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*

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Abstract

The crustacean hyperglycemic hormone (CHH) neuropeptide family has multiple functions in the regulation of hemolymph glucose levels, molting, ion, and water balance and reproduction. In crab species, three neuroendocrine tissues: the eyestalk ganglia (medulla terminalis X-organ and -sinus gland= ES), the pericardial organ (PO), and guts synthesize a tissue-specific isoforms of CHH neuropeptides. Recently the presence of the mandibular organ-inhibiting hormone (MOIH) was reported in the stomatogastric nervous system (STNS) that regulates the rhythmic muscle movements in esophagus, cardiac sac, gastric and pyloric ports of the foregut. In this study, we aimed to determine the presence of a tissue-specific CHH isoform in the Jonah crab, *Cancer borealis* using PCR with degenerate primers and 5', 3' rapid amplification of cDNA ends (RACE) in the ES. PO, and STNS. The analysis of CHH sequences shows that *C. borealis* has one type of CHH isoform, unlike other crab species. We also isolated the cDNA sequence of molt-inhibiting hormone (MIH) in the ES and MOIH in the ES and STNS. The presence of CHH, MOIH and MIH in the sinus gland of adult females and males is confirmed by using a dot-blot assay with the putative peaks collected from RP-HPLC and anti-*Cancer* sera for CHH, MIH, and MOIH. The present of crustacean female sex hormone (CFSH) in the sinus gland of adult females was examined with a dot-blot assay with anti-*Callinectes* CFSH serum. Levels of CHH, MOIH, and MIH in the sinus gland and their expressions in the eyestalk ganglia are estimated in the adult males, where CHH is the predominant form among these neuropeptides.

1. Introduction

Crustacean hyperglycemic hormone (CHH) is commonly found in all the crustaceans, since its first structural isolation from the sinus gland of the European green crab, *Carcinus maenas* [23]. CHH neuropeptides form the CHH neuropeptides family together with molt-inhibiting hormone (MIH), and mandibular organ-inhibiting hormone (MOIH), and vitellogenesis or gonad-inhibiting hormone (V/GIH) [2, 5]. CHH family neuropeptides possess similar primary structures, and their number of amino acid residues range from 71 to 78 amino acids, containing three intradisulphide bridges. Based on the putative amino acid sequence of an open reading frame (ORF) of cDNA either with or without CHH-precursor related peptide (CPRP), the CHH family is further divided into two subgroups: the first subgroup with CHH neuropeptides with CPRP and the other with MIHs and MOIHs without CPRP. Moreover, depending on a species, it appears that the CHHs are subject to post-translational modification processes including cyclization at N-terminus, isomerization of F_3 from L to D amino acid and amidation at C-terminus [9, 13, 23, 31], resulting in multiple isoforms of CHH neuropeptides in the sinus gland.

The structural isoforms of CHH neuropeptides are found in eyestalk (ES) and pericardial organs (PO) of several crab species while they are also present in fore- and hindguts in crab species which are possibly derived from alternative splicing of CHH gene(s) [3, 8, 17, 18]. The two isoforms of CHH neuropeptides (1 and 2) present in the sinus gland or the PO are derived from the post-translational modification, cyclization at N-terminus from Q to <Q, respectively [9, 18]. The levels of conversion from Q to <Q of N terminus of CHH is not complete to be a 100 %; hence, there are always two forms of CHH1 and CHH2 present in the sinus gland and the PO. In any case, both CHH1 and CHH2 are present in the hemolymph [9], while CHH2 being the primary form having 4 -5 times more than CHH1 [15] is referred to as the ES-CHH.

The function of a hormone is intimately associated with its presence in hemolymph, while the levels of expression are indicative of the amounts of a protein. The expression of ES- and PO-CHH is molt-stage independent [10, 14], whereas the gut-CHH is exclusively present during premolt stage [8]. The full-length cDNA of CHH is encoded in four exons, the first two of which are common in the CHHs of three tissues, while the third is alternatively spliced in a tissue-specific manner [17].

The eyestalk that shows exhibiting the presence of these hormones first in the embryonic stages [11, 32], is considered as the primary tissue source for CHH neuropeptides family. CHHs and MIHs are also found in ventral nerve cord of *Metapenaeus ensis* [20]. The ES also serves for the source of MOIH as the primary structural isolation of MOIH is first reported in the sinus gland of *C. pagurus* [39]. The intense immunostaining with anti-MOIH serum in the stomatogastric nervous system (SNTS) of *C. productus* [21] suggests that STNS may be an additional site of these CHH neuropeptides.

Most hormones transduce their signals at the optimal concentration throughout the life cycle, as seen in the case of molting hormone ecdysteroid levels in the hemolymph of three different sized juvenile and adult females of *C. sapidus* [6]. Despite the size variation, the ecdysteroid levels are kept at the tight range of 230-330 ng/ml. Hence, it is plausible to infer that the larger size of *C. borealis* may produce more neuropeptides or hormone to achieve the optimal concentration as they grow. The information regarding how the hormone levels vary during the life cycle of a species, especially, by size is still missing in general. The levels of CHH neuropeptide expression in the eyestalk ganglia and of the corresponding proteins in the sinus gland may differ by size.

In the present study, we aimed to isolate the cDNA sequences of CHH family members including CHH, MIH and MOIH from ES, CHH and MOIH from STNS, and CHH from PO of intermolt stage adult male *C. borealis*. To this end, PCR with degenerate primers and 5', 3' rapid amplification of cDNA ends (RACE) were employed. The presence of the neuropeptides in the sinus gland was examined using an RP-HPLC combined with dot-blot assays. We then measured the expression levels of these neuropeptides in the ES of adult males using qPCR assays and the corresponding neuropeptide contents in the sinus gland of the same animals using a reversed phase-high performance liquid chromatography (RP-HPLC). For the first time in decapod crustaceans, we have isolated a CHH isoform from the STNS. Interestingly *C. borealis* may have only one type of CHH in the tissues examined, unlike other portunid crab species.

