coding sequences for the matured peptide of the previously reported *HaCHH-A* (GenBank no. P198063) and *HaCHH-B* (GenBank no. 2105187B). The 5' end of the larger cDNA carried a coding sequence identical to the corresponding *HaCHH-A* or *HaCHH-B*, but the 3' end carried a peptide sequence different from the original *HaCHH-A* and *HaCHH-B* cDNA (see below).

HaCHH-A and *HaCHH-B* Gene Organization

To study the *HaCHH-A* and *CHH-B* genes, PCR amplification of genomic DNA was performed with the DNA templates and cDNAs from a single individual (Figures 1B, C and Table 1). Two major DNA bands with sizes of 1,600 and 1,450 bp were amplified. Because the intensity of the smaller band in the agarose gel was stronger, more copy numbers for the smaller genomic DNA were expected. Sequence determination of the bands revealed that each DNA fragment carries the coding and non-coding sequences for the *HaCHH-A* (1.4 kb) or the *HaCHH-B* (1.6 kb) gene (Figures 1B, C). These fragments carry coding sequences for partial exon 2, exon 3, and exon 4 and the interrupting non-coding introns 2 and 3. We employed genomic PCR to attempt to recover the partial exon 1 coding sequence and non-coding intron 1 of both genes, without success. As a comparative study of these CHH genes, genomic information of the four-exon CHH genes from other decapods was collected (Figure 2). Except for the complete CHH gene

structure of *L. vannamei* (from the *L. vannamei* genome project), the other CHH gene structures were incomplete. Most of these genes lacked information on the first intron. In the *L. vannamei* CHH gene, intron 1 consisted of several highly repeated microsatellite sequences [i.e., (AT) n , (CT) n , and (GT) n] along the span of a 3.34 intronic region (*L. vannamei* genomic database, GenBank no. LOC113815764).

The RT-PCR and 5' and 3'RACE cloning approaches were used to clone the large and small fragments, as described above. The four cDNA sequences were cloned and the sequences determined. The amino acid sequences of two smaller cDNAs were identical to the previously reported *HaCHH-A* and *HaCHH-B*. The two larger cDNAs (i.e., *sHaCHH-A* and *sHaCHH-B*) represented the newly discovered transcript variants (Figure 3). As expected, *HaCHH-A* and *sHaCHH-A* shared identical amino acids in the N-terminal end, but major amino acid sequence variations occurred in the C-terminal end. Similar results were found for *HaCHH-B* and *sHaCHH-B*. For the multiple alignment and phylogenetic study, other CHH subtype I sequences and MIH/GIH subtype II group neuropeptides were included in the ClustalW analysis together with the four *HaCHH* variants. Within the same color block, a much higher degree of sequence identity was observed in the signal mature peptide region (Figure 4). For the mature peptide, the degree of homology was much higher in the coding sequence of exon 2 and was much lower in the C-terminal end of the protein encoded by exon 3. The amino acid similarity at the N-terminal end of the mature peptide for *HaCHH-A* and *HaCHH-B* was much higher, i.e., >97% amino acid identity, but the homology decreased to only 63.5% at the C-terminal end.

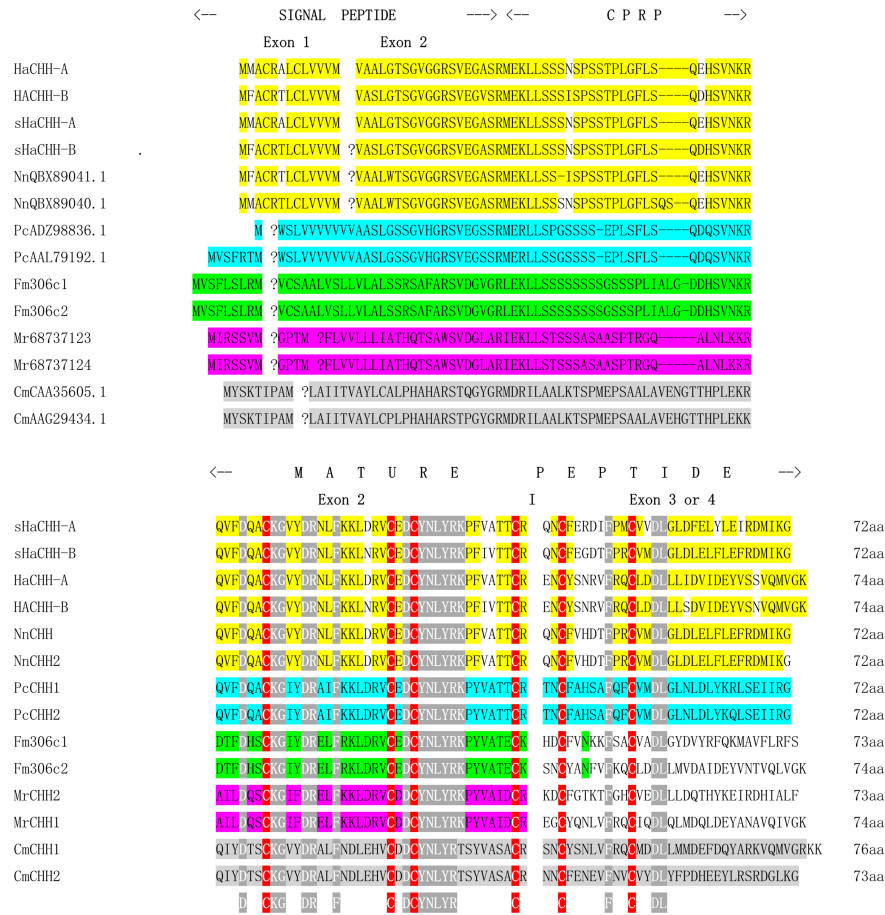


FIGURE 4 | Alignment of the lobster four-exon CHH genes with that of other decapods, including lobster (yellow: *HaCHH-A* and *HaCHH-B*; *NnCHH-1* and *NnCHH-2*), crab (gray: *CmCHH-1* and *CmCHH2*), shrimp (green: *Fm306-c1* and *Fm306-c2*) (Shi et al., 2013), freshwater shrimp (pink: *MrCHH1* and *MrCHH2*), and crayfish (blue: *PcCHH1*, *PcCHH2*). The same block color indicates the same groups of decapods. Signal peptides and mature peptides are indicated. Gaps are introduced in the boundary of the exons in lobster, shrimp, and crab CHH-like genes. A question mark is inserted in the locations of potential intron insertion sites in the CHH-like genes from other decapods. In addition to the conserved cysteine residues (highlighted in red), several conserved amino acids (highlighted in gray) may be important for motif structure formation of the peptide.

CPRP–mature peptide structure. The CHH-Like group is the more recently discovered CHHs having the signal peptide–mature peptide structure. The CHH-L/S group comprise the four-exon CHH genes that produce alternative transcripts, as described in this study. The lobster *sHaCHH-A* and *sHaCHH-B* were closely related to the crayfish CHH, followed by those of the shrimp and crab (Figure 5). As a reference, the MIH/GIH subtype evolved from a common ancestor, giving rise to the three-exon CHH and the four-exon CHH-L/S. It was observed that the four-exon CHH-L/S gene cluster represented a small number relative to the CHH group since only a few (i.e., *LvCHH2* and *MeCHH-B*) of the CHH type were selected for phylogenetic tree construction (Figure 5).

