

Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab, *Carcinus maenas*

From target to transcript

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The currently accepted model of moult control in crustaceans relies entirely on the hypothesis that moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) repress ecdysteroid synthesis of the target tissue (Y-organ) only during intermoult, and that changes in synthesis and/or release of these neurohormones are central to moult control. To further refine this model, we investigated the biological activities of these neuropeptides in the crab *Carcinus maenas*, at the target tissue, receptor and cellular level by bioassay (inhibition of ecdysteroid synthesis), radioligand (receptor) binding assays, and second messenger (cGMP) assays, at defined stages of the moult cycle. To investigate possible moult cycle-related changes in

neuropeptide biosynthesis, steady-state transcript levels of both neuropeptide mRNAs were measured by quantitative RT-PCR, and stored neuropeptide levels in the sinus gland were quantified during intermoult and premoult. The results show that the most important level of moult control lies within the signalling machinery of the target tissue, that expression and biosynthesis of both neuropeptides is constant during the moult cycle, and are not central to the currently accepted model of moult control.

Keywords: *Carcinus maenas*; moult cycle; neuropeptides; ecdysteroids; receptors.

It is now well established that a variety of structurally related neuropeptides, generically called members of the crustacean hyperglycaemic hormone (CHH) peptide family, control a diverse variety of physiological processes in crustaceans, such as moulting, carbohydrate metabolism, reproduction and hydromineral balance. Whilst the primary structures of over 50 of these peptides have been described, using a combination of microsequencing and cDNA cloning approaches [1,2], we still know remarkably little regarding the physiologically relevant roles of these neurohormones. In many cases, several processes appear to be regulated by single hormones, as might be expected, given the centrally important roles of these hormones in regulatory mechanisms, particularly those related to moulting and reproduction. This feature is vividly illustrated if the actions of the CHH neuropeptides on repression of ecdysteroid synthesis by the Y-organ (YO) are considered.

The most widely accepted paradigm of moult control in crustaceans concerns the inhibitory action of moult-inhibiting hormone on ecdysteroid synthesis. For crabs, the

moult-inhibiting hormone (MIH) is structurally distinct from CHHs [3], yet crab CHHs also repress ecdysteroid synthesis, albeit with a lower potency [4], which may suggest that CHH has a physiologically relevant role in moulting, at least for crabs. In lobsters, highly distinctive MIH type molecules do not seem to occur, but rather CHH-like molecules, which also have hyperglycaemic effects *in vivo* are functional MIHs. The variety of CHH-like molecules involved in both of these processes is exemplified in penaeids where several distinctive, yet very similar CHH-like molecules seem to be involved in carbohydrate mobilization, and in some instances, inhibition of ecdysteroid synthesis [5]. In *Penaeus japonicus*, distinctive MIH-like peptides, which have been implicated in repression of ecdysteroid synthesis, have also been identified [5,6]. Further complexity is added if the accepted model of moult control is revisited. It has been tacitly accepted that increases in ecdysteroid levels sufficient to drive proecdysis, and ultimately moulting, result from the reduced secretion/synthesis of MIH by the eyestalk neurosecretory tissues at the end of intermoult. However, this simplistic hypothesis remains untested, and it seems likely that both changes in target organ sensitivity and synthesis/release patterns of neuropeptides may be relevant. Evidence that MIH synthesis may be dramatically reduced during late premoult has been suggested from qualitative measurement of MIH transcript abundance in premoult *Callinectes sapidus* eyestalks [7], and a reduction in sinus gland MIH content during late premoult has been observed in *Procambarus clarkii* [8]. However, an alternative explanation might be that the YO becomes refractive to MIH during premoult, as has been suggested for *Penaeus*

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Abbreviations: AK, arginine kinase; CHH, crustacean hyperglycaemic hormone; MIH, moult-inhibiting hormone; MT, medulla terminalis; SG, sinus gland; XO, X-organ; YO, Y-organ.