2. Methods and Materials

2.1 Dissection and cDNA synthesis

Adult male *C. borealis* were purchased from a local crabber (Maine) and kept in a flow-through natural seawater system at 10^{o}C and fed mussels (5% of body weight) twice a week. Tissues (eyestalk ganglia, STNS and PO) were dissected from the ice-chilled animals for total RNA extraction using a stereo dissection microscope. Tissues: ES, PO, and STNS were dissected from the ice-chilled *C. borealis* and immediately processed for total RNA or mRNA extraction using total RNA extraction kit or Oligotex mRNA mini kit, respectively (Promega). The quantity of RNAs was estimated using a NanoDrop spectrometer (Fishersci).

2.2 Profiles of CHH neuropeptides in the sinus gland of different sized adult males

The extract of sinus glands that were dissected from individual adult males with 111.9 ± 3.0 mm CW (n=9) was separated on a Jupiter C18 column (4.6 x 150 mm, 5 µm, Phenomenex), connected to a RP-HPLC (HP1100) using a gradient 30-70% B over 60 min (A= 0.1% in 100%

127 water and B= 0.11% TFA in 60% acetonitrile and 40% water). The flow rate was 0.6 ml/min. The
128 absorption was monitored using a photodiode array detector and the chromatogram was
129 extracted at 210 nm and presented. The peaks were manually collected for dot-blot assay. The
130 peak area (milli-Absorption Units*sec =mAU*s) was used for comparing the relative amount of
131 each neuropeptide/sinus gland after normalizing the initial peak area of the all the
132 neuropeptides to the size of CHH.
133
134 *2.3 Dot blot analysis of neuropeptides*
135 As alluded to earlier, the peaks that were manually collected and dried in a SpeedVac
136 were re-dissolved in 100 µl of 2 M acetic acid. One microliter of each peak was spotted on a
137 nitrocellulose membrane and air-dried. Then, the membranes were incubated with the following
138 antisera at a final dilution of 2,000 in 5% NFM in PBST for overnight at 4^{o}C: rabbit anti-*Cancer*
139 CHH, MOIH, and MIH sera [21] and anti-*Callinectes* CFSH [44]. After washing the membranes
140 with 5% non-fat milk in the PBS containing 0.5% Tween 20 (PBST) three times for 5 min each,
141 the membranes were incubated with an horseradish peroxidase (HRP)-conjugated anti-rabbit
142 IgG at the final dilution of 2000 in 5% NFM in PBST for an hour at room temperature. Stable
143 3,3′-Diaminobenzidine (DAB) was used to develop the membranes after repeating the washing
144 step as above.
145
146 *2.4 PCR cloning with degenerate primers and 5', 3' rapid amplification of cDNA ends (RACE)*
147 The degenerate primers were generated based on the conserved regions of CHH, MIH,
148 and MOIH sequences that were identified using Clustal W (www.genome.ad.jp). 5' and 3' RACE
149 cDNAs were synthesized using SMART cDNA synthesis kit (BD Biosciences) by following the
150 manufacturer's instructions.
151 The first touchdown (TD) PCR was carried out with Advantage Taq (BD Bioscience) and
152 a combination of CHHdF1 (5'TGYAARGGNGTNTAYGA3'), MIHdF1 (5'

GNGTNATHAAYGAYGA3') or MOIHdF1 (5' TGYCARAAYTTYATHGGNAA 3') and Universal Primer (UMP, BD Biosciences). The TD-PCR product once 20-fold diluted in water was served for the nested PCR with the dF2 and dR1 primers of CHH, MIH, and MOIH (listed in Table 1). The nested PCR products were run on a 1.5% agarose gel and the band located approximately ~140 bp was excised for DNA extraction using the Qiagen gel extraction kit (Qiagen). The purified DNA was then inserted into a TOPO-TA vector for cloning and sequencing analysis. Based on the initial sequence of CHH, MIH, and MOIH, gene-specific primers (listed in Table 1) were generated for 5', 3' RACE. Two-step PCRs were first employed using a procedure similar to that previously described [7]. *C. borealis* gene-specific primers listed in Table 1 as stated above. The sequencing results were compared to the Non-redundant protein sequences (nr) database using blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the encoded protein sequences were aligned by ClustalW (www.genome.ad.jp) employing the default parameters. The ORF sequence of CHH, MIH, and MOIH was verified by cloning of the PCR product that was amplified with the start and end primer. The accession number of the sequences are as follows: MN382372 ES-crustacean hyperglycemic hormone (CHH); MN382373 ES-Molt-inhibiting hormone (MIH); MN382374 ES-Mandibular organ-inhibiting hormone (MOIH); MN382375 PO-crustacean hyperglycemic hormone (CHH); MN382376 STNS-crustacean hyperglycemic hormone (CHH)-ES type mRNA;
MN382377 STNS-crustacean hyperglycemic hormone (CHH)-PO type mRNA; and MN382378 STNS-Mandibular organ-inhibiting hormone (MOIH) mRNA.

2.5 Sequence analysis

The ORF was found using ORF finder (www.ncbi.nlm.nih.gov/orffinder/). The putative signal peptide of CHH, MIH, and MOIH was examined using Signal P 5.0 (http://www.cbs.dtu.dk/services/SignalP/).