Expression of Lobster *sHaCHH-A* and *sHaCHH-B* Transcripts

Both semi-quantitative RT-PCR and RT-qPCR were used to study the expression of the *HaCHH* gene. For semi-quantitative PCR,

multiple DNA fragments were amplified by F1A/R4 and F1B/R4 primers from different tissues. The smaller transcripts (i.e., 158 bp) represent the originally reported *HaCHH-A* or *HaCHH-B* cDNA, and they can be detected in the eyestalks of non-reproductive females. No specific pattern of these short transcripts was observed during the different stages of the reproductive cycle. The larger transcripts (i.e., 317 bp, which produced *sHaCHH-A* and *sHaCHH-B*) can be detected mainly in the Hp and thoracic ganglion. However, DNA fragments of different sizes were also amplified (i.e., especially in the thoracic ganglion) (Figure 6A). Further study by qPCR was performed using females in the early reproductive stages. The results confirmed that *HaCHH-A* and *HaCHH-B* were mainly expressed in the eyestalk. Also, *sHaCHH-A* and *sHaCHH-B* transcripts were detected in the non-eyestalk neuronal and in non-neuronal tissues, including the Hp, ovary, brain, and thoracic ganglion. However, the transcripts were not detected in the Hp (Figure 6B). The 158- and 317-bp PCR products were also subcloned and DNA sequencing was

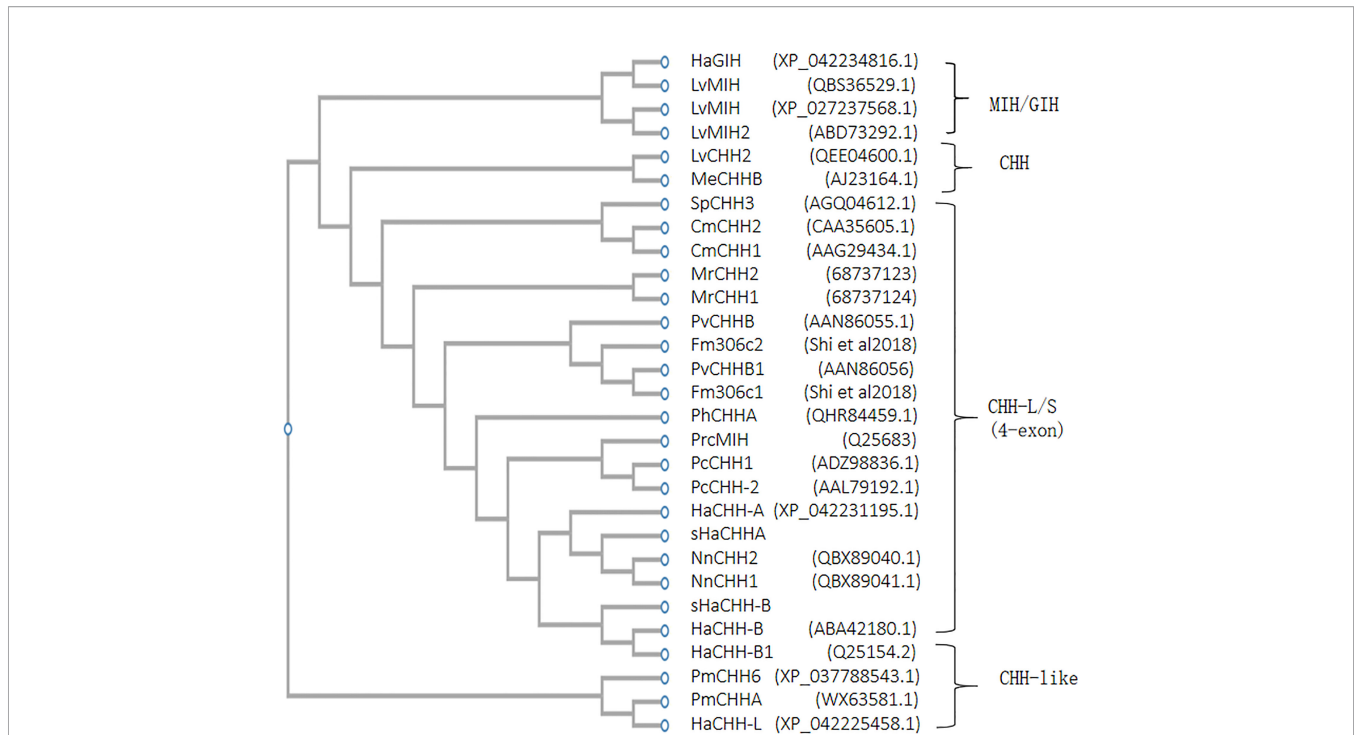


FIGURE 5 | Phylogenetic tree analysis of lobster *HaCHH-A* and *HaCHH-B* cDNA within the major subfamilies of decapod CHH/MIH/GIH. The sequences included the lobster (*Homarus americanus*, *Ha*) PhCHH-A (QHR84459.1), *HaCHH-L* (XP_042225458.1), *HaGIH* (XP_042234816.1), *HaCHH-A* (XP_042231195.1), *sHaCHH-A*, *NnCHH2* (QBX89040.1), *NnCHH1* (QBX89041.1), *sHaCHH-B*, (ABA42180.1), and *HaCHH-B1* (Q25154.2); crab (*Carcinus maenas*, *Cm*) *CmCHH2* (CAA35605.1), *CmCHH1* (AAG29434.1), and *SpCHH3* (AGQ04612.1); shrimp (*Litopenaeus vannamei*, *Lv*) *LvMIH* (QBS36529.1), *LvMIH* (XP_027237568.1), *LvMIH2* (ABD73292.1), *LvCHH2* (QEE04600.1), *PvCHHB* (AAN86055.1), *Fm306c2* (Shi et al., 2018), *PvCHHB1* (AAN86056), *Fm306c1* (Shi et al., 2018), *PmCHH6* (XP_037788543.1) *PmCHHA* (WX63581.1), and *MeCHHB* (AJ23164.1); freshwater shrimp (*Macrobrachium rosenbergii*, *Mr*) *MrCHH2* (68737123) and *MrCHH1*(68737124); and crayfish (*Procambarus clarkii*, *Prc*) *PrcMIH* (Q25683), *PcCHH1* (ADZ98836.1), and *PcCHH-2* (AAL79192.1). GenBank accession numbers are shown in *parentheses*.

performed, which confirmed that they were specific for the *HaCHH-A* and *HaCHH-B* and the *sHaCHH-A* and *sHaCHH-B* isoform sequences, respectively. Nucleotide sequence analysis of 12 clones generated from the PCR products revealed five *HaCHH-A* and two *sHaCHH-A* clones and two *HaCHH-B* and one *sHaCHH-B* clone, confirming that the large sized clones were from the *sHaCHH-A* and/or *sHaCHH-B* and the small sized clones were from the *HaCHH-A* and/or *HaCHH-B*.

