General and Comparative Endocrinology 178 (2012) 227–236

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Stimulation of molt by RNA interference of the molt-inhibiting hormone in the crayfish *Cherax quadricarinatus*

Ramachandra R. Pamuru [a,b,1], Ohad Rosen [a,1], Rivka Manor [a], J. Sook Chung [c], Nilli Zmora [c], Lilah Glazer [a], Eliahu D. Aflalo [a], Simy Weil [a], Sherry L. Tamone [d], Amir Sagi [a,*]

[a] Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University, Beer-Sheva, Israel
[b] Department of Biochemistry, Yogi Vemana University, Vemanapuram, Kadapa, Andhra Pradesh, India
[c] Institute of Marine and Environmental Technology, Baltimore, MD, USA
[d] University of Alaska Southeast, Juneau, AK, USA

ARTICLE INFO

Article history:
Received 11 March 2012
Revised 13 May 2012
Accepted 23 May 2012
Available online 1 June 2012

Keywords:
Cherax quadricarinatus
Molt-inhibiting hormone
RNAi
dsRNA
Molt cycle
Ecdysone

ABSTRACT

In crustaceans, molting is known to be under the control of neuropeptide hormones synthesized and secreted from the eyestalk ganglia. While the role of molt-inhibiting hormone (MIH) in regulating molting has been described in several species using classical methods, an *in vivo* specific MIH targeted manipulation has not been described yet. In the present study, an MIH cDNA was isolated and sequenced from the eyestalk ganglia of the Australian freshwater red claw crayfish *Cherax quadricarinatus (Cq)* by 5′ and 3′ RACE. We analyzed the putative Cq-MIH based on sequence homology, a three dimensional structure model and transcript's tissue specificity. We further examined the involvement of *Cq-MIH* in the control of molt in the crayfish through RNAi by *in vivo* injections of *Cq-MIH* double-stranded RNA, which resulted in, similarly to eyestalk ablation, acceleration of molt cycles. This acceleration was reflected by a significant reduction (up to 32%) in molt interval and an increased rate in molt mineralization index (MMI), which correlated with the induction of ecdysteroid hormones compared to control. Altogether, this study provides a proof of function for the involvement of the *Cq-MIH* gene in molt regulation in the crayfish.

1. Introduction

In crustaceans, growth which periodically occurs by molting is under the control of neuropeptide hormones and ecdysteroids. The paired Y-organs are endocrine glands that secrete the steroid hormone ecdysone, which promotes the molting process [47]. Ecdysteroid synthesis in the Y-organ is negatively controlled by molt-inhibiting hormone (MIH), which is synthesized and released by a cluster of neurosecretory cells (collectively called the medulla terminalis X-organ sinus gland (XO-SG) complex) located within the eyestalks [47,49,63]. Circulating MIH inhibits ecdysteroid synthesis by the Y-organs throughout most of the molt cycle (intermolt), and provides later negative feedback to the XO-SG that results in decreased circulating MIH levels that is thought to initiate premolt, as suggested in studies with the American crayfish *Procambarus clarkii* [36]. In addition to their primary role in the inhibition of the synthesis and release of ecdysteroids, some MIHs are also involved in reproduction [16,54,69].

MIH belongs to the crustacean hyperglycemic hormone (CHH) family, the members of which share amino acid sequence similarity, and some have overlapping biological functions [4,17,40,54,56]. CHH-family peptides are categorized into two groups based on amino acid homology and whether the CHH-precursor-related peptide (CPRP) is present or absent. MIH belongs to class II CHH family peptides, which usually lack the CPRP and the amidated C-terminus (exceptions are the MIHs of *P. clarkii* [34] and the African spiny lobster *Jasus lalandii* [33]). In addition, class II peptides contain an additional glycine residue at position 12 between the first and second cysteine residues. This finding initially led to the assumption that the additional glycine is associated with the molt-inhibiting function. However, recent studies showing that MIH activity may be executed by distinct peptides [10,28,35,62,64] or that CHHs possess molt-inhibiting capability [1,7,12,65] refute this assumption. Putative MIH-like cDNAs have been cloned and sequenced in different crustacean decapods including crabs [24,27–29,57], crayfish [2] and shrimps [8,15,25,37,38,50,51,60]. Nevertheless, there is a dearth of information regarding MIH structure and activity in Southern Hemisphere crayfish, specifically in the Australian freshwater crayfish. Moreover, an *in vivo* study in which the specific effect of an MIH encoding gene on molt cycles in crustaceans has not been reported yet.

* Corresponding author. Address: Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University, P.O. Box 653, Beer-Sheva 84105, Israel. Fax: +972 8 6479062.
E-mail address: sagia@bgu.ac.il (A. Sagi).
[1] These authors contributed equally to this work.

0016-6480/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2012.05.007

In most crustaceans, the molt cycle is a perpetual cyclical process composed of four main stages: post molt, intermolt, premolt and ecdysis [5,48]. In freshwater crayfish, including *C. quadricarinatus*, calcium is stored in the form of calcium carbonate containing organs called gastroliths during the premolt. As the molt cycle progresses toward ecdysis, the gastroliths increase in size and immediately after ecdysis are digested in the stomach releasing calcium, which is mobilized through the hemolymph to harden the new exoskeleton. Molt cycle durations vary among crustaceans and are strongly influenced by ecdysteroid secretion [14,47]. A precise molt stage specific profile for gastrolith development has been established previously [44,45] using the biometric parameter molt mineralization index (MMI), which correlated with the size of the gastroliths. Thus, this tool can be used to non-invasively monitor molt cycle progression by observing gastroliths growth (represented as MMI) using X-ray imaging.

Utilization of RNA interference (RNAi) to reveal a specific gene function was successfully applied in several decapod crustaceans. In *Litopenaeus schimitti* CHH expression was inhibited by *Ls-CHH* double stranded RNA (dsRNA) injections [30]. In *Metapenaeus ensis*, *Me-MIH*-B dsRNA injections caused specific reduction of this transcript's levels in the thoracic ganglia and in the eyestalk, which in turn led to a reduced expression of the *vitellogenin* gene in the hepatopancreas and ovary [54]. More recently, vitellogenesis-inhibiting hormone (VIH) was silenced by *VIH* dsRNA injections in *Penaeus monodon* [55], resulting in a decrease in *VIH* expression levels accompanied by increased vitellogenesis in the ovary. Gene silencing studies in crustaceans and their direct effects on growth and reproduction are still in their infancy and thus far were not executed *in vivo* in the context of MIH regulating molt cycle.

In the present study, an *MIH*-like gene was identified in the eyestalk ganglia of the freshwater crayfish *C. quadricarinatus*. The full length cDNA sequence was obtained and a signal sequence and mature peptide sequence were predicted. Sequence homology of the deduced mature peptide for Cq-MIH was studied in comparison to selected MIH peptides of other crustaceans. A predicted 3 dimensional (3D) structure of Cq-MIH was generated and compared with the NMR structure of *Marsupenaeus japonicus* MIH [21]. *Cq-MIH* tissue specific expression was validated. Induced alterations of ecdysteroids concentrations molt mineralization index (MMI) and molt cycle duration were measured following *in vivo* injections of *Cq-MIH* dsRNA.

2. Materials and methods

2.1. Animals

Male *C. quadricarinatus* crayfish were obtained from the Fish and Aquaculture Research Station, Dor, Israel, and were grown in artificial ponds at Ben-Gurion University of the Negev. Food pellets comprising dried shrimp (30% protein; Rangen, Inc., Buhl, ID) were supplied *ad libitum* three times a week. Water temperature was held at 28 ± 2 °C under a photoperiod of 14 h light: 10 h dark. Water quality was maintained by circulating the entire volume of water through a plastic bed biofilter.

2.2. Amplification, cloning and sequencing of the full length cDNA of Cq-MIH

Several primers were used for PCR amplification and RNA synthesis of the *Cq-MIH* gene in the present study (Table 1). Total RNA was extracted from eyestalk ganglia using EZ-RNA (Biological Industries, Beit Haemek Ltd.) according to the manufacturer's protocol and quantified using a NanoDrop Spectrophotometer (GeneQuant Pro, Amersham Pharmacia Biotech Ltd.). The first strand of cDNA was synthesized using 1 μg total RNA, RT-Expand (BD, Biosciences) and an oligo $dT_{(18)}$ primer (Syntezza). The initial degenerate primers were designed according to the conserved amino acid domains CPGVMGNRA, RVCEDCYN, and FPDFKRW of MIH from *Orconectes limosus* and *P. clarkii* (GenBank accession numbers P83636 and 2209245A, respectively). An amplicon of 186 bp was initially amplified from eyestalk ganglia cDNA by PCR with degenerate primers-*Cq*MIH F1 and *Cq*MIH F2 (Table 1) and the Advantage cDNA Polymerase Mix (BD Biosciences). In the first amplification, *Cq*MIH F1 and *Cq*MIH R1 were used followed by a semi-nested PCR using *Cq*MIH F2 and *Cq*MIH R1. PCR products were separated by agarose gel electrophoresis and bands were excised and extracted (Ultrafree-DA, Millipore). The resulting fragment was cloned into a TOPO TA cloning vector (Invitrogen) and sequenced.