179 *2.6 Expression analysis*

180 The cDNA samples of eyestalk ganglia obtained from adult males sized with 111.9 ± 3.0
181 mm carapace width (CW, n=9) assayed were assayed for CHH, MOIH, and MIH expression.
182 These animals at the intermolt stage and sexually matured (i.e., presence of spermatophores in
183 the testis and vas deferens) that were obtained from a commercial crabber were caught off the
184 Rhode Island area (US) in April 2019. The animals were shipped overnight to the Institute of
185 Marine and Environmental Technology (IMET, Baltimore, MD) and acclimated in 30 ppt artificial
186 seawater for two weeks at 10^{o}C. The animals were fed as described above. The samples were
187 assayed in duplicate with each gene standard ranging 2E6 to 2E2 copies. The data were
188 presented as mean ± 1 SE (n) copies/µg eyestalk total RNA.
189
190 *2.7 Statistical analysis*

191 Statistical analyses were carried out with SPSS 17.0 software. Data were tested for
192 normality (Kolmogorov-Smirnov test) and homogeneity of variances using the Levene's test.
193 Non-parametric one-way analysis of variance (ANOVA, Kruskal-Wallis test) was used to
194 determine the statistical significance. Statistical differences at $P<0.05$ were indicated with
195 letters.
196
197 **3. Results**
198 *3.1 Neuropeptide profiles of a single sinus of adult males and females by RP-HPLC*

199 The chromatograms were obtained from the separation of neuropeptides present in
200 single sinus gland of adult males and females (Figs. 1A and B). Dot blot analysis using
201 corresponding *C. pagurus* anti-MOIH, CHH, and MIH sera showed that four peaks collected
202 from male and female SG and noted to 1 to 4 were identified as MOIH, CHH1, CHH2, and MIH.

In adult female SG, the presence of crustacean female sex hormone (CFSH) that was noted "*' in Fig. 1B was confirmed using *C. sapidus* anti-CFSH serum.

3.2 CHH sequences from eyestalk ganglia (ES), pericardial organs (PO), and stomatogastric nervous system (STNS)

The sequences of CHH obtained from ES, PO and STNS are shown in Fig. 2A. ES and PO had the same CHH ORF sequences. The ORF of ES and PO cDNAs (140 amino acids) consists of the signal peptide, CPRP, a dibasic cleavage site and CHH at C terminus with amidation site and tribasic cleavage site in this order. The first 24 putative amino acid sequence (MLTTRTLLLGVMCVYLSTLPYVHA, boxed in solid black line) was identified as the signal peptide using Signal P 5.0 (*P*= 0.947). The dibasic cleavage site (KR) was flanked between 38 amino acid of CPRP and 72 amino acid of CHH which started with Q and ended with amidation site, G137 and triple cleave site, KKK138-140 (squared in red in Fig. 2A).

The signal peptide of STNS-CHH cDNAs was not predicted (P= 0.0071, Signal P 5.0), while the rest of sequence of ORF sequence showed the presence of the same CPRP, dibasic cleavage site and CHH sequence with the amidation site G_{137} but dibasic cleave site, $KK_{138\text{-}139}$.

The cDNA sequences of *C. borealis* CHH were aligned with those isolated from *C. productus* (Fig. 2B). The Cab-ES- and PO- CHH signal peptides were three amino acid residues shorter than Cap-CHHs. Interestingly, the sequences of CPRP and CHH vary greatly in their C-terminal regions, compared to N-terminal regions.

3. 2 MIH and MOIH sequences from ES

MIH cDNA was found only in the ES. The ORF of MIH cDNA sequence was aligned with *C. pagurus* MIH sequence (Fig. 3A) and consisted of 113 amino acids starting with 35 amino acids comprising the signal peptide (P= 0.9827, Signal P 5.0) and 78 amino acids of MIH starting with R_1 and ending with K_{78}.

Two MOIH cDNAs were isolated from the ES and STNS. Both sequences were identical and shared the same ORF sequences shown as Fig. 3B. The ORF sequence consisted of a 34-amino acid signal peptide (P= 0.784) and 78 amino acid MOIH.

3.3 Neuropeptide levels in the sinus gland and their expression in the eyestalk ganglia of adult males

Fig. 4A shows the relative peak area of the following neuropeptide present in the sinus gland of the adult males: CPRP, MOIH, CHH1, CHH2 and MIH in the order of RP-HPLC elution. CPRP and CHH2 are predominant, followed by CHH1, MIH and MOIH. The ratio CHH1: CHH2 was 0.275 ± 0.019 (n=9).

The expression levels of these neuropeptides in the eyestalk ganglia (Fig. 4B) were similar to neuropeptides (Fig. 4A). CHH expression was the greatest with 2.6 ± 0.4E8 copies/µg total RNA (n=9), followed by MIH with 7.5 ± 1.0 E7 copies/µg total RNA (n=9) and MOIH with 5.3 ± 0.75 E7 copies/µg total RNA (n=9).

4. Discussion

The present study describes the isolation of cDNA sequences of neuropeptides: CHH, MIH and MOIH in the eyestalk ganglia, CHH from the PO, and STNS and MOIH from STNS of the Jonah crab, *C. borealis,* together with the presence of the sinus gland neuropeptides: CHH, MIH, and MOIH. The expression levels of CHH, MOIH, and MIH are in agreement with the neuropeptide levels in the sinus gland.

We employed PCR with degenerate primers and 5', 3' RACE cloning to isolate CHH cDNAs from ES, PO, and STNS of the Jonah crab, *C. borealis*. A notable finding was the presence of CHH in the STNS, in addition to the previous report stating STNS exhibited MOIH-like substance using immunohistochemistry [21]. The sequence identity between the CHHs of

ES, PO, and STNS is unexpected, particularly the amino acid position 41- to the end, which contrasts to the previous reports of the crustacean hyperglycemic hormone in other crab species [14, 18]. Overall, as expected, *C. borealis* CHHs show the highest identity (>90%) with ES-CHH of *C. pagurus* and *C. productus* [13, 22].

In the sinus gland of adult *C. borealis*, four peaks are common in both genders. Females show another peak, identified as CFSH using *Callinectes* CFSH antiserum [44], suggesting that the CFSH presence in females of crab species may be a common feature [25]. Two structural isoforms of CHH are present as two peaks that are cross-reacted to *Cancer* CHH antiserum. This suggests that CHH2, the largest peak is derived from post translational modification at the N-terminus same as in the other crab species [7, 9, 13, 14, 38, 41]. *C. borealis* has one MOIH form which differs from *C. pagurus* with two structural isoforms: CapMOIH 1and CapMOIH 2: K_{32} to Q_{32} [39].