Because previous tissue expression results suggested that neuronal tissues express relatively large amounts of *sHaCHH-A* and *sHaCHH-B*, we studied their expressions at different gonad maturation stages of the female lobster. The RT-PCR results indicated that they are the predominant transcripts during the different stages of maturation (i.e., stages I to IV). Although both *sHaCHH-A* and *sHaCHH-B* were expressed, the transcript level for *sHaCHH-B* was much lower than that of the original *HaCHH-A* and *HaCHH-B* transcripts. In contrast, the other non-eyestalk neuronal tissues (i.e., brain, thoracic ganglion, and nerve cord) expressed mainly the larger transcripts consisting of exons 1–4 (**Figure 7A**). In the thoracic ganglion and the nerve cord, the expression level of *sHaCHH-A* was consistently low. The expression level of *HaCHH-B* appeared higher during the early and active stages of vitellogenesis (**Figure 7B**). However, no specific expression pattern for *sHaCHH-A/sHaCHH-B* was

recognized in the neuronal tissues. In summary, the expression study indicated that *sHaCHH-A* and *sHaCHH-B* are both expressed in non-eyestalk neuronal tissues, including the brain, thoracic ganglion, and ventral nerve cord. The expressions of *sHaCHH-A* and *sHaCHH-B* in other non-neuronal tissues, such as the Hp and ovary, during ovarian maturation suggested that these proteins are important in ovary maturation. Moreover, the expressions of these genes are stage-specific as lower transcript levels were detected during the immature stage, but much higher levels of expression in lobster undergo reproduction.

Functional Study of *sHaCHH-A* and *sHaCHH-B* by *In Vitro* Tissue Culture of the Hepatopancreas and Ovary Fragments

Purified recombinant protein and dsRNA were added to the nutrient medium in the tissue explant bioassay experiments (**Figure 8**). In stage I Hp, recombinant *sHaCHH-A* (i.e., 2 μ g) caused a decrease in the transcript level of *HaVg1*. Despite the lower expression of *HaVg1* in the ovary compared to that in the hepatopancreas, an increase in the concentration of *rsHaCHH-A* caused a decrease in the expression of *HaVg1* in a dose-dependent manner ($p < 0.05$). In stage I Hp and ovary, the dsRNAs for *sHaCHH-A* caused a significant increase in the expression of *HaVg1* ($p < 0.05$) (**Figure 9A**). In stage III Hp,

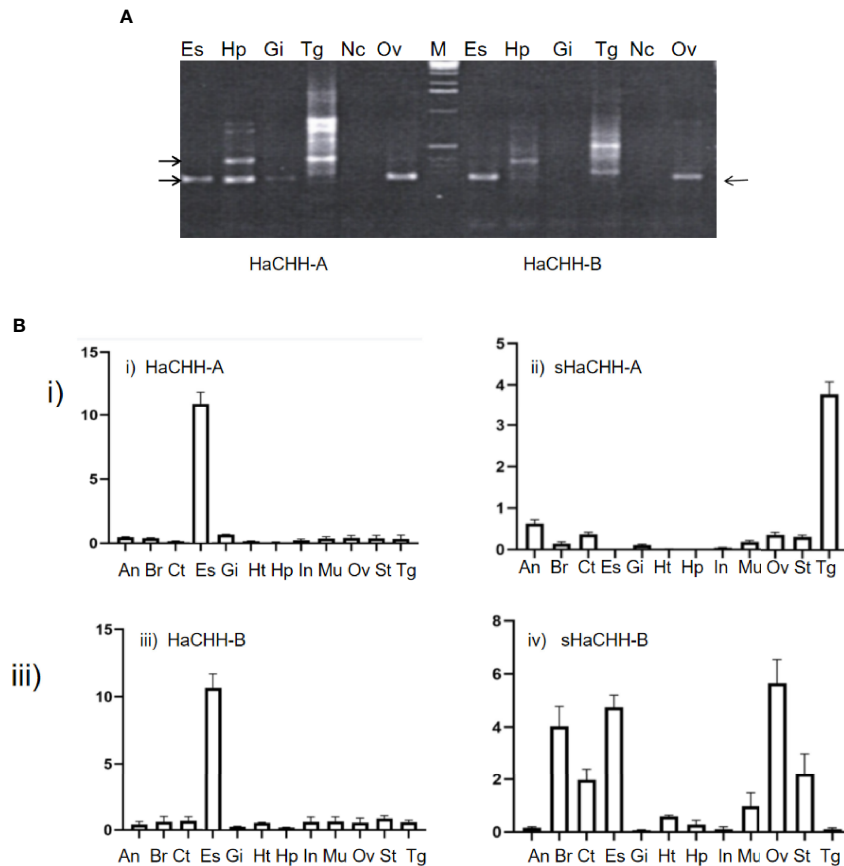


FIGURE 6 | (A) RT-PCR expression study of the *HaCHH-A*, *sHaCHH-A*, *HaCHH-B*, and *sHaCHH-B* genes in different tissues of the lobster female. The primers F1A and RP were used to amplify *HaCHH-A* and *sHaCHH-A*, while primers F1B and RP were used to detect the transcripts of *HaCHH-B* and *sHaCHH-B* in the different tissues [eyestalk (*Es*), hepatopancreas (*Hp*), gill, (*Gi*), thoracic ganglion (*Tg*), nerve cord (*Nc*), and ovary (*Ov*)] of lobster during the early reproductive stage (stage I). **(A)** Representative agarose gels showing the detection of *HaCHH-A*, *HaCHH-B*, *sHaCHH-A*, and *sHaCHH-B*. The larger (317 bp) fragment is the *sHaCHH-A* or *sHaCHH-B* and the smaller (158 bp) fragment is the amplified partial *HaCHH-A* and *HaCHH-B* cDNA. **(B)** qPCR expression study of the i) *HaCHH-A*, ii) *sHaCHH-A*, iii) *HaCHH-B*, and iv) *sHaCHH-B* genes in different tissues of the female lobster. Primers HaCHH-AF/*HaCHH-AR* and *sHaCHHAF/sHaCHHAR* were used to amplify *HaCHH-A* and *sHaCHH-A*. Primers HaCHHAF/*HaCHHAR* and *sHaCHH-BF/sHaCHH-BR* were used to detect the transcripts of *HaCHH-B* and *sHaCHH-B* in the different tissues [abdominal nerve (*An*), brain (*Br*), cuticle (*Ct*), eyestalk (*Es*), hepatopancreas (*Hp*), heart (*Ht*), gill (*Gi*), intestine (*In*), muscle (*Mu*), stomach (*St*), thoracic ganglion (*Tg*), nerve cord (*Nc*), and ovary (*Ov*)] of lobster during the middle stage of reproduction (II).

rsHaCHH-A caused a decrease of *HaVg1* in a dose-dependent manner ($p < 0.05$). However, the expression level of *HaVg1* in the ovary was not affected. When dssHaCHH-A was added to the medium, the expression levels of *HaVg1* in the Hp and ovary increased (Figure 9B).