Table 1
Primers used for *Cq-MIH* PCR amplification and RNA synthesis.

Nucleotide sequence (5′–3′)	Primer
TGYCCNGGNGTNATGGGNAAYC	*CqMIH F1*
GTNTGYGARGAYTGYTAYAA	*CqMIH F2*
ATNYSNAYCCANCKYTTRAANTC	*CqMIH R1*
CCACGTTCAATAAGCTCCAGACACG	MIH RACE R1
AGAGGAAGCTGAACAAGCTCCTCCTTTC	*Cq-MIH begin-F*
TTCAAGTCGTGTCTGGAGCTT	MIH RACE F1
GCTCCAGACACGACTTGAATGTGTC	*Cq-MIH R2*
TAATACGACTCACTATAGG**TCTAGA***ATGGTTAA ACACGCAAATCAGTC*	MIH dsRNA F
GGGCCCTAATACGACTCACTATAGGGTCGAC*TC ACTTGCCTCCAGCGTTG*	MIH dsRNA R

2.3. 5′ & 3′ RACE and sequence confirmation

First strand cDNA for 5′ and 3′ RACE was generated from 1 μg total RNA of the eyestalk ganglia using the SMART™ RACE cDNA Amplification kit (BD Biosciences), according to the manufacturer's protocol. A 5′ RACE fragment was amplified using the reverse primer MIH RACE R1 (Table 1) against a Universal Primer Mix (supplied in the kit) using the 5′ RACE eyestalk ganglia cDNA as a template. To confirm the 5′ sequence of 635 bp that was obtained, a forward primer from the 5′ end was designed (based on the 5′ RACE results)—*Cq*-MIH begin-F, nt 30–59 against the reverse primer MIH RACE R1, and a 605 bp amplicon was obtained. An 824-bp 3'-RACE fragment was amplified using primer MIH RACE F1 (Table 1) against oligo $dT_{(18)}$ primer.

All PCR products were cloned into the pGEM-T Easy Vector System (Promega) and transformed into *Escherichia coli*. Clones were isolated and grown overnight, plasmid DNA was purified (HiYield Plasmid Mini Kit, RBC Bioscience) and sequenced.

2.4. Tissue specific expression of Cq-MIH

The distribution of the *Cq-MIH* transcript was determined using RT-PCR in selected tissues. Total RNA was isolated from three intermolt crayfish, as described above, from eyestalk ganglia, hepatopancreas, thoracic-ganglion, hind gut, testis and muscle. The RT reaction was carried out with 1 μg total RNA of each tissue separately. The primer *Cq*-MIH begin-F and the custom-designed reverse primer *Cq*-MIH R2 were used to obtain a 592 bp product in the PCR reaction. *Elongation factor-II* (*EFT-II*) cDNA was amplified in the same cDNA samples using its gene specific primers [32] as a quality control. PCR products were separated on a 1.2% agarose gel, visualized by ethidium bromide staining, and photographed

by ultraviolet illumination with a gel image documentation system (Bio Imaging System, Chemi Genius, Syngene).

2.5. Cq-MIH dsRNA synthesis and its in vivo activity

2.5.1. In vitro transcription of Cq-MIH

The template for *Cq-MIH* dsRNA synthesis was prepared by amplifying eyestalk cDNA (synthesized as mentioned above) with the primers MIH dsRNA F and MIH dsRNA R. The forward primer, MIH dsRNA F (5′ TAATACGACTCACTATAGG**TCTAGA***ATGGTTAAACAC GCAAATCAGTC* 3′), consisted of 19 b of T7 promoter (underlined), 6 b of *Xba* I restriction site (bold) and 23 b (located between 369 to 392 b from the beginning) of *Cq-MIH* sequence (italic). The reverse primer MIH dsRNA R (5′ **GGGCCC**TAATACGACTCACTATAGGGTC-GAC*TCACTTGCCTCCAGCGTTG* 3′), consisted of 6 b of *Apa* I restriction site (bold), 19 b of T7 promoter (underlined), 6 b of *Sac* II restriction site (5′ GTCGAC 3′) and 19 b (located in between 674 to 693 b from the beginning) of *Cq*-MIH sequence (italic). This pair of forward and reverse primers was used to amplify the *Cq-MIH* template. A PCR product of approximately 340 bp was ligated, and cloned as mentioned above. The plasmid containing the desired *Cq-MIH* sequence was digested using restriction enzymes *Xba I* or *Sac II* (New England Biolabs, Inc.,) in two separate reactions to generate the two complementary strands of *Cq-MIH*. Single-stranded RNA was synthesized with MEGAscript T7 kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. The resulting single stranded RNAs were hybridized in one tube by incubation at 70 °C for 15 min, 65 °C for 15 min and room temperature for 30 min then stored at −70 °C until used.

2.5.2. In vivo Cq-MIH loss of function through dsRNA injection

Juvenile, intermolt *C. quadricarinatus* males with body weights ranging from 100 to 600 mg were selected for the *Cq-MIH* dsRNA injection experiment. Sexing was done under a dissecting microscope based on the presence or absence (male or female, respectively) of gonopores on the base of the fifth pair of pereiopods (walking legs). Four groups, each containing 12 juvenile males, were used in the experiment, three of which were controls (one standard and two positive) and the fourth served as the experimental group. Based on our previous experience *vitellogenin*-based dsRNA was not different from saline injection [44]. Thus, the standard control group was injected with *Cherax* saline (194 mM NaCl; 5 mM KCl; 12 mM KH_2PO_4; 33 mM $NaHCO_3$ at pH 7.5) at volumes equivalent to those applied to the experimental group (based on gram body weight). Treatment of one positive control group comprised bilateral eyestalk ablation. The second positive control group was injected with ecdysone at a final concentration of 1000 pg/μl as per [44]. The treatment group was injected with *Cq-MIH* dsRNA at a dose of 5 μg/g

```
TACGCGGGGATCACCACCAGCATCATCCTCAGAGGAAGCTGAACAAGCTCCTCCTTTCTCCTTGCTTCTTCACTGCCACT
CTTCTCCCCAGACTCACGCCAGACGAGTGTCTTGTTCACCCAGGTGTTAGGATTGACGTGAACGAGACAGACATACTTTG
AACATCACTGCCAACACTTACATTCCTGGTGTATTATCGTCAGTCTCTTGAAAGCCCGAGGATTCCTCTTCAGAGCTCCA
CTCGTCACTGTACTGGCATCTCAAGAAAATAAAAACATTTGGATTTCTCTCTGAGTTCCGAGAAACTAAGTAAGGATACG
          330       340       350       360       370       380       390       400
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
AGAGTAACAAGCAGAGAGGATAACACTGTGTCTCCAAGTGTCTCAAGAGATGGTTAAACACGCAAATCAGTCTTGCTCTG
                                                  M  V  K  H  A  N  Q  S  C  S  A -20
          410       420       430       440       450       460       470       480
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
CGCTGAGGCCGTGGCTGATAGTGATGGTGGTGGGTCTTCTTGTTCACCAGACAACGCCAACGTTAACGGACGACTGTCCT
 L  R  P  W  L  I  V  M  V  V  G  L  L  V  H  Q  T  T  P  T  L  T  D  D  C  P    7
          490       500       510       520       530       540       550       560
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
GGAGCCATGGGCAACAGACATATACACACCATGTTGTTGAGAGTATGTGGAGACTGCTATAATGTCCTCAGAGATCCTGA
G  A  M  G  N  R  H  I  H  T  M  L  L  R  V  C  G  D  C  Y  N  V  L  R  D  P  E  34
          570       580       590       600       610       620       630       640
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
AATAGAGGTTGACTGCAGGAGCGGCTGCTTCACCAGTGACACATTCAAGTCGTGTCTGGAGCTTATTGAACGTGGGGATG
 I  E  V  D  C  R  S  G  C  F  T  S  D  T  F  K  S  C  L  E  L  I  E  R  G  D  E 61
          650       660       670       680       690       700       710       720
----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
AGTTCTTTGATTTCATGAGAAGGGTTGGCATTCTCAACGCTGGAGGCAAGTGATGAGATCCATGCCGTTTGCTACCGTTG
  F  F  D  F  M  R  R  V  G  I  L  N  A  G  G  K  *                           77
TACCGTTGGTCTGAAACTCTTCTCACAAGCATCACACATCCCGCTTCACGGACACCCGTCGGTTGACCATAAATCACTCT
GCATCAGACATGTGTTTATGACCGTGCCAATTCTTCTGCACTCACTCTTCATCTAAGCTATATCTTTGTGAAAGATCCTC
TCACAAAGACTTAAATATATAGGAACCAATTGGAAATACCGTAAATAAGAGTTCCAAACTACCCCTCAATTTGGAGAGGA
AATGATACAACATTTCTAGGTGAACCTAATAGTAACTATCTCTATAACCACTTCCTAGTAGTTAGAGGAATAGTGCTTAT
GTTGATAGTATCATTGTTAATCCCAGTTGCTTGAGGATACCTTCTCTTCAGATTTTATCATAACCATCAACTACTTATGA
AAAGATTTTTCAAGTATCCTTTTAATACCGCTTCTTACATTCTTTAAATAATTGCTTATTTTTCTCTGTTATTGCCCCAA
AAATATCGCAGTCCACTCGCACTTCTATTGTTTCTTTTTAATACGCAAATCGATAATTATCATATTTACACTTGTATTTA
TATTTCTTATCCATATCAAATTCATAGTTCATCAACATAATAAAATTACCAACTATAAGAGAAAAAAAAAAAAAAAAAA
AAA
```