Two brachyuran species show the tissue specific isoforms of CHH in ES and PO the sequences of which only share ~ 60-70% identity [14, 18, 23]. This major difference is located in the last ~ 30 residues of amino acids that are encoded in the third exon. To our surprise, the CHH sequences obtained from the different tissues of *C. borealis* exhibits 100% sequence identity, contrasting to the finding of *C. productus* where CHH polymorphism is observed at individual levels [22]. Our finding of one CPRP form (type II based on the sequence reported [19] contrasts to the earlier report of the presence of four CPRP sequences in the pooled sinus gland extracts (n=30) of *C. borealis* using nanoscale liquid chromatography tandem mass spectrometry [19]. However, we observed one CPRP peak from the sinus gland of an individual animal.

Both the MIH and MOIH of all three *Cancer* species exhibit higher sequence identity than CHHs. The putative MIH sequences of three *Cancer* species including *C. borealis* show the highest identity as only four aa of 113 aa encompassing the entire ORF differ in the main MIH neuropeptide [28, 37]. The MOIH sequences of these animals also differ by four aa of 112 aa of the entire ORF region (two aa in the signal peptide and the other two in MOIH neuropeptide region).

MOIH is found only in the crabs belonging to the genus *Cancer* and *Metacarcinus* [21, 39]. It is unknown why the brachyuran crabs belonging to the family Cancridae including genus *Cancer* and *Metacarcinus* possess the MOIH. Since MIH is found in all crab species, MOIH could be a product of gene duplication of MIH. In the family Cancridae, MOIH has acquired a separate function for the inhibition of mandibular-organ activity i.e., suppressing the synthesis of methyl farnersoate [39] that stimulates vitellogenesis [33, 34]. Hence, MOIH acts as a VIH in this animal group. The other crab species do not have a separate neuropeptide form of the MOIH; for example, in the spider crab *Labinia emarginata*, CHH with hyperglycemic action exhibits MOIH action [26, 27].

Eyestalk ganglia developing in early embryonic stages of decapod crustaceans are the hub of the most important endocrine tissues. Lobster, crayfish, and shrimp have multiple isoforms of CHH peaks, while crab species exhibits two types of CHH neuropeptides: CHH type with CPRP and the other MIH and MOIH without CPRP. It is noted that the sinus gland of all crab species contains fewer neuropeptide peaks than those of shrimp and lobster species, where each of these peaks are assigned to a specific type of a neuropeptide with a respective function. Two peaks are two isoforms of CHH together with one or multiple forms of CPRP and MIH while *Cancer* species display additional one or two forms of MOIH [7, 9, 13, 40]. In the tiger prawn, *Metapenaeus japonicus* and the whiteleg shrimp*, Litopenaeus vannamei*, the sinus

306 gland reveals the presence of 6-7 neuropeptides that are all related to the CHH type, with each
307 showing the activity of hyperglycemic, molt-inhibiting, or vitellogenin-inhibiting hormone
308 functions [35, 36, 43] . For example, the sinus gland of the American lobster, *Homarus*
309 *americanus*, contains at least three CHH neuropeptide types with each showing two isoforms [4,
310 31]: a predominant peak with hyperglycemic activity; the other two with MIH activity and
311 VIH/GIH activity. On the other hand, shrimp and lobsters contain VIHs that structurally differ
312 from CHHs [16, 36].
313
314 Though little is known about the CHH gene arrangement, relatively high sequence
315 homology among CHH neuropeptides suggests that CHH gene is well-conserved among the
316 arthropods. *C. borealis* has most extensively used for studying decapod neuromodulation [1, 24,
317 29, 30]. It will be interesting to examine if the STNS of other decapod crustaceans may produce
318 a tissue-specific isoform of CHH. The STNS-CHH sequence does not contain a signal peptide,
319 indicating presumably it is not released. The functional significance of CHH present in the STNS
320 needs to be studied in terms of the role of STNS in two central pattern-generating networks
321 controlling feeding behaviors in crustaceans.
322
323 The distribution and isoform of CHH neuropeptides vary by species. Whilst MIH is
324 predominantly present in the eyestalk ganglia of crab species, MOIH is also found in eyestalk
325 ganglia and STNS [21]. A tissue-specific isoform of CHH is found in ES, PO, and fore- and
326 hindgut of *C. maneas* [8, 9, 18]. Interestingly, this is the first time we report that in *C. borealis*,
327 the same form of CHH is found in multiple tissues including ES, PO, and STNS.
328
329 The sinus gland functions as the storage and release site of neuropeptides (CHH, CPRP,
330 and MIH) produced by neurosecretory cells of eyestalk ganglia of decapod crustaceans. The
331 translation of CHH mRNA encoding one CPRP and CHH yields the equal amount of these

neuropeptides. The post-translational modification is incomplete; the sinus gland always contains two CHHs: 1 and 2 with CHH2 as the major form [7, 13, 14]. The ratio of CHH1: CHH2 (~0.27) could reflect the efficiency of Q_1 to $<Q_1$ at the N-terminus and translation rate.

The presence of CPRP in the hemolymph predisposes its release from the sinus gland [42]. Notably, a 1:1 stoichiometric ratio of CHH and CPRP release is noted in *C. pagurus* [42]. Thus, it is plausible to suggest that the sinus gland should have contained the same amount of these two neuropeptides. In *C. borealis*, the ratio of CPRP and CHH1 and CHH2 present in the sinus gland is skewed at 1.0: 1.6, suggesting that these peptides may have been differentially released. The function of CPRP remains to be defined. However, the sinus gland with the amounts of CPRP less than those of CHH suggesting the higher amounts of CPRP secretion, together with the longer half-life of CPRP ($t_{1/2}$ = ~60 min) in hemolymph than CHH ($t_{1/2}$ = ~5-10 min) [12, 42] indicates the presence of a high concentration of this peptide in hemolymph, which points out the importance of an additional study.

Overall, the expression of each of these CHH neuropeptides reflects the neuropeptide levels in the sinus gland: highest expression of CHH, supporting the predominant CHH, and CPRP levels in the sinus gland. The adult animals > 110 mm CW are used for this study. We are currently investigating to examine if there is a relationship between the size and neuropeptide levels in ES (both transcripts and protein).