When the recombinant protein for *sHaCHH-B* was added to the medium, a decrease in the level of *HaVg1* was observed in the Hp and ovary from stages I and III (Figures 9C, D). When the dsRNAs for *sHaCHH-B* were added, the expression levels of *HaVg1* also increased in the Hp and ovary fragments. It appears that the level of stimulation was much higher for the dssHaCHH-B-treated Hp. In summary, the results indicated that the recombinant proteins of *sHaCHH-A* and *sHaCHH-B* inhibited the expression of *HaVg1*, but gene knockdown of *sHaCHH-A* and *sHaCHH-B* stimulated the expression of *HaVg1* in the Hp and ovary fragments (Figure 9).

DISCUSSION

Transcript Variants of CHH in Lobsters and Other Decapods

Major advancement occurred in crustacean endocrinology in the recent application of the next-generation sequencing technique in transcriptome and genome studies. As a result, the number of CHH family genes identified in the same species and the total number of CHH in crustaceans have greatly increased (Havird and Santos, 2016; Chang and Lai, 2018; Oliphant et al., 2018; Hyde et al., 2020; Mykles and Chang, 2020). Therefore, many new CHH sequences have been reported in recent years. However, most CHH sequencing projects mainly focused on economically important species. Therefore, the number of CHH family members identified in lobster is fewer than that of the shrimps and other decapods (Hyde et al., 2020). Based on

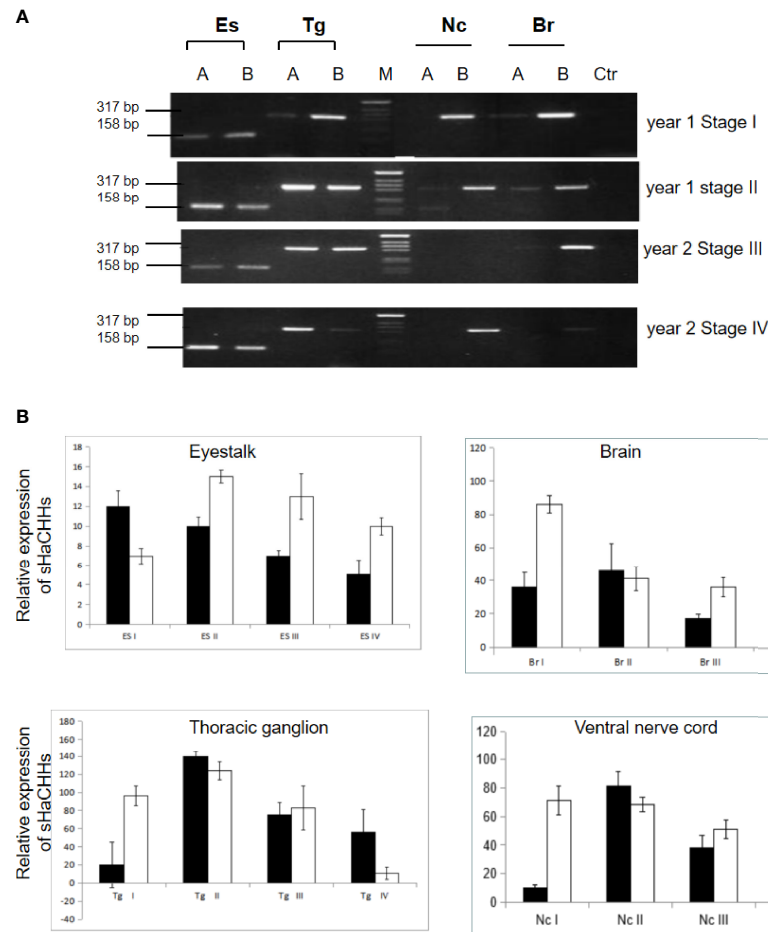


FIGURE 7 | Expression study of sHaCHH-A and sHaCHH-B by Semi-quantitative **(A)** and **(B)** qPCR. **(A)** Agarose gel analysis of the PCR products and detection of the sHaCHH-A, HaCHH-A sHaCHH-B, HaCHH-B. The lanes A used primers for HaCHH-A gene and lane B used primers for HaCHH-B for cDNA from eyestalk (Es), thoracic ganglion (Tg), ventral nerve cord (Nc) and brain (Br).

genomic and transcriptomic sequence data from *L. vannamei* and *P. monodon*, it is evident that the CHH family consists of multiple gene members. In *L. vannamei*, it is estimated that at least >65 CHH family genes are present in the genome (Zhang et al., 2019). However only a small portion of the CHH genes had the four-exon gene organization. Therefore, the four-exon CHH genes from the decapods shown in the phylogenetic tree in **Figure 4** represent a very small portion of the same species within the same gene family. Although the number of CHH genes reported in lobster was fewer, it is unlikely that *H. americanus* consists of only two highly homologous four-exon CHH genes. From the recent lobster genome project (Polinski et al., 2021), we only identified <5 CHH-related genes. Moreover, no positive sequence was returned when we used several lobster genes (from our lobster transcriptome project) as the query sequence to the BLAST search against the lobster genome. Therefore, additional lobster CHH-like sequences may exist, but were missed in the lobster genome project. Despite the ambiguity, the lobster genome should consist of fewer number of CHH/MIH/GIH genes compared to the shrimps. From the

expression and multiple alignment results (**Figure 5**), some generalizations can be proposed. The non-eyestalk expressed transcript is usually the larger CHH variant derived from the four-exon transcript. In addition, due to the broad distribution of the transcripts in the non-eyestalk and non-neuronal tissues, it is suggested that they may have a broad spectrum of functions.

Structurally, the *sHaCHH-A* and *sHaCHH-B* identified in this study had the major features of the CHH neuropeptide, and they shared identical lengths with the mature peptides and are conserved in other lobsters. In the lobster *N. norvegicus*, despite two *NnCHH* being cloned, the two cDNAs shared >98% amino acid identity in the mature peptide region; a second CHH that shares similar exon 3 has not been reported. In *L. vannamei* (GenBank no. AAK69346.1) and *P. monodon* (GenBank no. XP_037787978) CHH-like, they also consisted of identical amino acid residues in the mature peptide region. All the short/smaller CHH-like variants were derived from longer transcripts consisting of exons 1–4. For example, in *Fenneropenaeus merguensis*, the gill form CHH-like was derived from a longer transcript (i.e., exons 1–4) and the larger