Fig. 1. Nucleotide and deduced amino acid sequence of Cq-MIH. The deduced amino acid sequence, including signal peptide (open box) and mature peptide are shown in single-letter code below the relevant nucleotide sequence. The putative signal sequence cleavage site is marked with an arrow head, the stop codon is indicated with an asterisk (∗) and the polyadenylation signal is underlined by a solid line.

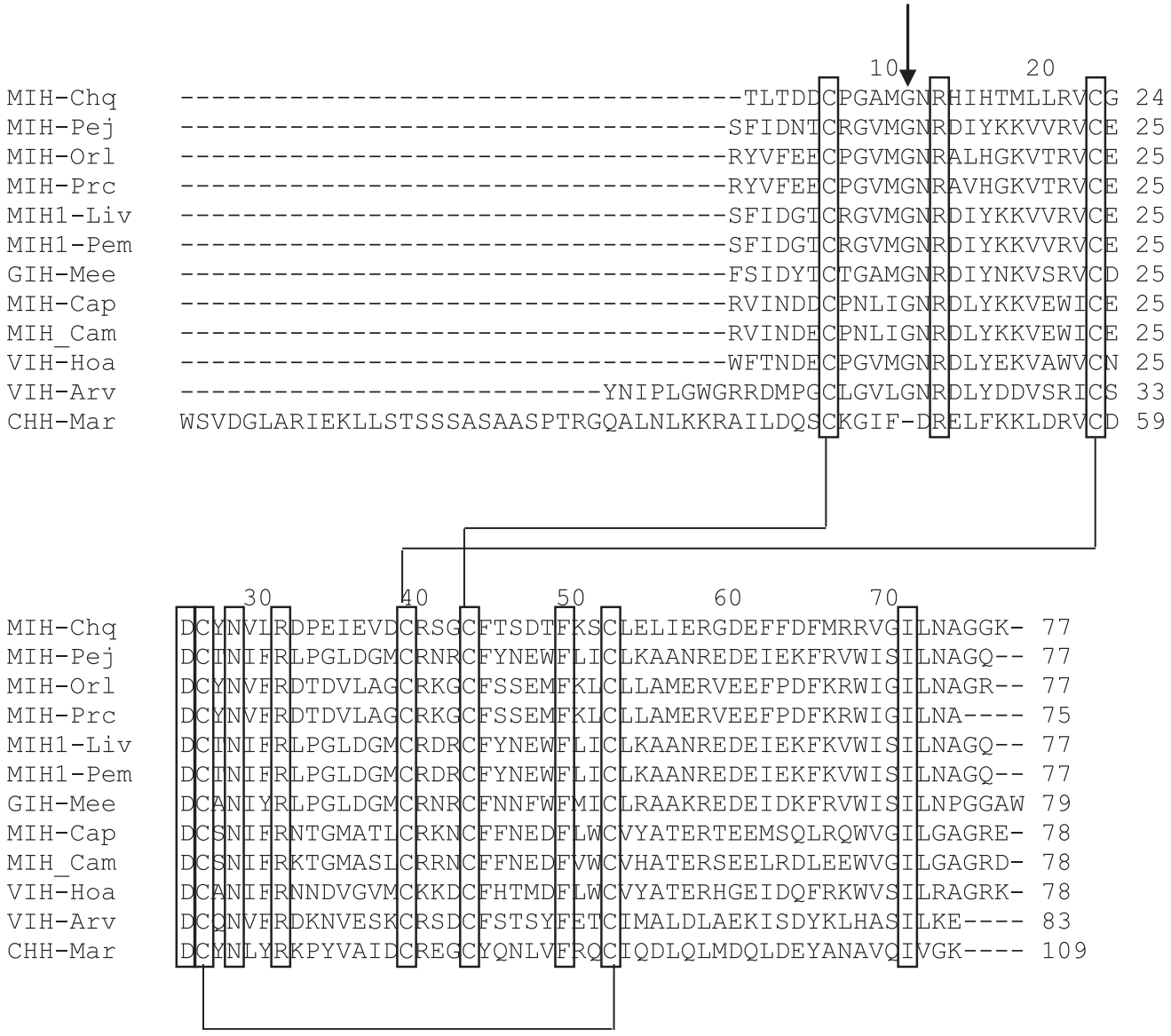

Fig. 2. Multiple alignment of amino acid sequence of the putative *C. quadricarinatus* (Chq) MIH with other CHH-family peptides. The presented sequences which were obtained from the NCBI and UniProtKB databases were: *M. japonicus* (Pej); *O. limosus* (Orl); *P. clarkii* (Prc); *L. vannamei* (Liv); *P. monodon* (Pem); *M. ensis* (Mee); *C. pagurus* (Cap); *C. maenas* (Cam); *H. americanus* (Hoa); *A. vulgare* (Arv) and *M. rosenbergii* (Mar). Six cysteine (C), two arginine (R), one aspartic acid (D), one asparagine (N), one phenylalanine (F) and one isoleucine (I), which are conserved across all of the presented CHH-family peptides, are highlighted in rectangular frames. The glycine residue in the 12th position in all MIH/VIH family peptides is indicated by an arrow. Putative disulfide bonds, formed between cysteine residues, appear as black lines.

animal. The different agents were injected into the fifth pereiopod sinus every other day and continued until the animal completed two molt cycles. Molt cycle progression in each individual was recorded between the two molt cycles by calculating gastrolith MMI values on alternate days during the experiment using X-ray digital imaging [45].

2.5.3. Cq-MIH dsRNA effects on circulating ecdysteroids levels

Intermolt mature *C. quadricarinatus* males with body weights averaging 11.4 ± 2.2 g were injected biweekly with either *Cq-MIH* dsRNA ($n = 5$) or an exogenous RB^{TM} dsRNA ($n = 4$; Beeologics, Israel) as a negative control. In a second control group eyestalk ablation was performed ($n = 4$). From each animal, 20 μl of hemolymph samples were collected twice a week to be further analyzed for ecdysteroid concentrations. In addition, molt progression was monitored and MMI values were calculated as mentioned above. For each animal, hemolymph was sampled and MMI was monitored until the animal completed a single molt cycle.

2.6. Ecdysteroid Assay

Circulating levels of ecdysteroids were analyzed using an enzyme-linked immunosorbent assay (ELISA) developed by Kingan [23] and modified for crustaceans by Tamone et al. [52]. Briefly, 96-well plates were coated overnight at room temperature with goat anti-rabbit IgG (Jackson Immunological Research Laboratories) and blocked for one hour using assay buffer (0.025 mM Tris, pH 7.5; 0.15 M NaCl; 0.5% BSA). Hemolymph samples were extracted in 90% methanol, supernatants evaporated to dryness using an evaporator (Thermo Scientific Savant), and samples reconstituted with water to their original volume. Standards of 20-hydroxyecdysone and diluted hemolymph samples (total volume of 50 μl) were incubated with 50 μl primary antiserum (1:100,000) and 50 μl HRP-conjugated ecdysone (1:4000) at 4 °C overnight. The conjugated steroid and the primary antiserum were obtained from Dr. T. Kingan. After washing three times for five minutes with PBS containing 0.05% Tween-20, the plates were

developed for 15 min at room temperature using a two part TMB substrate (Kirkegaard and Perry Laboratories, Inc.,) and color development was stopped using 1 M phosphoric acid. Absorbance was measured at 450 nm (Bio-Rad plate reader Model 680XR). The detection limit of this assay is 4 fmol. The inter- and intra-assay variation for this ELISA is 12% and 5% respectively.