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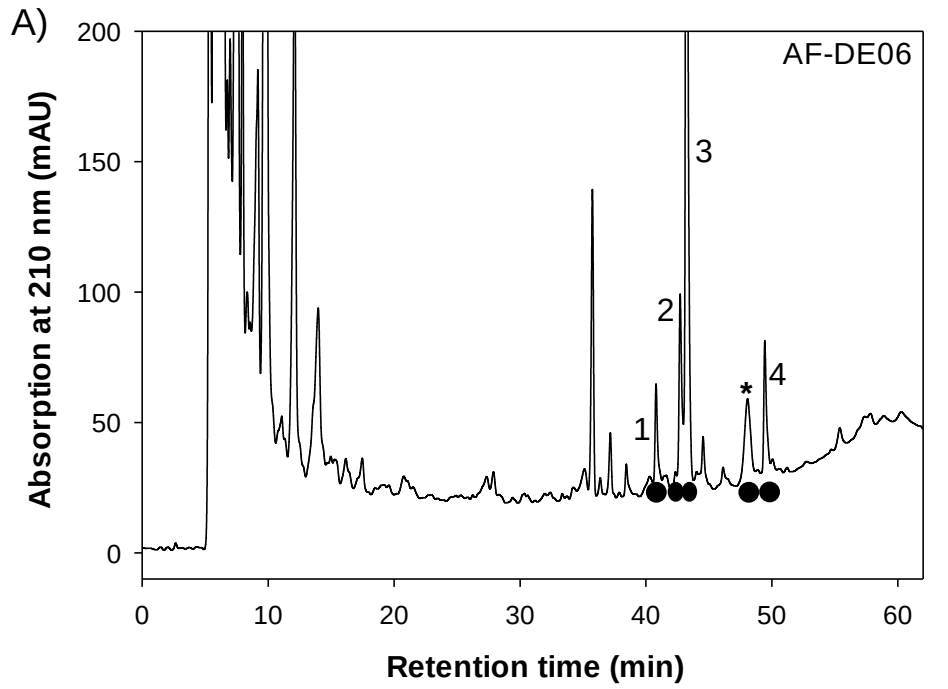
505 **Figure legends**
506
507
508 Figure 1. The neuropeptide profiles of sinus gland obtained from adult female (A) and male (B)
509 by RP-HPLC analysis. Two sinus gland extract was separated on a Gemini column (4.6
510 x 250 mm, 5 µm particle size with100 Å) using a gradient: 30-70% B over 60 min at the
511 flow rate of 0.6 ml/min. The chromatogram is shown with the absorption at 210 nm. 1:
512 MOIH; 2: CHH1; 3: CHH2; 4: MIH. *= CFSH. Peaks identified using dot blot assays with
513 specific antisera are noted with 'black dot'.
514
515 Figure 2. Clustal W alignment of the putative amino acid sequence of CHHs deduced from the
516 ES, PO, and STNS cDNA sequences of *C. borealis*, together with Cap ES-CHH.
517 Consensus amino acids are shown with '*'. Signal peptides of ES- and PO-CHH are
518 boxed. Dibasic cleavage site, KR, precedes CHH sequences. Putative amidation site (G)
519 and tribasic cleavage site (KKK) are boxed in red, respectively.
520
521 Figure 3. Clustal W alignment of putative amino acid sequence deduced from ES-MIH (A) and
522 ES- and STNS-MOIH (B) cDNA sequences, together with Cap-ES-MOIH 1 and 2.
523 Consensus amino acids are shown with '*'. Signal peptide is boxed.
524
525 Figure 4. Relative quantification of neuropeptide levels in the sinus gland of the adult males (A).
526 Single or two sinus glands were separated on a C18 column with a gradient condition of
527 30-70% B (0. 1% TFA in 60% acetonitirle + 40% water) over 60 min at 0.6 ml/min flow
528 rate. The detection was at 210 nm wavelength that monitors peptide bonds. To compare
529 the amount of each neuropeptide, the relative peak areas shown in Fig. 4A were derived
530 by normalizing the initial values to the size of CHH1 and CHH2 (72 aa). (B) Expression
531 levels of CHH and MIH in eyestalk ganglia of adult males. The data were presented as
532 mean ± 1 SE copies/µg eyestalk total RNA. Each cDNA sample was assayed in triplicate.
533 The data are presented as mean ± 1SE (n). Non-parametric one-way analysis of
534 variance (ANOVA) (Kruskal-Wallis test) was used to determine the statistical
535 significance. Statistical differences at $P<0.05$ were indicated with letters.
536
537

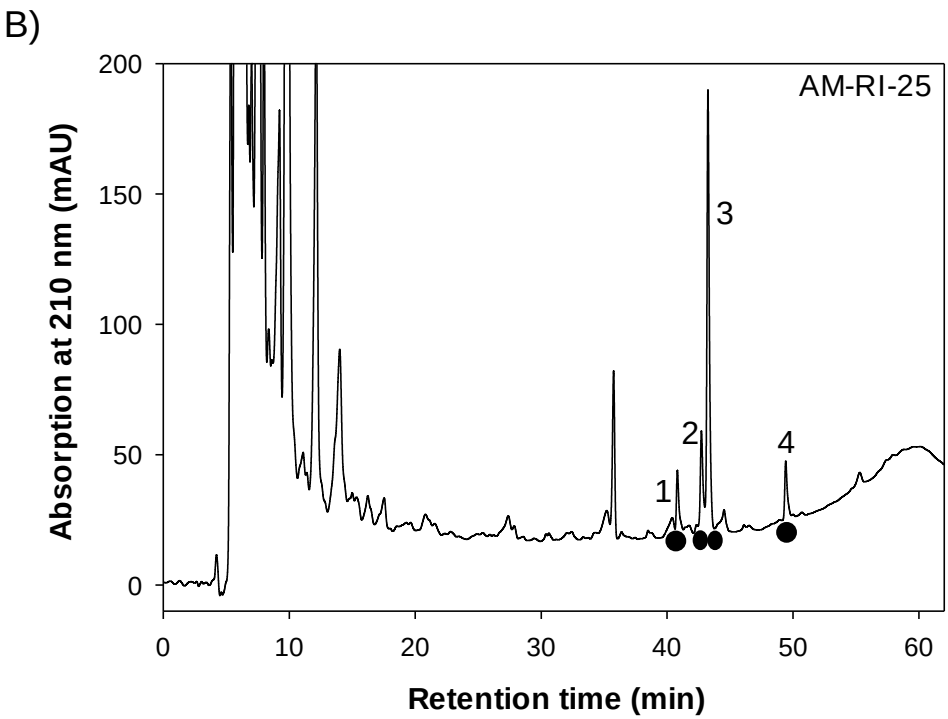
Table 1. Primer sequences for the initial PCR cloning with degenerate primers and for 5' and 3' RACEs and the expression analysis of CHH, MIH, and MOIH of *C. borealis*