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vannamei [9]. To address questions regarding the roles of MIH and CHH in moult control, we have used a broad approach. As either (or both) of the above-mentioned processes may be relevant to moult control, we first investigated the biological activity of MIH and CHH during precisely timed stages of the moult cycle of *Carcinus* to determine changes in: (a) potency of these peptides in repressing ecdysteroid synthesis; (b) receptor density and affinity; and (c) signal transduction (cGMP). Second, we measured quantitative changes in both peptide and transcript abundance in eyestalk neurosecretory tissues during intermoult and premoult.

Materials and methods

Animals and peptides

Carcinus maenas were collected using baited traps in the Menai Strait, UK, and maintained in a recirculating seawater system under ambient conditions. MIH and CHH were purified from sinus gland extracts by HPLC and quantified by amino acid analysis, as described previously [3]. Moult stages of experimental animals (carapace width 45–57 mm) were determined as previously described [10]. For these experiments female crabs were used, as these were (in contrast to males) available in large numbers over much of the moulting season (May to November). Crabs undergoing vitellogenesis were not used in experiments. All animals were anaesthetized on ice prior to dissection.

Bioassays

Inhibition of ecdysteroidogenesis by YO *in vitro* was performed as described previously [4]. Between five and 10 YO pairs were used for each experiment. YO were cultured for 24 h at 12 °C in 24-well culture plates (Corning) containing 400 µL of MIH (5 nM) or CHH (50 nM) in crustacean saline, or saline (controls). Normally, RIA measured total ecdysteroid content of the culture medium. However, to measure inhibition of ecdysone and 25-deoxyecdysone biosynthesis (these ecdysteroids are the major ones secreted by *Carcinus* YO *in vitro* [11]), pooled samples were separated by HPLC. Conditions were: Bakerbond C₁₈ column, 250 × 4.6 mm, solvent A: water; solvent B: methanol; 40–80% B over 30 min, 1 mL·min⁻¹. Under these conditions ecdysone eluted at ≈ 14–15 min, 25-deoxyecdysone, 25–26 min. Eluates corresponding to the retention times of these ecdysteroids (± 2 min) were collected, dried and quantified by RIA.

For measurement of cGMP production, YO pairs were incubated for 30 min, in the same conditions as above. To minimize phosphodiesterase(s) activity, incubation media were supplemented with 3-isobutylmethylxanthine (final concentration 500 µM). Incubations were terminated by freezing the tissues in liquid N₂ and stored at –80 °C. YO extracts were prepared by rapid ultrasonic disruption in ice-cold 200 µL 50 mM acetate buffer (pH 4.8) containing 20 mM EDTA and 1 mM 3-isobutylmethylxanthine, centrifuged and acetylated prior to RIA [12]. [¹²⁵I]cGMP (specific activity 27–37 TBq·mmol⁻¹) was prepared by Chloramine-T iodination of 0.3 nmol 2'-*O*-succinylguanosine 3',5'-cyclic

monophosphate tyrosyl methyl ester (Sigma) with 18.5 MBq [¹²⁵I]NaI (Amersham) [13]. Labelled product was purified on Sep-Pak C₁₈ (Waters) cartridges and eluted with 40% isopropanol. For RIA anti-cGMP serum (final dilution 1 : 24 000) was used. Separation of bound from free ligand was performed using solid-phase donkey anti-rabbit IgG (Immunodiagnostic Services, Tyne and Wear, UK).

Receptor binding assays

Batches of 100 YO were dissected from moult staged crabs (carapace width 45–57 mm) and immediately frozen in liquid N₂ and stored at –80 °C. Membrane rich fractions were prepared as described previously [14]. Receptor binding assays for MIH and CHH using ¹²⁵I-labelled ligands were performed using 'displacement' or 'saturation' type protocols, but modified so that the concentration of BSA in the binding buffer was increased to 1%; this dramatically reduced non-specific binding. Membrane quantities were reduced to 20–25% of those reported previously. Data reduction and analysis was carried out using a radioligand binding analysis program (Elsevier-BIOSOFT). Experiments were generally triplicated, where quantities of tissues allowed this, and for each experiment, parallel positive control binding assays using YO membranes from intermoult (Stage C₄) animals were included as quality controls.