2.7. Bioinformatic analyses

To enhance their quality, sequences were first stripped of low quality and vector sequences using Sequencher™ software (GeneCodes Corp.), which was also used to cluster and assemble the remaining sequences. The resulting contig was compared to the Uniprot database (Swiss-Prot + TrEMBL) using NCBI's BLASTx algorithm. The full length of *Cq-MIH* cDNA was computationally translated using the ExPASy Proteomics Server (http://ca.expasy.org/tools/dna.html), the most likely frame was selected (5′3′ Frame 3) and molecular weight was calculated using the compute pI/MW tool (http://web.expasy.org/compute_pi/). The deduced amino acid sequence was further analyzed using CBS Prediction Servers (http://www.cbs.dtu.dk/services). Using ClustalX [53], multiple sequence alignment of the predicted mature Cq-MIH sequence was performed with selected representative members of the CHH-family: *M. japonicus* (accession # P55847); *O. limosus* (accession # P83636); *P. clarkii* (accession # P55848); *Litopenaeus vannamei* (accession # AARO4348); *P. monodon* (accession # AAR89516.1); *M. ensis* (accession # AAL33882.1); *Cancer pagurus* (accession # CAC39425.1); *Carcinus maenas* (accession # Q27225); *Homarus americanus* (accession # P55320); *Armadillidium vulgare* (accession # P83627) and *Macrobrachium rosenbergii* (accession # AAL40915.1). Cq-MIH was predicted to be structurally similar to the MIH of *M. japonicus* according to FUGUE v2.s.07 (http://tardis.nibio.go.jp/fugue/ [46]. The 3D model of the Cq-MIH peptide was created by the ESyPred3D Web Server 1.0 (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred) [26] and compared with the NMR 3D structure of *M. japonicus* MIH [21] on the Swiss-Pdb Viewer 3.7 software [18].

2.8. Statistical analysis

Molt durations were analyzed with one-way analysis of variance (ANOVA) followed by Fisher LSD multiple comparison tests using the statistical program Statistica 6.1 (StaSoft Inc.). Values are expressed as means ± SE and are considered significant at $P < 0.05$.

3. Results

3.1. Molecular cloning of cDNA encoding Cq-MIH and sequence analysis

An amplicon of 186 bp was obtained by PCR with degenerate primers that were designed based on conserved regions of MIH sequences of *O. limosus* and *P. clarkii* (GenBank accession numbers P83636 and 2209245A, respectively). The full-length cDNA of *Cq-MIH* was subsequently generated using 5′ and 3′ RACE. The complete amino acid sequence of Cq-MIH was deduced from the nucleotide sequence of the MIH-cDNA clone (Fig. 1, accession number GQ926177). *Cq-MIH* cDNA consists of 1363 bp comprising a 369 bp 5'UTR, a 321 bp ORF, a TGA stop codon, and a 760 bp 3′UTR. The ORF sequence spans from positions 370 to 693. The polyadenylation signal (AATAAA) is located 17 bp upstream from the poly (A) tail and 625 bp downstream from the stop codon. The ORF of *Cq-MIH* was conceptually translated into a putative peptide consisting of 107 amino acid residues with a calculated molecular weight of 11,974.8 Da (ExPASy compute pI/MW tool). The sequence analysis using SignalP 3.0 predicted a signal peptide of 30 amino acids (Signal peptide probability: 0.988, Met^{1} to Pro^{30}) and yielded a mature peptide containing 77 amino acid residues with a calculated molecular weight of 8662.8 Da. The mature Cq-MIH peptide includes six cysteine residues at the positions 6,

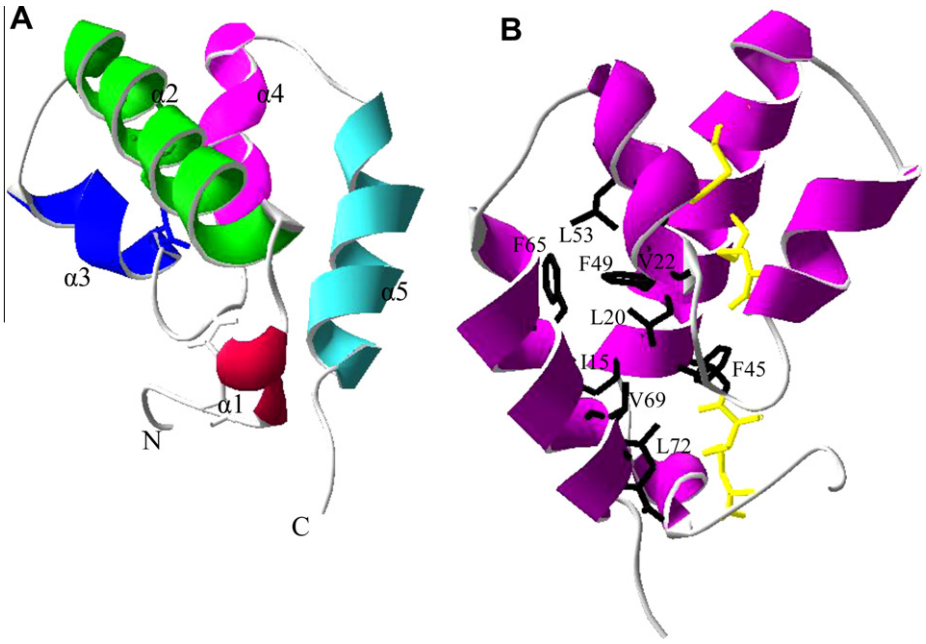

Fig. 3. A putative 3D structure of the predicted mature Cq-MIH. The 3D model was constructed based on its organizational similarity to the NMR structure of the mature MIH of *M. japonicus*. (A) The peptide backbone of 74 residues is presented in a ribbon diagram. The 5 α-helical regions are displayed in different colors, the N-terminal α-helix and the C-terminal tail, are predicted to be sterically close. (B) The 9 hydrophobic residues that are thought to stabilize the predicted 3D model through hydrophobic interactions are displayed (I^{15}, L^{20}, V^{22}, F^{45}, F^{49}, L^{53}, F^{65}, V^{69}, L^{72}). The side chains of cysteine residues, predicted to form three disulfide bridges, are displayed in yellow (6–43, 23–39, and 26–52).

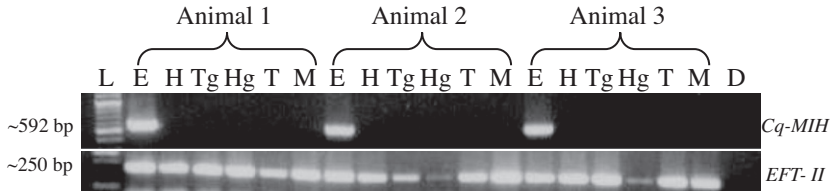

Fig. 4. *Cq-MIH* is exclusively expressed in the eyestalk ganglia of *C. quadricarinatus*. Six different tissues from three males at intermolt were analyzed: eyestalk ganglia (E); hepatopancreas (H); thoracic ganglia (Tg); hindgut (Hg); testis (T) and muscle (M). L, Molecular weight marker; D, distilled water control. *EFT-II* amplification in all the samples served as a positive control.