	Sequence (5'-3')
CHHdF1	TGYAARGGNGTNTAYGA
CHHdF2	CATRCAYTGNCKRAANAC
CHHdR1	GAYTTYATHGCNGCNGGNAT
CabCHH3F1	AGGACTCTTCAGTGAGCTTGAACA
CabCHH3F2	GAGTGCAGGAGGAACTGTTATAGC
CabCHH5R0	TACTTCTTCTTGCCAACGATCTGT
CabCHH5R1	GCTATAACAGTTCCTCCTGCACTC
CabCHH5R2	GTAGCAATCGTCACACACATATG
ES-CHH-srt	ATGCTGACTACAAGAACGCTACTTTTGGGCGT
ES-CHH-end	CTTCTTGCCAACGATCTGTACGGCTCTTGCA
MIHdF1	GNGTNATHAAYGAYGA
MIHdF2	TGYCCNAAYYTNATHGGNAA
MIHdR1	AARTCYTCRTTRAARAARCA
CabMIH3F1	AGACCTTTATAAGAAAGTAGAA
CabMIH3F2	CTCTCTGCAGAAAGAACTGTTTCT
CabMIH5R1	AGAAACAGTTCTTTCTGCAGAGAG
CabMIH5R2	ACAATCCTCACAGATCCATTCTAC
ES-MIH-srt	ATGATGTCACGAACGGAATCCAGATATTCTTCT
ES-MIH-end	TCACTTACTGCCTGCCCCGAGAATACCAACCCA
MOIHdF1	TGYCARAAYTTYATHGGNAA
MOIHdF2	ATGTAYGARAARGTNGAYTGG
MOIHdR1	TTCCANCCNGCNCCNARDAT
CabMOIH3F1	TCTGCAAAGACTGTGCA*AACAT*
CabMOIH3F2	AAACATATTCCGCCAAGATGGACT
CabMOIH5R1	GTTCCTTGTGTCTAGTGTTCTCC
CabMOIH5R2	ATGGCCGCCCATTGCTCCAGCTG
ES-MOIH-str	ATGATGTCACGTGCTAACTCCAAAGTGTTTCAG
ES-MOIH-end	TCAGTTCCAGCCGGCCCCGAGGATGGCCGCC
CabtubulinF	GGCAAGTATGTCCCCAGGGCCGTCTTAG
CabtubulinR	CTTGAGTGTACGGAAACAGATGTCATACAA
CabNaKF	TGCCAGTATGACAAGACTTCTGAAGGCT
CabNaKR	TTAGTCCAACAAATCGAAGGCCATGCAC
CabCHH-QF	TGAGCTTGAACATGTGTGTGACGATTGCTAC
CabCHH-QR	TTCTTCCATGCATTGTCGGAATACCACGTT
CabCHH-QR(STG)	CTCCGTCATTATCCCCTCACAGCATCCCTG
CabMIH-QF	GGATCTGTGAGGATTGTTCTAACATCT
CabMIHQR	GGAAGTCTTCGTTAAAGAAACAGTTC
CabMOIH-QF	TGGATCTGCAAAGACTGTGCAAACATA
CabMOIH-QR	TAGTGTTCTCCGTTGCGTCGATACACC
CabNa/K-QF	GATGTAGTCCGTAAGGAGGCTGAGAAG
CabNa/K-QR	TAGGAGACGGCACAACAGAGAAAGTGA

d: degenerate primer. QF and QR primers for qPCR analysis; str and end primers for producing standard for qPCR analysis

Chung et al Figures 1A and B





Chung et al Figures 2A and B

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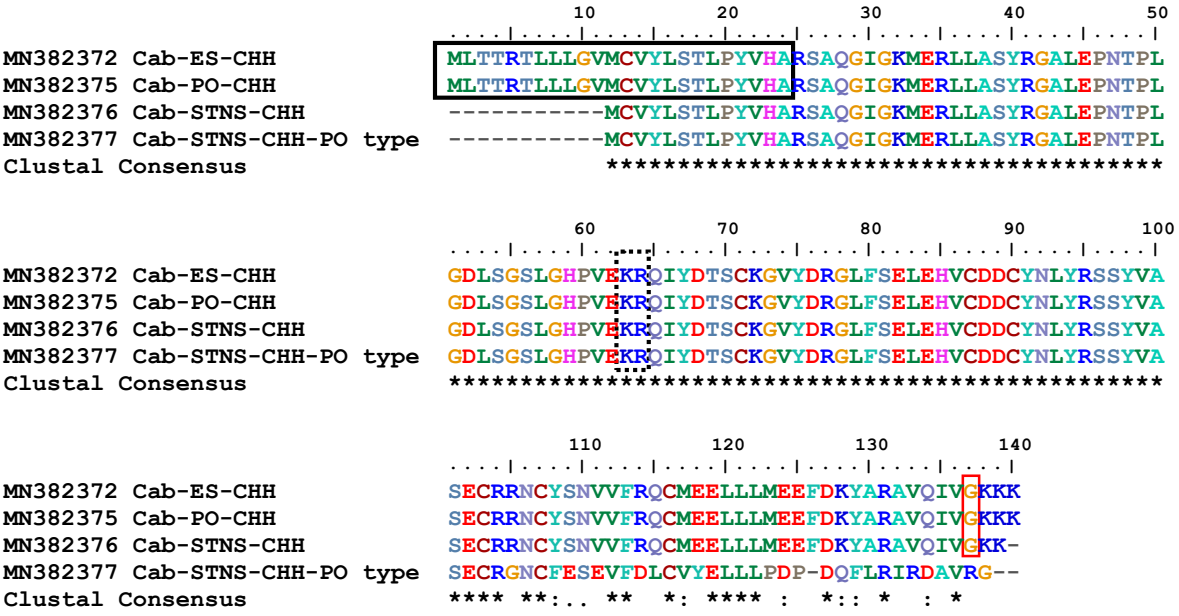