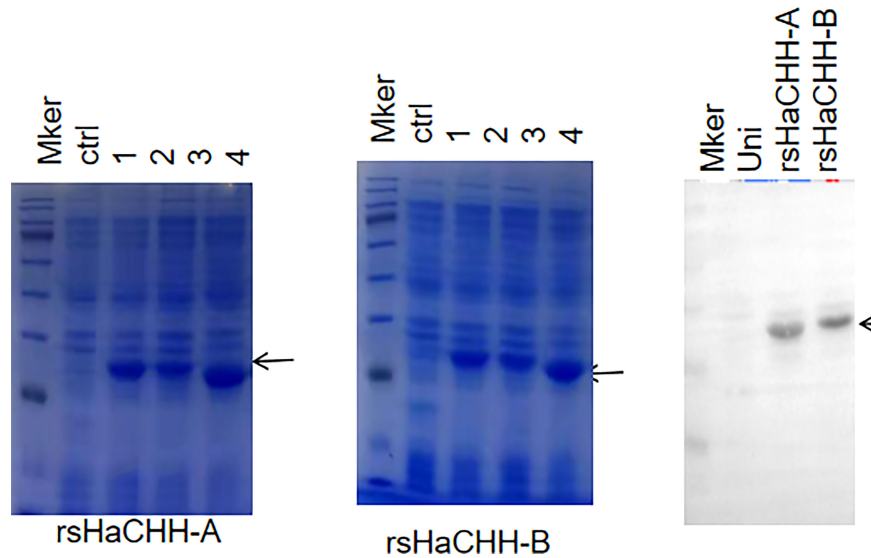


FIGURE 8 | SDS-PAGE (12.5%) analysis of the prokaryotic (*Escherichia coli*) expression of the recombinant proteins rsHaCHH-A (left) and rsHaCHH-B (middle) after induction by IPTG. *Ctrl*: uninduced (*uni*); lanes 1–4: induced and sampled at 2, 4, 6, and 8 h, respectively. Western blot detection of the poly-His-tagged recombinant proteins sHaCHH-A and sHaCHH-B (arrow). Right: The antibody used is the anti-polyhistidine peroxidase conjugate antibody (Sangon Biotech, Shanghai, China).

CHH-like was derived from a smaller transcript consisting of exons 1, 2, and 4 (Shi et al., 2018). The mature peptides of *sHaCHH-A* and *sHaCHH-B*, and *sNnCHH-A* and *sNnCHH-B* consisted of 73 and 74 amino acid sequences, respectively (Mettulio et al., 2004). The phylogenetic tree also revealed that the four-exon *CHH-L/S* gene (Figure 5) may be derived from the common ancestor gene that gave rise to the more abundant three-exon CHH genes (i.e., *LvCHH2*; GenBank no. QEE046000.1) and the *MIH/GIH* subtype. In the lobster *H. americanus*, a three-exon *HaCHH* gene was identified from the recent genome sequencing project (GenBank no. XP_042225458.1) and can be grouped into the CHH-like class, as indicated in Figure 5.

Four-Exon CHH Gene in Decapod Crustaceans

Although the four-exon CHH family genes have been reported in several decapods, but only the complete gene structure of *L. vannamei* is known. For the other decapods—the crab, freshwater shrimp, and crayfish—only the partial gene structure was revealed, and sequence information for the first intron was missing. We speculated that the gene organizations of these four-exon CHHs among decapods are highly similar. The sequence for the first intron of the CHH gene in *L. vannamei* was obtained from a genome sequencing project. The first intron of the CHH gene is 4.65 kb, and within this region, several highly repetitive simple sequence repeats (SSRs) exist. This highly repetitive sequence would affect the PCR reaction and, therefore, cause failure in genomic PCR (GenBank no. LOC113815764). Therefore, it is likely that the first exon of these decapod CHH genes is also made up of a large intron 1 and

may consist of highly repetitive SSRs within the intron that hinder/disrupt the PCR amplification.

CHH Sequence Comparison

In terms of the evolutionary relationship of the CHH family genes, it was speculated that their original prototype consisted of three exons and two introns. The evolution of the CHH family genes began from gene duplication and mutation (Chan et al., 2003; Montagné et al., 2008; Chung et al., 2010; Chen et al., 2020). Alignment of the four-exon CHH neuropeptide genes revealed both similarities and differences among decapods. The lobster *HaCHH-A* and *HaCHH-B* shared >95%–100% amino acid identity with the lobster CHHs. The similarity was much higher (i.e., 99% amino acid identity) in the mature peptide region. In the Norway lobster *Nephrops norvegicus*, two CHHs were cloned (i.e., GenBank nos. AY285782 and AY285783), which differed only in the signal peptide region, but the mature peptide showed 100% sequence identity (Mettulio et al., 2004). *HaCHH-A* and *HaCHH-B* shared only 77%–78% amino acid identity with the crayfish *P. clarkii* CHH-like (78.1%; GenBank no. Q25683) and *Astacus leptodactyl* (i.e., 77.3%; GenBank no. AAX09331). In earlier studies, the CHH family neuropeptides were reported to be expressed only in the eyestalks. Increasing evidence from recent expression studies has confirmed the expression of CHH family members in other non-eyestalk neuronal and non-neuronal tissues. So far, *H. americanus* is the only decapod consisting of two 4-exon CHH family genes, and these two genes could produce four different transcripts.

The original *HaCHH-A* and *HaCHH-B* transcripts are abundant in the eyestalk; therefore, RT-PCR cannot amplify

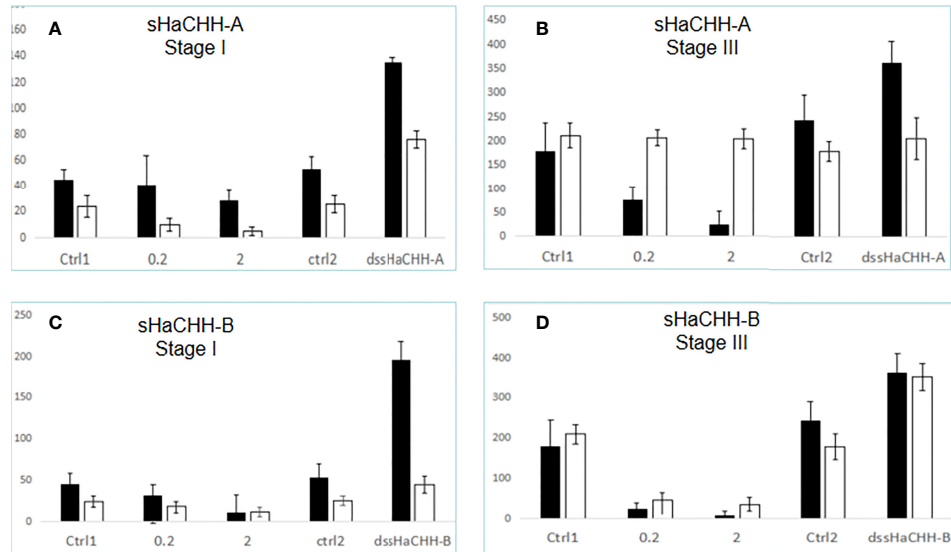


FIGURE 9 | Effect of recombinant protein and dsRNA for sHaCHH (A, B) and sHaCHH-B (C, D) on *HaVg1* gene expression. The Y-axis shows the relative expression level of the *HaVg1* gene in the hepatopancreas (black bar) and ovary (open white bar). Triplicate tests ($N = 4$ samples) were performed for each animal. The lobsters used were in the early stage of vitellogenesis (stage I) and middle-late stage (stage III) of the gonad maturation cycle. Tissue fragments were placed inside the well containing 1.5 ml of the nutrient medium M199 with either the control recombinant protein (0, 0.2, or 2.0 $\mu\text{g}/\text{PBS}$) and Ctrl2 dsTFV, 2 μg dssHaCHH-A, or dssHaCHH-B. After incubating for 3 h, the hepatopancreas and ovary fragments were analyzed for the expression of *HaVg1*. The vitellogenin gene-specific primer was derived from the *HaVg1* cDNA, as reported earlier (Tiu et al., 2008). Data are the mean \pm SE.

the larger transcripts in the eyestalk. In early studies, polyclonal antibodies for *HaCHH-A* and *HaCHH-B* were produced to determine the titer of CHH during the reproductive cycle of females. For example, in *N. norvegicus*, CHH antibodies have been generated to localize the neuropeptide by immunocytochemistry (Giulianini et al., 2002). Other antibodies for CHH were produced, and ELISA was performed to measure the concentration of CHH-A in the hemolymph (Chang et al., 1998; Chang et al., 1999). The cross-reactivity of the polyclonal antibody to *sHaCHH-A* and *sHaCHH-B* has not been evaluated. Therefore, it is necessary to reexamine the protein level of CHH during the reproductive cycle using more specific antisera against specific isoforms. The role of *sHaCHH-A* and *sHaCHH-B* in other neuronal tissues, i.e., brain, thoracic ganglion, and ventral nerve cord, has not been examined. In short, more specific antibody is needed to determine the protein expression patterns of sHaCHH during the lobster reproductive cycle.