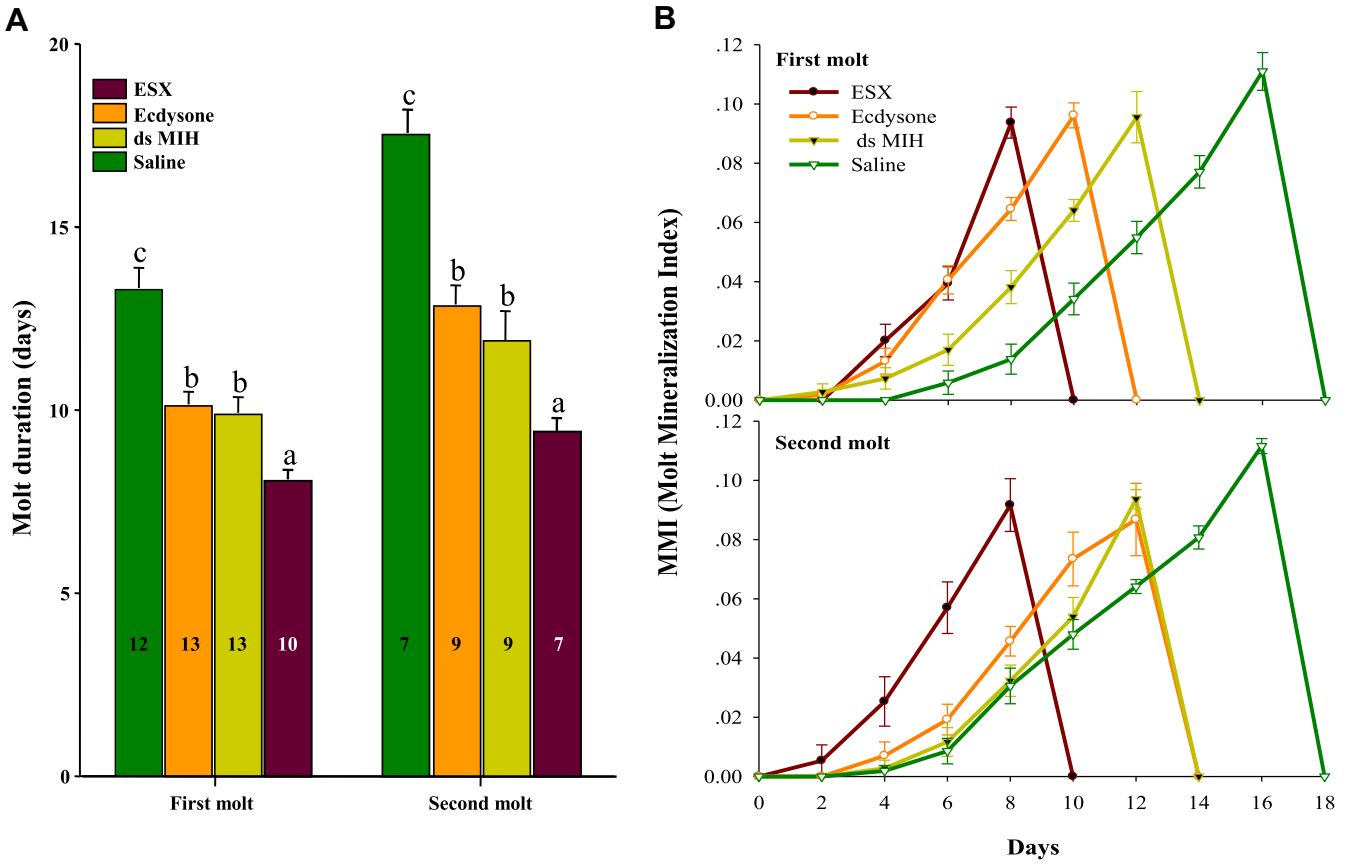

Fig. 5. *Cq-MIH* dsRNA injection shortens molt cycle duration in juvenile *C. quadricarinatus* males. (A) Molt durations were measured in two consecutive molt cycles and results are expressed as mean number of days ± SE. Numbers of animals are indicated on the bars. Letters represent statistically significant differences: 'a' from 'b' ($P < 0.05$), 'a' from 'c' ($P < 0.001$), and 'b' from 'c' ($P < 0.05$). Statistical analyses were performed using one-way ANOVA and post hoc Fisher LSD multiple comparison tests. (B) Progression of gastrolith growth (expressed as MMI values) in the two consecutive molt cycles. The values were synchronized and are presented as mean ± SE. ESX (eyestalk-ablated); ds MIH (*Cq-MIH* dsRNA-injected).

23, 26, 39, 43 and 52, all of which are conserved across the CHH-family of peptides in crustaceans. The deduced amino acid sequence of Cq-MIH was subjected to multiple alignment with selected MIHs, VIH/GIH (vitellogenesis/gonad-inhibiting hormone) and with a CHH amino acid sequence available at NCBI (Fig. 2). The Cq-MIH putative peptide showed less than 52% homology with the CHH family of peptides and its highest homologies were with the MIHs of *O. limosus* (51.9%) and *P. clarkii* (50.5%). These homologies to MIHs are considered relatively high compared to the lower homologies to known GIH (*M. ensis*, 41.8%) and VIHs (*H. americanus* and *A. vulgare*, 39.7% and 27.9%, respectively).

3.2. Three dimensional structure of Cq-MIH predicted peptide

The predicted Cq-MIH mature peptide sequence was used to construct a putative model of 3D structure that was found to be highly similar (ZSCORE = 16.53, 99% certainty) to the 3D structure of the *M. japonicus* MIH [21]. It suggested that the Cq-MIH includes 5α-helix regions as was shown for *M. japonicus* (Fig. 3A). The predicted peptide of Cq-MIH contains 9 hydrophobic residues at the same positions as in *M. japonicus* MIH [21], 7 of which are identical, as well as three intra disulfide bonds between the cysteine residues at positions 6 and 43, 23 and 39, and 26 and 52 (Fig. 3B). The predicted model suggests that the N-terminal α helix and the C-terminal tail are sterically adjacent to each other, similar to the NMR structure of *M. japonicus* MIH (Fig. 3A).

3.3. Tissue-specific expression of Cq-MIH

The tissue-specific gene expression of *Cq-MIH* was tested by RT-PCR in different tissues of three *C. quadricarinatus* males using gene-sequence-specific primers (Fig. 4). The PCR amplification

product appeared only in the eyestalk ganglia and not in any of the other tested tissues. Serving as a negative control in PCR, double distilled water used as a template did not yield any amplified product (lane D in Fig. 4). A positive control for the same cDNA samples was obtained by amplifying *EFT-II*, which resulted in a clear amplicon in all samples (Fig. 4). No specific amplification of the *Cq-MIH* transcript could be detected also in the ventral nerve cord and the supraesophageal ganglion (data not shown).

3.4. In vivo effects of Cq-MIH dsRNA on male C. quadricarinatus

To determine the role of *Cq-MIH*, dsRNA (generated based on its sequence) was injected into juvenile *C. quadricarinatus* males during two consecutive molt cycles. The duration of both molt cycles was significantly reduced by 25% in the first and 32% in the second cycle (Fig. 5A), relative to the corresponding durations measured in saline injected juvenile males. The molt interval for the second molt was 17.5 ± 0.6 days in the control versus 11.8 ± 0.8 days in the silenced group. A similar result was demonstrated with the two positive control groups, the ecdysone injected and eyestalk-ablated crayfish. Both of the two molt intervals in the ecdysone injected group were similar in duration to those of the *Cq-MIH* dsRNA-injected group (Fig. 5A). Molt intervals of the eyestalk-ablated group, however, were significantly shorter than the rest of the treatments: about 39% and 46% shorter than those of the control group (for first and second molts, respectively), and 20% shorter than the ecdysone injected and *Cq-MIH* dsRNA-injected groups (for both molt events).

The progression of the molt cycle was also monitored by measuring gastrolith growth, expressed as MMI, on alternate days throughout the molt cycle (Fig. 5B). *Cq-MIH* dsRNA-injected, eyestalk-ablated and ecdysone injected juvenile males reached maximum MMI values about 25–50% faster than the saline injected group in both molt cycles. We further examined *Cq-MIH*'s specific involvement in regulating ecdysteroidogenesis in a separate experiment. Using a specific ELISA, high levels of ecdysteroids (427.7 ± 93.0 ng/ml) were measured in the hemolymph of the eyestalk-ablated crayfish in days 10–14 post manipulation (Fig. 6A). In *Cq-MIH* silenced males, similar concentrations were detected, however with a slight delay compared with the eyestalk-ablated group. These elevated ecdysteroids levels were recorded at days 15 to 40 from first injection (Fig. 6B). The injection of the exogenous RB^{TM} dsRNA did not induce molt nor did it induce elevated concentrations of hemolymph ecdysteroids until day 40. Changes in MMI in manipulated animals correlated with measured ecdysteroid concentrations and were similar to indexes measured previously for intermolt and premolt animals (Fig. 6A′–C′).

4. Discussion

The function of MIH as a molt inhibitor was previously demonstrated by eyestalk removal and MIH injection in a few crustacean