Quantification of peptide contents of sinus glands

Sinus gland (SG) pairs were carefully dissected from moult staged crabs (carapace width 54–57 mm), and immediately frozen on liquid nitrogen. SG pairs were extracted by ultrasonic disruption in ice-cold 2 M acetic acid, briefly centrifuged, and immediately injected into the HPLC (This process was essential to avoid oxidation of CHH). Chromatography was performed on a 250 × 4.6 mm Bakerbond WP C₁₈ column, solvent A: 0.11% trifluoroacetic acid; solvent B 60% acetonitrile containing 0.1% trifluoroacetic acid; 40–80% B over 40 min, 1 mL·min⁻¹, detection at 210 nm. Peptides were quantified by peak area with reference to standard MIH and CHH. For CHH, both CHH-I (N-terminally unblocked) and CHH-II (N-terminally blocked) peak areas were combined as they are indistinguishable with respect to biological activity [15].

Quantification of neuropeptide mRNA

RNA isolation. Eyestalk tissues [medulla terminalis (MT) which contained the X-organ (XO)] were carefully dissected in diethyl pyrocarbonate (DEPC)-treated saline, rapidly transferred to RNAlater (Ambion) (4 °C overnight) and then stored at –80 °C. Total RNA was extracted from single (MT) using TRIzol (Invitrogen). Pellets were resuspended in 20 µL DEPC-treated water. Genomic DNA contamination was removed by incubation in 2 U DNase I (37 °C, 1 h) followed by clean-up on DNA-free (Ambion). Total RNA (per MT) was quantified using Ribogreen (Molecular Probes). Fluorescence was measured using a microplate format, on a Perkin Elmer Victor 1420. Yeast tRNA (Molecular Probes) was used as standard.

Table 1. Nucleotide sequences of primers. LF, LR primers were used during preparation of run-off transcripts, whilst SF, SR primers were those used for Q-RT-PCR. Product sizes using primer pair are shown on the right. AK, arginine kinase; CHH, crustacean hyperglycaemic hormone; MIH, moult-inhibiting hormone.

Primer name	Sequence (5' → 3')	Product size (bp)
CHH-LF	GCCATGCTAGCAATCATCACCGTAG	
CHH-LR	GTTGAGATCTGTTGTTTACTTCTTC	423
MIH-LF	GAGTTATCAACGACGAGTGTC	
MIH-LR	GAGACGACAAGGCTCAGTCC	249
AK-LF	AAAGGTTTCCTCCACCCTGT	
AK-LR	ACTTCCTCGAGCTTGTCACG	450
CHH-SF	GACTTGGAGCACGTGTGT	
CHH-SR	TATTGGTCAAACCTCGTCCAT	143
MIH-SF	AAGACAGGAATGGCGAGT	
MIH-SR	AATCTCTCAGCTCTTCGGGAC	100
AK-SF	AAACGGTCACCCTCCTTGA	
AK-SR	ACTTCCTCGAGCTTGTCACG	132

Standard RNA preparation. Total RNA (0.1–1 µg) was reverse transcribed with AMV reverse transcriptase (Roche Molecular Biochemicals), and cDNA amplified using the following gene specific primers for CHH (accession no. X17596), MIH (accession no. X75995) and for the control gene arginine kinase (AK; accession no. AF167313). Primers used are shown on Table 1. PCR amplification conditions were as previously described [16]. Products were electrophoresed on 1.2% agarose gels with ethidium bromide (EtBr) visualization. PCR products were purified on Microcon-PCR (Amicon) devices. *In vitro* ligations were carried out with 25 ng DNA with T7 promoter adaptors (Lig'n Scribe, Ambion), amplified using forward (sense) gene specific primers and T7 adapter primers. Ligated DNA was again purified (Microcon) and precipitated in 0.5 M ammonium acetate in 3 volumes of EtOH. Transcriptions were performed on 100–200 ng quantities of ligated PCR products using a MEGAshortscript kit (Ambion). Following treatment in DNA-free, run-offs were briefly denatured (95 °C, 3 min), incubated with 4 U DNase I (37 °C, 2 h) and retreated with DNA-free. Aliquots of the run-offs were purified by denaturing PAGE (5%). Transcripts of correct size were excised and eluted overnight in elution buffer (Ambion), precipitated in ethanol, dried and redissolved in 1 × Tris/EDTA. RNA was quantified using Ribogreen, diluted, aliquoted and stored in silanized PCR tubes at concentrations of 10¹¹ copies·µL⁻¹ at –80 °C. Under these conditions, the samples were stable for at least 6 months.