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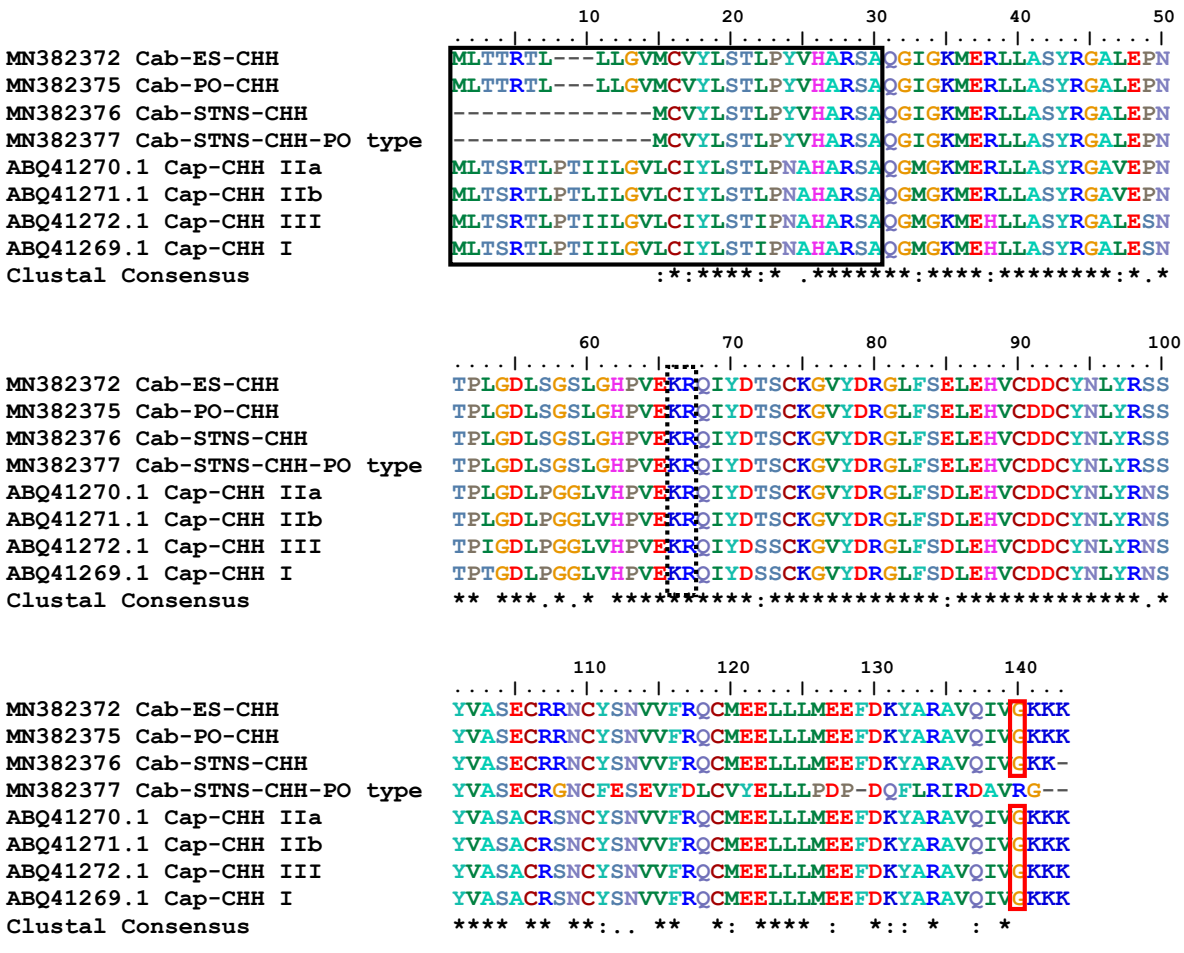
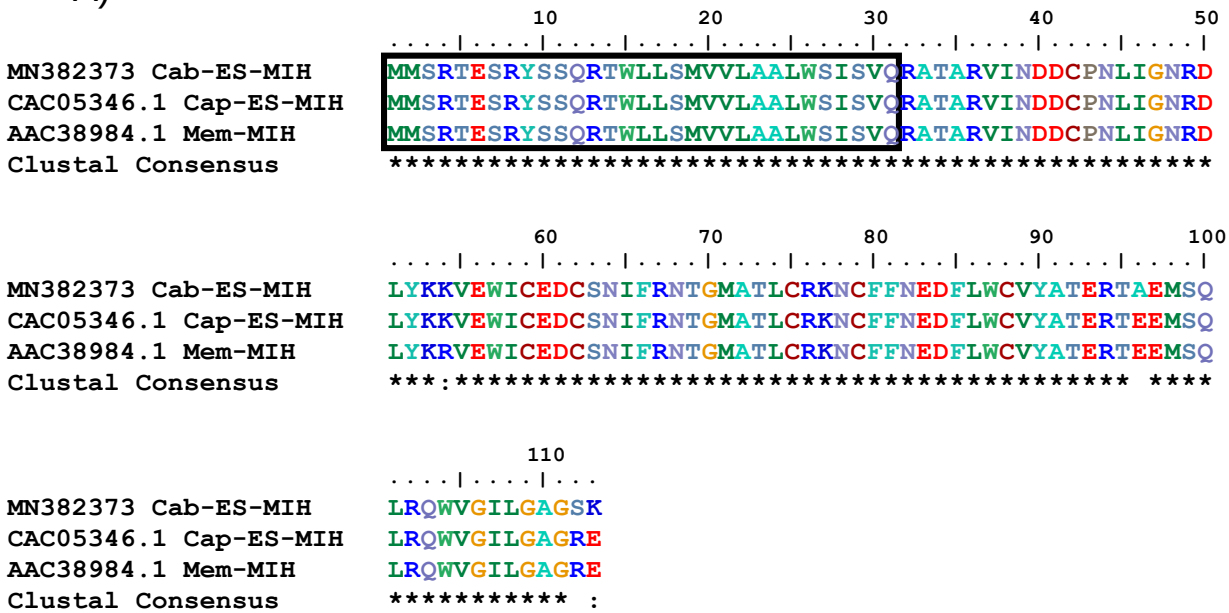