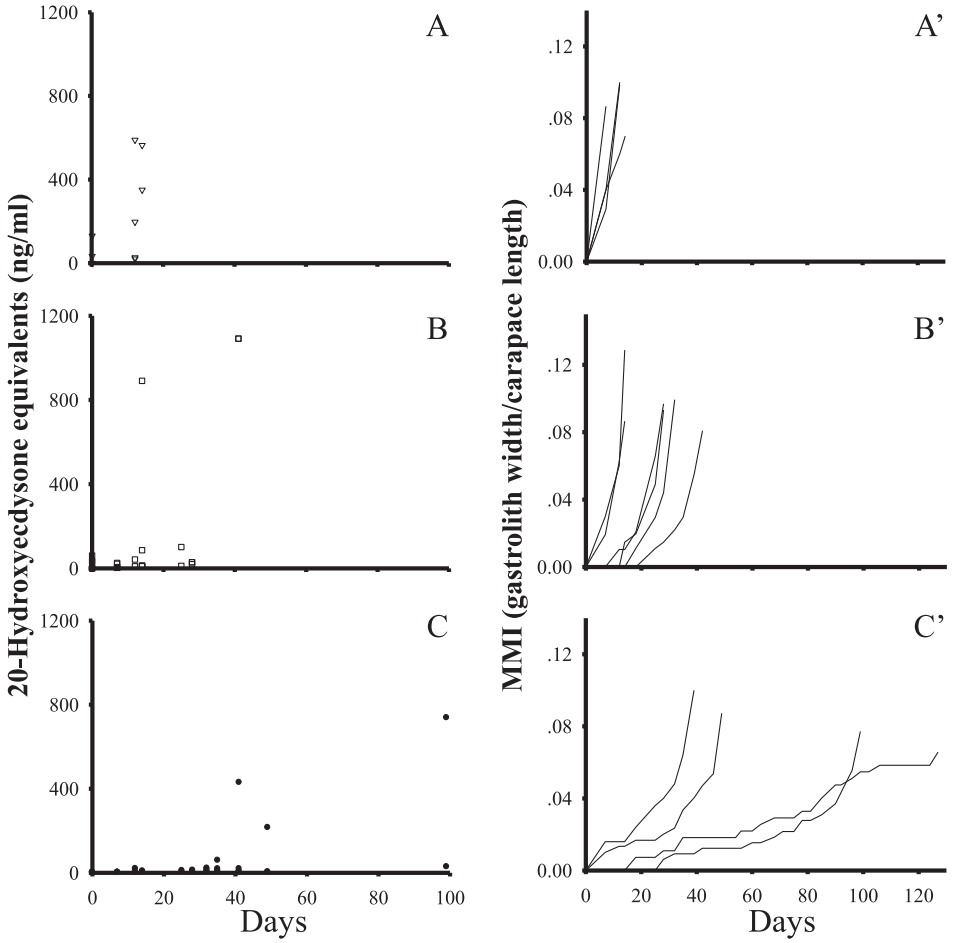

Fig. 6. *Cq-MIH* dsRNA *in vivo* injections affect molt related parameters. Ecdysteroid levels were measured using an ELISA. Hemolymph samples were collected from eyestalk-ablated (A), ds*Cq-MIH*-injected (B) and control (exogenous dsRNA-injected) (C) male crayfish. Samples were collected routinely and each individual was removed from the experiment once molted. Additionally, in the same specimens, molt progression was assessed by monitoring MMI (A′–C′). Each animal is represented individually, either by a unique symbol (A–C) or by a line (A′–C′).

species [6,39,42,47]. Furthermore, it was shown in the Chinese mitten crab *Eriocheir sinensis* that *in vitro* incubation of Y-organs with a recombinant MIH protein inhibited both ecdyseroidogenesis and ecdysteroid secretion [68]. To expand our knowledge and improve our understanding of molt regulation in males of a Southern Hemisphere crayfish, *C. quadricarinatus*, we investigated the role of Cq-MIH in the regulation of the molt cycle by the elimination of MIH using a molecular approach, i.e., RNAi. This approach, unlike eyestalk ablation, that also removes additional possible crucial factors [9,52] allows specific targeting of the gene of interest *in vivo* [13,41,44]. On the other hand, *in vitro* experiments do not reflect the precise, complex, and specific action of the tested factor [68]. Thus, *in vivo* gene silencing, such as described here, addresses both disadvantages and has a better potential to provide accurate biological data.

The regulatory mechanism of molting is a complex process that includes a network of signals involving the tissues comprising the endocrine molt axis (eyestalks, hemolymph and Y-organs). Characteristic events of the molt cycle include the synthesis and storage (in the eyestalk), transport (through the hemolymph) of molt-inhibiting hormone, and eventually the suppression of ecdysone synthesis and secretion (from the Y-organs) [6,19]. The full-length cDNA of *Cq-MIH* was isolated, sequenced and used to characterize the MIH gene and its expression. In addition, this sequence was used to generate MIH dsRNA to study the effect of this transcript on molt cycle duration and progress (the latter determined by gastrolith growth expressed as MMI values and ecdysteroid concentrations) *in vivo*.

Multiple sequence alignment of the deduced amino acid sequence of Cq-MIH with selected crustacean hyperglycemic hormone neuropeptides (CHH-family), confirmed that Cq-MIH is a member of the CHH-family. The predicted mature peptide is most homologous to MIHs of other species, specifically *O. limosus* [2] and *P. clarkii* [34] with 51.9 and 50.5% similarity, respectively. Like other MIHs, the deduced Cq-MIH contains multiple cysteine residues at positions 6, 23, 26, 39, 43 and 52, which are highly conserved in all CHH-family peptides [3,15,17,21,22,37,62] and are predicted to form disulfide bonds. A 3D model of Cq-MIH showing its high degree of similarity to the NMR structure of MIH of *M. japonicus* [21] affirmed the pattern of the disulfide bonds. Katayama et al. [21] further suggested that conformation of the 3D structure of *M. japonicus,* in addition to the three disulfide bonds, is stabilized by a number of hydrophobic interactions within the cluster of nine hydrophobic residues. Our findings, in the Cq-MIH sequence, are in accordance with this suggestion. In addition, it was suggested that in the *M. japonicus* MIH, the N-terminal α-helix and the C-terminal tail, which were sterically adjacent to each other, comprise a functionally important site for the molt-inhibiting activity since this region is absent from the CHH of *M. japonicus* [21]. This presumed functionally important 3D region also exists in the predicted 3D structural model of Cq-MIH shown in the present study.

RT-PCR analysis showed the specific amplification of a single band in the eyestalk of *C. quadricarinatus*, but not in the other tissues examined. Similarly, the tissue-specific expression pattern of *MIH* mRNA was identified in *Callinectes sapidus* [61] and *Cancer magister* [57]. In contrast, Chan et al. [3] observed *MIH* mRNA expression not only in the eyestalk, but also in the brain of the crab *Charybdis feriatus*. The expression of MIH in "non-traditional" sites raises the intriguing possibility that it may have alternate modes of action that influence molting and/or other physiological processes.

Cq-MIH dsRNA treatment caused a significant reduction in molt cycle duration, probably due to a precocious ecdysteroid synthesis triggered by a reduction in the MIH titer in the hemolymph. The observed reduction in molt duration after *Cq-MIH* dsRNA injection resembles the results of ecdysone injection reported earlier in our laboratory for the adult *C. quadricarinatus* [44] and also in this study, in which increasing levels of circulating ecdysteroids induces gastroliths growth (represented by MMI) until the animal molts. Eyestalk ablation had a greater effect probably due to the complete elimination of the MIH source (both the X-organ and the sinus gland that synthesize and store MIH, respectively, are removed). This result suggests that the *MIH* gene silencing only partially depletes MIH activity as its mRNA, and in turn the newly synthesized MIH is being affected. It is thus possible that the previously-produced MIH, which may remain in the SG, is functional and when secreted is responsible for the different response compared to the eyestalk ablation treatment, although MIH inhibitory effect over ecdysteroidogenesis in the Y-organs was shown to last only for several days *in vitro* [6]. Moreover, molt inhibition may also be induced by other CHH-family members, which compensate for the depleted MIH during the silencing intervention, but are eliminated once the eyestalk has been completely removed [9,67]. This study uses a loss of function approach (RNAi) to support previous knowledge in crustaceans [48], that MIH activity negatively affects molt by reducing the levels of circulating ecdysteroids.

The quantification of exact molt intervals and periodicity are of great importance in basic studies on crustacean growth. Moreover, such understandings can advance our ability to manipulate the molt cycle and promote valuable technologies that can be utilized. One such potential application includes biomineralization using crustacean models in which molt cycle duration and therefore gastrolith growth is manipulated [43]. In addition, induction of molting through specific action on MIH bioactivity could be applied in the food industry since post-molt animals could be consumed as soft-shell crayfish [20]. This high-valued product justifies specifically designed systems to maximize molting frequency [11,31]. Thus, the ability to specifically induce molt using MIH as a target could serve as a platform the aquaculture industry needs. On this note, as most commercialized species demonstrate cannibalism, such knowledge could also be harnessed for molt synchronization, thus increasing produce while minimizing cannibalism. The major drawback of such an idea lies in the high-price and lack of appropriate delivery for specific gene silencing-agents. With regard to the aquaculture industry this technology is in its infancy [58,59].

This study describes the identification and sequencing of *Cq-MIH* cDNA and its involvement in molt regulation in a Southern Hemisphere crayfish. Further studies are needed to identify the gene's translated protein product and its receptor, which is probably involved in the very early regulation of the ecdysteroidogenesis cascade. In general, the findings of the present study join ongoing advances elucidating regulation of molt and reproduction processes in crustaceans throughout the globe and specifically in southern species such as *C. quadricarinatus* at the single and multigenic levels [32,43,44,66].

Acknowledgments

We would like to thank Dr. Ofer Ovadia for his guidance in statistical analysis, Mr. Oren Levy and Mr. Ofek Landvirt for crayfish maintenance, Dr. Isam Khalaila, Dr. Shmuel Parnes, Dr. Assaf Shechter, Mr. Ohad Gafni and Mr. Shauli Raviv for their advice and help in completing this research. This research was supported by a Research Grant Award No. MB 8714 08 from the UMBI-BARD Program - the University of Maryland Biotechnology Institute and the United States-Israel Binational Agricultural Research and Development Fund and in part by a Planning & Budget Commission of the Council for Higher Education (VATAT) fellowship for post-doctoral students to R.R.P.