The two CHH genes reported in this study can produce a total of four different transcripts (i.e., *HaCHH-A*, *sHaCHH-A*, *HaCHH-B*, and *sHaCHH-B*). However, the expressions of these transcript variants appeared to be tightly regulated as they may exist in a specific temporal and spatial manner. One of the major controversies in the study of *HaCHH-A* expression and protein level was the lack of correlation in both the mRNA and peptide levels in the hemolymph during the pre-vitellogenic stage (de Kleijn et al., 1998). For *HaCHH-B*, the total mRNA and hemolymph CHH peptide levels increased in the mature stage, and there were no differences in peptide storage that may suggest that synthesis is not related to storage (de Kleijn et al., 1998). The

interpretation was hampered by the lack of information for these transcripts' variants. The expression study was focused only on the mRNA in the eyestalk, and the antibody used to detect *HaCHH-A* and *HaCHH-B* may not be able to differentiate *sHaCHH-A* and/or *sHaCHH-B*. Therefore, a more systematic approach for the study of the titer and expression patterns must be reinvestigated to fully elucidate the roles of these hormones in lobster reproduction control.

Alternative splicing is a method used to create more proteins from the same gene (Baralle and Giudice, 2017; Gallego-Paez et al., 2017; Modrek and Lee, 2002). It is an essential mechanism increasing the complexity of gene expression, and it plays an important role in cellular differentiation and organism development. There are several reports for the alternative splicing of the CHH gene in crustaceans (Dirksen et al., 2003; Chen et al., 2004; Jeon et al., 2012; Shi et al., 2018; Sun et al., 2019). In the Morotoge shrimp *Pandalopsis japonica*, an eyestalk form and a pericardial organ isoform were identified. Although the reproductive function of the CHH variant was not studied, eyestalk ablation would reduce the expression of *Pj-CHH1ES* in the brain and *Pj-CHH1PO* and *Pj-CHH2* in the thoracic ganglion (Jeon et al., 2012). The *Mar-CHH* gene of *M. rosenbergii* also consists of four exons. The eyestalk transcript (*chh*) contains exons I, II, and IV, whereas the *CHH-l* transcript in the heart, gills, antennal glands, and thoracic ganglion contains all four exons. Similarly, the *sHaCHH-A* and *sHaCHH-B* transcripts are mainly expressed in non-eyestalk tissues. The production of alternatively spliced variants from a single gene, therefore, would increase the number of proteins in a cell and increase the overall proteome content within the cell. Additionally, these

spliced variants may be produced at different stages of the life cycle (Wang et al., 2015). The differential stage-specific expression of the transcripts in lobster might contribute to its regulation during the reproductive cycle in the biannual reproductive pattern of *H. americanus*. Despite that a similar 3D structure was predicted between HaCHH-A vs. *HaCHH-B* and *sHaCHH-A* vs. *sHaCHH-B*, the pI values (i.e., hydrophobicity) of the corresponding proteins were quite different. The differences in the hydrophobicity may have allowed different isoforms to work optimally in different pH conditions of the Hp and ovary. Therefore, we hypothesized that, with a lower pI value (i.e., 5.27), *sHaCHH-A* may work optimally under a more acidic condition in the Hp, which has a lower pH for the function of the acidic digestive enzyme.

A major challenge in the research on the CHH/MIH/GIH family is the lack of functional studies for most of the members. Therefore, the bioassay/functional confirmation of most CHHs was not performed. The use of recombinant protein and RNAi technique to study the function of the CHH family genes has been reported for the MIH-like in the sand shrimp and the MIH-like in the banana shrimp *F. merguensis* type II neuropeptide (Gu et al., 2000; Tiu et al., 2008; Liang et al., 2019). Opposing results of the dsRNAs and recombinant proteins were demonstrated in the sand shrimp and in the lobster in this study. Since there are multiple numbers of genes in the CHH/MIH/GIH family, the antagonistic results from the recombinant proteins and dsRNAs may provide a tool to investigate the role of this neuropeptide superfamily.

CONCLUSION

The present study has confirmed that the previously reported *HaCHH-A* and *HaCHH-B* cDNAs were derived from alternative

splicing of two 4-exon CHH genes. The retention of intron 3 and exon 4 produced the transcript variants *sHaCHH-A* and *sHaCHH-B*, as reported in this study. Results of the functional study indicated that *sHaCHH-A* and *sHaCHH-B* can regulate the gene expression of vitellogenin. The findings in this study have provided new information on the gene structure organization, precise expression patterns for the transcript variants, and the vitellogenin-inhibiting function of *sHaCHH-A* and *sHaCHH-B*. The results of this report have also provided a basis for reassessing the expression patterns and hemolymph titers of these transcripts during the reproductive cycle of the female lobster.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SC: conceptualization, writing—original draft preparation, project administration, and funding acquisition. SC, CGW, and YS: methodology. CGW: software and resources. WW: validation. YS and WW: formal analysis. WG and SC: writing—review and editing. LLS: visualization. YS and SC: supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded in part by the National Natural Science Foundation of China (#31572606) and Zhanjiang City Fund.