Chung et al Figures 3A &B

A)



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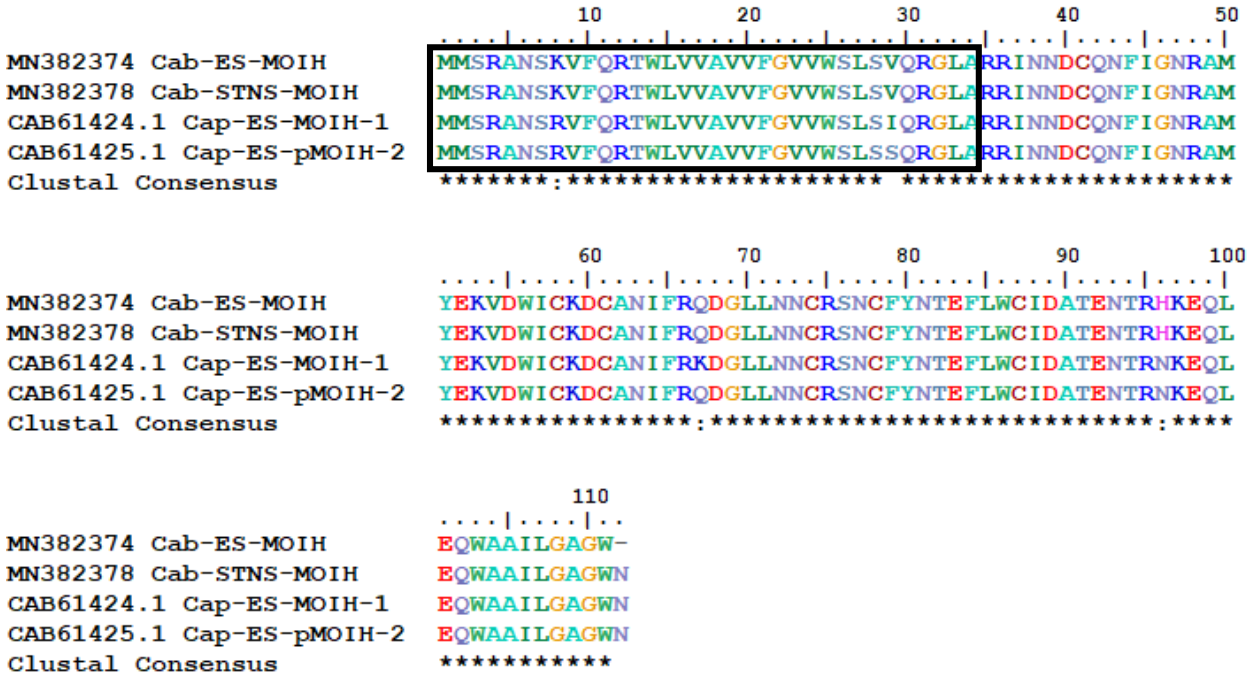
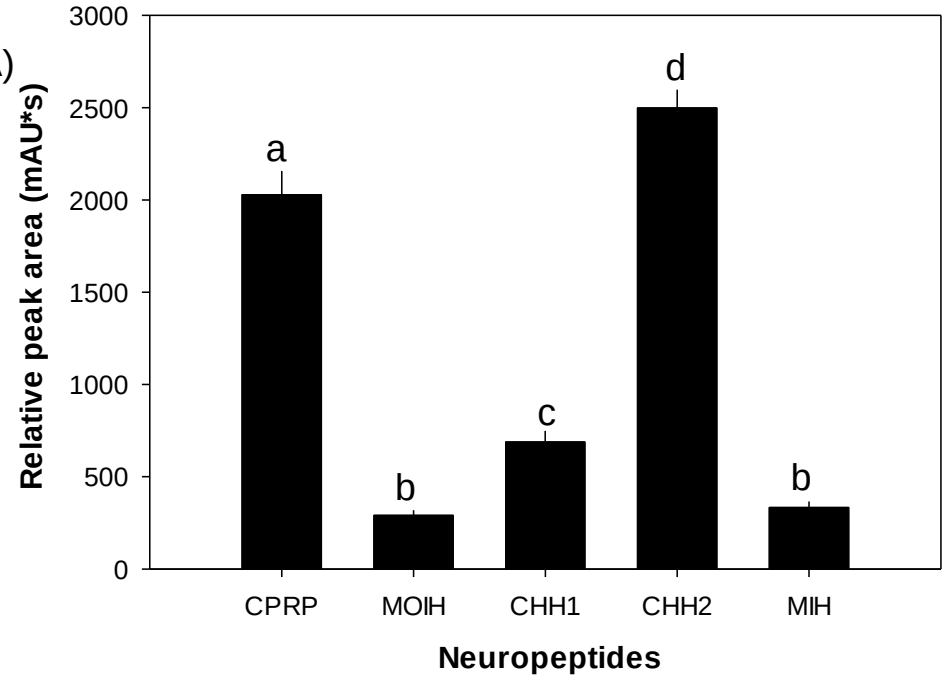


Chung et al Figures 4A & B

A)



B)