References

[1] M.B. Aguilar, D. Soyez, R. Falchetto, D. Arnott, J. Shabanowitz, D.F. Hunt, et al., Amino acid sequence of the minor isomorph of the crustacean hyperglycemic hormone (CHH-II) of the Mexican crayfish *Procambarus bouvieri* (Ortmann): presence of a D-amino acid, Peptides 16 (1995) 1375–1383.

[2] P. Bulau, A. Okuno, E. Thome, T. Schmitz, J. Peter-Katalinic, R. Keller, Characterization of a molt-inhibiting hormone (MIH) of the crayfish, *Orconectes limosus*, by cDNA cloning and mass spectrometric analysis, Peptides 26 (2005) 2129–2136.

[3] S.M. Chan, X.G. Chen, P.L. Gu, PCR cloning and expression of the molt-inhibiting hormone gene for the crab (*Charybdis feriatus*), Gene 224 (1998) 23–33.

[4] S.M. Chan, P.L. Gu, K.H. Chu, S.S. Tobe, Crustacean neuropeptide genes of the CHH/MIH/GIH family: implications from molecular studies, Gen. Comp. Endocrinol. 134 (2003) 214–219.

[5] E.S. Chang, Comparative endocrinology of molting and reproduction: insects and crustaceans, Annu. Rev. Entomol. 38 (1993) 161–180.

[6] E.S. Chang, D.L. Mykles, Regulation of crustacean molting: a review and our perspectives, Gen. Comp. Endocrinol. 172 (2011) 323–330.

[7] E.S. Chang, G.D. Prestwich, M.J. Bruce, Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster, *Homarus americanus*, Biochem. Biophys. Res. Commun. 171 (1990) 818–826.

[8] H.Y. Chen, R.D. Watson, J.C. Chen, H.F. Liu, C.Y. Lee, Molecular characterization and gene expression pattern of two putative molt-inhibiting hormones from *Litopenaeus vannamei*, Gen. Comp. Endocrinol. 151 (2007) 72–81.

[9] J.S. Chung, Hemolymph ecdysteroids during the last three molt cycles of the blue crab, *Callinectes sapidus*: quantitative and qualitative analyses and regulation, Arch. Insect Biochem. Physiol. 73 (2010) 1–13.

[10] J.S. Chung, M.C. Wilkinson, S.G. Webster, Determination of the amino acid sequence of the moult-inhibiting hormone from the edible crab, *Cancer pagurus*, Neuropeptides 30 (1996) 95–101.

[11] D.D. Culley, L. Duobinisgray, 24-Hour molting pattern of the red swamp crawfish (*Procambarus clarkii*), Aquaculture 64 (1987) 343–346.

[12] D.P.V. De Kleijn, K.P.C. Janssen, S.L. Waddy, R. Hegeman, W.Y. Lai, G.J.M. Martens, et al., 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 (1998) 291–298.

[13] L. Glazer, A. Shechter, M. Tom, Y. Yudkovski, S. Weil, E.D. Aflalo, et al., A protein involved in the assembly of an extracellular calcium storage matrix, J. Biol. Chem. 285 (2010) 12831–12839.

[14] M.E. Gramitto, Molt pattern identification through gastrolith examination on Nephrops norvegicus (L.) in the Mediterranean Sea, Sci. Mar. 62 (1998) 17–23.

[15] P.L. Gu, S.M. Chan, Cloning of a cDNA encoding a putative molt-inhibiting hormone from the eyestalk of the sand shrimp *Metapenaeus ensis*, Mol. Mar. Biol. Biotechnol. 7 (1998) 214–220.

[16] P.L. Gu, S.S. Tobe, B.K. Chow, K.H. Chu, J.G. He, S.M. Chan, Characterization of an additional molt inhibiting hormone-like neuropeptide from the shrimp *Metapenaeus ensis*, Peptides 23 (2002) 1875–1883.

[17] P.L. Gu, K.L. Yu, S.M. Chan, Molecular characterization of an additional shrimp hyperglycemic hormone: cDNA cloning, gene organization, expression and biological assay of recombinant proteins, FEBS Lett. 472 (2000) 122–128.

[18] N. Guex, M.C. Peitsch, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling, Electrophoresis 18 (1997) 2714–2723.

[19] P.M. Hopkins, The eyes have it: a brief history of crustacean neuroendocrinology, Gen. Comp. Endocrinol. 175 (2012) 357–366.

[20] C. Jones, The biology and aquaculture potential of the tropical freshwater crayfish *Cherax quadricarinatus*. Department of Primary Industries Queensland, Queensland Government, Information Series Q 190028 (1997).

[21] H. Katayama, K. Nagata, T. Ohira, F. Yumoto, M. Tanokura, H. Nagasawa, The solution structure of molt-inhibiting hormone from the Kuruma prawn *Marsupenaeus japonicus*, J. Biol. Chem. 278 (2003) 9620–9623.

[22] H. Katayama, T. Ohira, S. Nagata, H. Nagasawa, Structure-activity relationship of crustacean molt-inhibiting hormone from the kuruma prawn *Marsupenaeus japonicus*, Biochemistry 43 (2004) 9629–9635.

[23] T.G. Kingan, A competitive enzyme-linked immunosorbent assay: applications in the assay of peptides, steroids, and cyclic nucleotides, Anal. Biochem. 183 (1989) 283–289.

[24] J.M. Klein, S. Mangerich, D.P. de Kleijn, R. Keller, W.M. Weidemann, Molecular cloning of crustacean putative molt-inhibiting hormone (MIH) precursor, FEBS Lett. 334 (1993) 139–142.

[25] A. Lago-Leston, E. Ponce, M.E. Munoz, Cloning and expression of hyperglycemic (CHH) and molt-inhibiting (MIH) hormones mRNAs from the eyestalk of shrimps of *Litopenaeus vannamei* grown in different temperature and salinity conditions, Aquaculture 270 (2007) 343–357.

[26] C. Lambert, N. Leonard, X. De Bolle, E. Depiereux, ESyPred3D: Prediction of proteins 3D structures, Bioinformatics 18 (2002) 1250–1256.

[27] K.J. Lee, T.S. Elton, A.K. Bej, S.A. Watts, R.D. Watson, Molecular cloning of a cDNA encoding putative molt-inhibiting hormone from the blue crab *Callinectes sapidus*, Biochem. Biophys. Res. Commun. 209 (1995) 1126–1131.

[28] K.J. Lee, H.W. Kim, A.M. Gomez, E.S. Chang, J.A. Covi, D.L. Mykles, Molt-inhibiting hormone from the tropical land crab, *Gecarcinus lateralis*: cloning, tissue expression, and expression of biologically active recombinant peptide in yeast, Gen. Comp. Endocrinol. 150 (2007) 505–513.

[29] W. Lu, G. Wainwright, L.A. Olohan, S.G. Webster, H.H. Rees, P.C. Turner, Characterization of cDNA encoding molt-inhibiting hormone of the crab, *Cancer pagurus*; expression of MIH in non-X-organ tissues, Gene 278 (2001) 149–159.

[30] J.M. Lugo, Y. Morera, T. Rodriguez, A. Huberman, L. Ramos, M.P. Estrada, Molecular cloning and characterization of the crustacean hyperglycemic hormone cDNA from *Litopenaeus schmitti*. Functional analysis by double-stranded RNA interference technique, FEBS J. 273 (2006) 5669–5677.

[31] R.F. Malone, D.G. Burden, Design of recirculating soft crawfish shedding systems, Louisiana Sea Grant College Program, Center for Wetland Resources, Louisiana State University1988.

[32] R. Manor, S. Weil, S. Oren, L. Glazer, E.D. Aflalo, T. Ventura, et al., Insulin and gender: an insulin-like gene expressed exclusively in the androgenic gland of the male crayfish *Cherax quadricarinatus*, Gen. Comp. Endocrinol. 150 (2007) 326–336.

[33] H.G. Marco, S. Stoeva, W. Voelter, G. Gade, Characterization and sequence elucidation of a novel peptide with molt-inhibiting activity from the South African spiny lobster *Jasus lalandii*, Peptides 21 (2000) 1313–1321.

[34] H. Nagasawa, W.J. Yang, H. Shimizu, K. Aida, H. Tsutsumi, A. Terauchi, et al., Isolation and amino acid sequence of a molt-inhibiting hormone from the American crayfish *Procambarus clarkii*, Biosci. Biotechnol. Biochem. 60 (1996) 554–556.

[35] T. Nakatsuji, C.Y. Lee, R.D. Watson, Crustacean molt-inhibiting hormone: structure, function, and cellular mode of action, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 152 (2009) 139–148.

[36] T. Nakatsuji, H. Sonobe, Regulation of ecdysteroid secretion from the Y-organ by molt-inhibiting hormone in the American crayfish *Procambarus clarkii*, Gen. Comp. Endocrinol. 135 (2004) 358–364.