REFERENCES

- Baralle, F. E., and Giudice, J. (2017). Alternative Splicing as a Regulator of Development and Tissue Identity. *Nat. Rev. Mol. Cell Biol.* 18, 437–451. doi: 10.1038/nrm
- Chang, E. S., Chang, S. A., Beltz, B. S., and Kravitz, E. A. (1999). Crustacean Hyperglycemic Hormone in the Lobster Nervous System: Localization and Release From Cells in the Subesophageal Ganglion and Thoracic Second Roots. *J. Comp. Neurol.* 414, 50–56. doi: 10.1002/(SICI)1096-9861(19991108)414:1<50::AID-CNE4>3.0.CO;2-Q
- Chang, E. S., Keller, R., and Chang, S. A. (1998). Quantification of Crustacean Hyperglycemic Hormone by ELISA in Hemolymph of the Lobster, *Homarus Americanus*, Following Various Stresses. *Gen. Comp. Endocrinol.* 111, 359–366. doi: 10.1006/gen.1998.7120
- Chang, W. H., and Lai, A. G. (2018). Comparative Genomic Analysis of Crustacean Hyperglycemic Hormone (CHH) Neuropeptide Genes Across Diverse Crustacean Species. *F1000Res* 7, 100. doi: 10.12688/f1000research.13732.1
- Chan, S. M., Gu, P. L., and Chu, K. H. (2003). Crustacean Neuropeptide Genes of the CHH/MIH/GIH Family: Implications From Molecular Studies. *Gen. Comp. Endocrinol.* 134, 214–219. doi: 10.1016/S0016-6480(03)00263-6
- Chen, S. H., Lin, C. Y., and Kuo, C. M. (2004). Cloning of Two Crustacean Hyperglycemic Hormone Isoforms in Freshwater Giant Prawn (*Macrobrachium Rosenbergii*): Evidence of Alternative Splicing. *Mar. Biotechnol. (NY)* 6, 83–94. doi: 10.1007/s10126-003-0014-8
- Chen, H. Y., Toullec, J. Y., and Lee, C. Y. (2020). The Crustacean Hyperglycemic Hormone Superfamily: Progress Made in the Past Decade. *Front. Endocrinol. (Lausanne)* 11. doi: 10.3389/fendo.2020.578958
- Chung, J. S., Christie, A., and Flynn, E. (2020). Molecular Cloning of Crustacean Hyperglycemic Hormone (CHH) Family Members (CHH, Molt-Inhibiting Hormone and Mandibular Organ-Inhibiting Hormone) and Their Expression Levels in the Jonah Crab. *Cancer Borealis Gen. Comp. Endocrinol.* 1 (295), 113522. doi: 10.1016/j.ygcen.2020.113522
- Chung, J. S., Zmora, N., Katayama, H., and Tsutsui, N. (2010). Review. Crustacean Hyperglycemic Hormone (CHH) Neuropeptides Family: Functions, Titer, and Binding to Target Tissues. *Gen. Comp. Endocrinol.* 166, 447–454. doi: 10.1016/j.ygcen.2009.12.011
- De Kleijn, D. P., De Leeuw, E. P., Van den Berg, M. C., Martens, G. J., and Van Herp, F. (1995). Cloning and Expression of Two mRNAs Encoding Structurally Different Crustacean Hyperglycemic Hormone Precursors in the Lobster. *Homarus Americanus Biochim. Biophys. Acta* 1260, 62–66.
- de Kleijn, D. P., Janssen, K. P., Waddy, S. L., Hegeman, R., Lai, W. Y., Martens, G. J., and Van Herp, F. (1998). Expression of the Crustacean Hyperglycemic Hormones and the Gonad-Inhibiting Hormone During the Reproductive Cycle of the Female American Lobster *Homarus Americanus*. *J. Endocrinol.* 156 (2), 291–298. doi: 10.1677/joe.0.1560291

- De Kleijn, D. P., Janssen, K. P., Martens, G. J., and Van Herp, F. (1994). Cloning and Expression of Two Crustacean Hyperglycemic-Hormone mRNAs in the Eyestalk of the Crayfish. *Orconectes Limosus Eur. J. Biochem.* 224, 623–629. doi: 10.1111/j.1432-1033.1994.00623.x
- De Kleijn, D. P., and Van Herp, F. (1995). Molecular Biology of Neurohormone Precursors in the Eyestalk of Crustacea. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 112, 573–579. doi: 10.1016/0305-0491(95)00126-3
- Dirksen, H., Böcking, D., Heyn, U., Mandel, C., Chung, J. S., Baggerman, G., et al. (2003). Crustacean Hyperglycaemic Hormone (CHH)-Like Peptides and CHH-Precursor-Related Peptides From Pericardial Organ Neurosecretory Cells in the Shore Crab, *Carcinus Maenas*, are Putatively Spliced and Modified Products of Multiple Genes. *Biochem. J.* 356, 159–170. doi: 10.1042/bj3560159
- Diwan, A. D. (2005). Current Progress in Shrimp Endocrinology. *Indian J. Exp. Biol.* 43, 209–223.
- Fingerman, M. (1987). The Endocrine Mechanisms of Crustaceans. *J. Crustacean Biol.* 7, 1–24. doi: 10.2307/1548622
- Gallego-Paez, L. M., Bordone, M. C., Leote, A. C., Saraiva-Agostinho, N., Ascensão-Ferreira, M., and Barbosa-Morais, N. L. (2017). Alternative Splicing: The Pledge, the Turn, and the Prestige: The Key Role of Alternative Splicing in Human Biological Systems. *Hum. Genet.* 136, 1015–1042. doi: 10.1007/s00439-017-1790-y
- Giulianini, P. G., Pandolfelli, N., Lorenzon, S., Ferrero, E. A., and Edomi, P. (2002). An Antibody to Recombinant Crustacean Hyperglycaemic Hormone of *Nephrops Norvegicus* Cross-React With Neuroendocrine Organs of Several Taxa of Malacostracan Crustacea. *Cell Tissue Res.* 307, 243–254. doi: 10.1007/s00441-001-0484-9
- Gu, P. L., Yu, K. L., and Chan, S. M. (2000). Molecular Characterization of an Additional Shrimp Hyperglycemic Hormone: cDNA Cloning, Gene Organization, Expression and Biological Assay of Recombinant Proteins. *FEBS Lett.* 472, 122–128. doi: 10.1016/S0014-5793(00)01420-4
- Havird, J. C., and Santos, S. R. (2016). Here We Are, But Where Do We Go? A Systematic Review of Crustacean Transcriptomic Studies From 2014–2015. *Integr. Comp. Biol.* 56 (6), 1055–1066. doi: 10.1093/icb/icw061
- Helluy, S. M., and Beltz, B. S. (1991). Embryonic Development of the American Lobster (*Homarus Americanus*): Quantitative Staging and Characterization of an Embryonic Molt Cycle. *Biol. Bull.* 180, 355–371. doi: 10.2307/1542337
- Huberman, A. (2000). Shrimp Endocrinology: A Review. *Aquaculture* 191, 191–208. doi: 10.1016/S0044-8486(00)00428-2
- Hyde, C. J., Fitzgibbon, Q. P., Elizur, A., Smith, G. G., and Ventura, T. (2020). CrustyBase: An Interactive Online Database for Crustacean Transcriptomes. *BMC Genomics* 21, 637. doi: 10.1186/s12864-020-07063-2
- 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. (Lausanne)* 11. doi: 10.3389/fendo.2020.577925
- Jeon, J.-M., Kim, B.-K., Lee, J. H., Kim, H. K., Kang, C.-K., Mykles, D. L., et al. (2012). Two Type I Crustacean Hyperglycemic Hormone (CHH) Genes in Morotoge Shrimp (*Pandalopsis Japonica*): Cloning and Expression of Eyestalk and Pericardial Organ Isoforms Produced by Alternative Splicing and a Novel Type I CHH With Predicted Structure Shared With Type II CHH Peptides Comp. *Biochem. Physiol. B Biochem. Mol. Biol.* 162 (4), 88–99. doi: 10.1016/j.cbpb.2012.04.003
- Keller, R. (1992). Crustacean Neuropeptides: Structures, Functions and Comparative Aspects. *Experientia* 48, 439–448. doi: 10.1007/BF01928162
- Liang, H. F., Liu, Y., Zhou, T. T., Li, X. Y., Li, B., Chan, S. F., et al. (2019). Molecular Characterization, RNA Interference and Recombinant Protein Approach to Study the Function of the Putative Molt Inhibiting Hormone (FmMIH1) Gene From the Shrimp *Fenneropenaeus Merquiensis*. *Peptides* 122, 1–12. doi: 10.1016/j.peptides.2017.10.017
- Loredó-Ranjel, R., Fanjul-Moles, M. L., and Escamilla-Chimal, E. G. (2017). Crustacean Hyperglycemic Hormone Is Synthesized in the Eyestalk and Brain of the Crayfish *Procambarus Clarkii*. *PLoS One* 12 (4), e0175046. doi: 10.1371/journal.pone.0175046
- Magaña-Gallegos, E., Arévalo, M., Cuzon, G., and Gaxiola, G. (2021). Effects of Using the Biofloc System and Eyestalk Ablation on Reproductive Performance and Egg Quality of *Litopenaeus Vannamei* (Boon) (Decapoda: Dendrobranchiata: Penaeidae). *Anim. Reprod. Sci.* 228, 106749. doi: 10.1016/j.anireprosci.2021.106749
- Mettulio, R., Edomi, P., Ferrero, E. A., Lorenzon, S., and Giulianini, P. G. (2004). The Crustacean Hyperglycemic Hormone Precursors a and B of the Norway Lobster Differ in the Preprohormone But Not in the Mature Peptide. *Peptides* 25, 1899–1907. doi: 10.1016/j.peptides.2004.06.013
- Modrek, B., and Lee, C. (2002). A Genomic View of Alternative Splicing. *Nat. Genet.* 30, 13–19. doi: 10.1038/ng0102-13
- Montagné, N., Soyez, D., Céline, D., Jean-Yves, G., and Toullec, O. (2008). New Insights Into Evolution of Crustacean Hyperglycaemic Hormone in Decapods—First Characterization in Anomura. *FEBS J.* 275, 1039–1052. doi: 10.1111/j.1742-4658.2007.06245.x
- Mykles, D. L., and Chang, E. S. (2020). Hormonal Control of the Crustacean Molting Gland: Insights From Transcriptomics and Proteomics. *Gen. Comp. Endocrinol.* 294, 113493. doi: 10.1016/j.ygcen.2020.113493
- Okumura, T., and Aida, K. (2001). Effects of Bilateral Eyestalk Ablation on Molting and Ovarian Development in the Giant Freshwater Prawn. *Macrobrachium Rosenbergii Fish Sci.* 67, 1125–1135. doi: 10.1046/j.1444-2906.2001.00370.x
- Oliphant, A., Alexander, J. L., Swain, M. T., Webster, S. G., and Wilcockson, D. C. (2018). Transcriptomic Analysis of Crustacean Neuropeptide Signaling During the Molt Cycle in the Green Shore Crab, *Carcinus Maenas*. *BMC Genomics* 19, 711–720. doi: 10.1186/s12864-018-5057-3
- Panouse, J. B. (1943). Influence De L'ablation Du Pédoncle Oculaire Sur La Croissance De L'ovaire Chez La Crevette *Leander Serratus*. *Comptes Rendus l'Académie Des. Sci.* 217, 553–555.
- Polinski, J. M., Zimin, A. V., Clark, K. F., Kohn, A. B., Sadowski, N., and Timp, W. (2021). The American Lobster Genome Reveals Insights on Longevity, Neural, and Immune Adaptations. *Sci. Adv.* 7 (26). doi: 10.1126/sciadv.abe8290
- Primavera, J. H. (1978). Induced Maturation and Spawning in Five-Month-Old *Penaeus Monodon* Fabricius by Eyestalk Ablation. *Aquacult* 13, 355–359. doi: 10.1016/0044-8486(78)90184-9
- Reddy, P. R., and Kishori, B. (2017). Mini Review. Crustacean Endocrinology: Intriguing Towards Quality Protein Production. *J. Endocrinol. Thyroid Res.* 1 (2), 555556. doi: 10.19080/JETR.2017.01.555556
- Shi, L., Li, B., Zhou, T. T., Wang, W., and Chan, S. F. (2018). Functional and Evolutionary Implications From the Molecular Characterization of Five Spermatophore CHH/MIH/GIH Genes in the Shrimp *Fenneropenaeus Merquiensis*. *PLoS One* 13(3):e0193375. doi: 10.1371/journal.pone.0193375
- Sun, D., Lv, J., Gao, B., Liu, P., and Li, J. (2019). Crustacean Hyperglycemic Hormone of *Portunus Trituberculatus*: Evidence of Alternative Splicing and Potential Roles in Osmoregulation. *Cell Stress Chaperones* 24, 517–525. doi: 10.1007/s12192-019-00980-6
- Tensen, C. P., Verhoeven, A. H., Gaus, G., Janssen, K. P., Keller, R., and Van Herp, F. (1991). Isolation and Amino Acid Sequence of Crustacean Hyperglycemic Hormone Precursor-Related Peptides. *Peptides* 12, 673–681. doi: 10.1016/0196-9781(91)90119-A
- Tiu, S. H. K., and Chan, S.-M. (2007). The Use of Recombinant Protein and RNA Interference Approaches to Study the Reproductive Functions of a Gonad-Stimulating Hormone From the Shrimp *Metapenaeus Ensis*. *FEBS J.* 274, 4385–4395. doi: 10.1111/j.1742-4658.2007.05968.x
- Tiu, S. H., Hui, H. L., Tsukimura, B., Tobe, S. S., He, J. G., and Chan, S. M. (2009). Cloning and Expression Study of the Lobster (*Homarus Americanus*) Vitellogenin: Conservation in Gene Structure Among Decapods. *Gen. Comp. Endocrinol.* 160, 36–46. doi: 10.1016/j.ygcen.2008.10.014
- Uawisetwanathana, 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
- Wang, Y., Liu, J., Huang, B. O., Xu, Y. M., Li, J., Huang, L. F., et al. (2015). Mechanism of Alternative Splicing and its Regulation. *Biomed. Rep.* 3, 152–158. doi: 10.3892/br.2014.407
- Webster, S. G. (2001). Crustacean Hyperglycaemic Hormone (CHH)-Like Peptides and CHH-Precursor-Related Peptides From Pericardial Organ Neurosecretory Cells in the Shore Crab, *Carcinus Maenas*, are Putatively Spliced and Modified Products of Multiple Genes. *Biochem. J.* 356, 159–170. doi: 10.1042/bj3560159

- Webster, S. G., Keller, R., and Dirksen, H. (2012). The CHH-Superfamily of Multifunctional Peptide Hormones Controlling Crustacean Metabolism, Osmoregulation, Moulting, and Reproduction. *Gen. Comp. Endocrinol.* 175, 217–233. doi: 10.1016/j.ygcen.2011.11.035
- Zhang, X., Yuan, J., Sun, Y., Li, S., Gao, Y., Yu, Y., et al. (2019). Penaeid Shrimp Genome Provides Insights Into Benthic Adaptation and Frequent Molting. *Nat. Commun.* 10, 356. doi: 10.1038/s41467-018-08197-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Wang, Wang, Shi, Shen and Chan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.