[37] T. Ohira, H. Katayama, S. Tominaga, T. Takasuka, T. Nakatsuji, H. Sonobe, et al., Cloning and characterization of a molt-inhibiting hormone-like peptide from the prawn *Marsupenaeus japonicus*, Peptides 26 (2005) 259–268.

[38] T. Ohira, T. Watanabe, H. Nagasawa, K. Aida, Molecular cloning of a molt-inhibiting hormone cDNA from the kuruma prawn *Penaeus japonicus*, Zoolog. Sci. 14 (1997) 785–789.

[39] T. Okumura, T. Ohira, H. Katayama, H. Nagasawa, *In vivo* effects of a recombinant molt-inhibiting hormone on molt interval and hemolymph ecdysteroid level in the kuruma prawn *Marsupenaeus japonicus*, Zoolog. Sci. 22 (2005) 317–320.

[40] P.R. Reddy, R.S. Reddy, Isolation of peptide hormones with pleiotropic activities in the freshwater crab *Oziotelphusa senex senex*, Aquaculture 259 (2006) 424–431.

[41] O. Rosen, R. Manor, S. Weil, O. Gafni, A. Linial, E.D. Aflalo, et al., A sexual shift induced by silencing of a single insulin-like gene in crayfish: ovarian upregulation and testicular degeneration, PLoS ONE 5 (2010) e15281.

[42] G. Rotllant, P. Takac, L. Liu, G.L. Scott, H. Laufer, Role of ecdysteroids and methyl farnesoate in morphogenesis and terminal moult in polymorphic males of the spider crab *Libinia emarginata*, Aquaculture 190 (2000) 103–118.

[43] A. Shechter, A. Berman, A. Singer, A. Freiman, M. Grinstein, J. Erez, et al., Reciprocal changes in calcification of the gastrolith and cuticle during the molt cycle of the red claw crayfish *Cherax quadricarinatus*, Biol. Bull. 214 (2008) 122–134.

[44] A. Shechter, L. Glazer, S. Cheled, E. Mor, S. Weil, A. Berman, et al., A gastrolith protein serving a dual role in the formation of an amorphous mineral containing extracellular matrix, Proc. Natl. Acad. Sci. USA 105 (2008) 7129–7134.

[45] A. Shechter, M. Tom, Y. Yudkovski, S. Weil, S.A. Chang, E.S. Chang, et al., Search for hepatopancreatic ecdysteroid-responsive genes during the crayfish molt cycle: from a single gene to multigenicity, J. Exp. Biol. 210 (2007) 3525–3537.

[46] J. Shi, T.L. Blundell, K. Mizuguchi, FUGUE: sequence-structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties, J. Mol. Biol. 310 (2001) 243–257.

[47] D.M. Skinner, Molting and regeneration, in: D.E. Bliss, L.H. Mantel (Eds.), The Biology of Crustacea, Academic Press, New York, 1985, pp. 43–146.

[48] M.J. Snyder, E.S. Chang, Ecdysteroids in relation to the molt cycle of the American lobster, *Homarus americanus*. I. Hemolymph titers and metabolites, Gen. Comp. Endocrinol. 81 (1991) 133–145.

[49] E. Spaziani, W.L. Wang, Biosynthesis of ecdysteroid hormones by crustacean Y-organs: conversion of cholesterol to 7-dehydrocholesterol is suppressed by a steroid 5 alpha-reductase inhibitor, Mol. Cell. Endocrinol. 95 (1993) 111–114.

[50] P.S. Sun, Molecular cloning and sequence analysis of a cDNA encoding a molt-inhibiting hormone-like neuropeptide from the white shrimp *Penaeus vannamei*, Mol. Mar. Biol. Biotechnol. 3 (1994) 1–6.

[51] P.S. Sun, Expression of the molt-inhibiting hormone-like gene in the eyestalk and brain of the white shrimp *Penaeus vannamei*, Mol. Mar. Biol. Biotechnol. 4 (1995) 262–268.

[52] S.L. Tamone, M.M. Adams, J.M. Dutton, Effect of eyestalk-ablation on circulating ecdysteroids in hemolymph of snow crabs, *Chionoecetes opilio*: physiological evidence for a terminal molt, Integr. Comp. Biol. 45 (2005) 166–171.

[53] J.D. Thompson, T.J. Gibson, P. Frédéric, J. François, D.G. Higgins, The CLUSTAL-X windows interface. flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res. 25 (1997) 4876–4882.

[54] S.H. Tiu, S.M. Chan, 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 (2007) 4385–4395.
[55] S. Treerattrakool, S. Panyim, S.M. Chan, B. Withyachumnarnkul, A. Udomkit, Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference, FEBS J. 275 (2008) 970–980.
[56] N. Tsutsui, T. Ohira, I. Kawazoe, A. Takahashi, M.N. Wilder, Purification of sinus gland peptides having vitellogenesis-inhibiting activity from the whiteleg shrimp *Litopenaeus vannamei*, Mar. Biotechnol. 9 (2007) 360–369.
[57] H.R. Umphrey, K.J. Lee, R.D. Watson, E. Spaziani, Molecular cloning of a cDNA encoding molt-inhibiting hormone of the crab *Cancer magister*, Mol. Cell. Endocrinol. 136 (1998) 145–149.
[58] T. Ventura, R. Manor, E.D. Aflalo, S. Weil, O. Rosen, A. Sagi, Timing sexual differentiation: full functional sex reversal achieved through silencing of a single insulin-like gene in the prawn *Macrobrachium rosenbergii*, Biol. Reprod. 86 (2012) 90.
[59] T. Ventura, A. Sagi, The insulin-like androgenic gland hormone in crustaceans From a single gene silencing to a wide array of sexual manipulation-based biotechnologies, Biotechnol. Adv. (2012), http://dx.doi.org/10.1016/j.biotechadv.2012.04.008.
[60] Z.Z. Wang, C.Z. Jiao, X.J. Zhang, J.H. Xiang, Molecular cloning and sequence analysis of full length cDNA encoding molt-inhibiting hormone from *Fennropenaeus chinensis*, Yi Chuan Xue Bao 30 (2003) 128–134.
[61] R.D. Watson, K.J. Lee, K.J. Borders, H. Dircksen, K.Y. Lilly, Molt-inhibiting hormone immunoreactive neurons in the eyestalk neuroendocrine system of the blue crab *Callinectes sapidus*, Arthropod. Struct. Dev. 30 (2001) 69–76.
[62] S.G. Webster, Amino acid sequence of putative moult-inhibiting from the crab Carcinus maenas, Proc. R. Soc. Lond. B Biol. Sci. 244 (1991) 247–252.
[63] S.G. Webster, Neuropeptides inhibiting growth and reproduction in crustaceans, in: G.M. Coast, S.G. Webster (Eds.), Recent Advances in Arthropod Endocrinology, Cambridge University Press, Cambridge, 1998, pp. 33–52.
[64] W.J. Yang, K. Aida, A. Terauchi, H. Sonobe, H. Nagasawa, Amino acid sequence of a peptide with molt-inhibiting activity from the kuruma prawn *Penaeus japonicus*, Peptides 17 (1996) 197–202.
[65] A. Yasuda, Y. Yasuda, T. Fujita, Y. Naya, Characterization of crustacean hyperglycemic hormone from the crayfish (*Procambarus clarkii*): multiplicity of molecular forms by stereoinversion and diverse functions, Gen. Comp. Endocrinol. 95 (1994) 387–398.
[66] Y. Yudkovski, A. Shechter, V. Chalifa-Caspi, M. Auslander, R. Ophir, C. Dauphin-Villemant, et al., Hepatopancreatic multi-transcript expression patterns in the crayfish *Cherax quadricarinatus* during the moult cycle, Insect Mol. Biol. 16 (2007) 661–674.
[67] T.P. Zarubin, E.S. Chang, D.L. Mykles, Expression of recombinant eyestalk crustacean hyperglycemic hormone from the tropical land crab, *Gecarcinus lateralis*, that inhibits Y-organ ecdysteroidogenesis *in vitro*, Mol. Biol. Rep. 36 (2009) 1231–1237.
[68] Y. Zhang, Y. Sun, Y. Liu, X. Geng, X. Wang, Y. Wang, et al., Molt-inhibiting hormone from Chinese mitten crab (*Eriocheir sinensis*): cloning, tissue expression and effects of recombinant peptide on ecdysteroid secretion of YOs, Gen. Comp. Endocrinol. 173 (2011) 467–474.
[69] N. Zmora, A. Sagi, Y. Zohar, J.S. Chung, Molt-inhibiting hormone stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *Callinectes sapidus* 2: novel specific binding sites in hepatopancreas and cAMP as a second messenger, Saline Systems 5 (2009) 6.