Neuropeptide mRNA quantification. This was performed by 'real-time' RT-PCR using a Roche Light Cycler and RNA Master kits (Roche Diagnostics), with SYBR green detection. As this method suffers inherently from potential primer–dimer amplification, primers were carefully designed to give short (≈ 100–140 bp) products, which gave no detectable primer–dimer formation within 35 amplification cycles (as evidenced by melt-curve analysis). Additionally, for MIH and CHH, primers were designed to span intron II, thus potential gDNA contamination could be easily identified by melt-curve analysis. Primer sequences used are

shown on Table 1. For the one-step reverse transcription and amplification, 10 µL volumes were used in the capillaries, adjusting reagent volumes accordingly. Mg²⁺ concentration was 5 mM, primer concentration 500 nM. Standard curves were constructed from 10⁸ to 10⁴ copies·µL⁻¹, run in duplicate. MT samples were 0.05 MT equivalents µL⁻¹. RT-PCR conditions were: reverse transcription 55 °C, 10 min, initial denaturation 95 °C, 30 s, 20 °C·s⁻¹; annealing 55 °C, 10 s, 20 °C·s⁻¹; extension 72 °C, 13 s, 2 °C·s⁻¹, denaturation 95 °C, 0 s, 20 °C·s⁻¹, 40 cycles. Melt curve data acquisition was from 65 to 95 °C, 0.1 °C·s⁻¹.

Results

Bioassays

For intermoult (stage C₄) YO, inhibition of total ecdysteroid synthesis by 5 nM MIH or 50 nM CHH was between 55 and 60% as previously reported [4]. During premoult, both neuropeptides became markedly less effective in this respect, and particularly for MIH, where its inhibitory activity was absent during late premoult and early postmoult (Fig. 1). However, during postmoult, the YO rapidly regained competence to respond to both neuropeptides; during early intermoult (C₁) the YO seemed to be particularly sensitive, as evidenced by somewhat greater (but not statistically significant) inhibition of ecdysteroid synthesis by both 5 and 50 nM concentrations of MIH and CHH. When pooled extracts of incubation media (from these experiments) were analyzed by HPLC-RIA, a reduction in the ability of MIH (5 nM) to repress synthesis of both ecdysone and 25-deoxyecdysone synthesis during premoult could be observed (Fig. 1B). A similar situation was also found using 50 nM CHH (data not shown), but as these experiments clearly showed reduction in biosynthesis of both ecdysteroids in the presence of either neuropeptide, reiterating the findings seen for total ecdysteroids, further studies were not attempted.

For YO taken from intermoult animals, 5 nM MIH elicited a notable 30- to 40-fold increase in cGMP levels during a 30-min incubation (Fig. 2); indeed a doubling of cGMP levels could be observed within 2 min of application of hormone (results not shown). For YO taken from premoult and early postmoult animals, this response was dramatically reduced – only a five- to 10-fold increase was observed, but in early intermoult animals (C_{1–3}) competence was restored to levels seen in intermoult animals. Whilst CHH (50 nM) exhibited a qualitatively similar response, this was attenuated.

Receptor binding studies

The results of receptor binding studies, using displacement (K_d) and saturation experiments (B_{max}) are shown on Table 2. With regard to displacement experiments (receptor affinity), very little variation was observed during the moult cycle for MIH: K_d s were around 4 × 10⁻¹⁰ M·mg⁻¹ protein (intermoult), increasing to around 10 × 10⁻¹⁰ M·mg⁻¹ protein during premoult. For CHH somewhat greater variability was observed, but again, taking this variability into account, results were not significantly different from means at any stage of the moult cycle. For saturation experiments

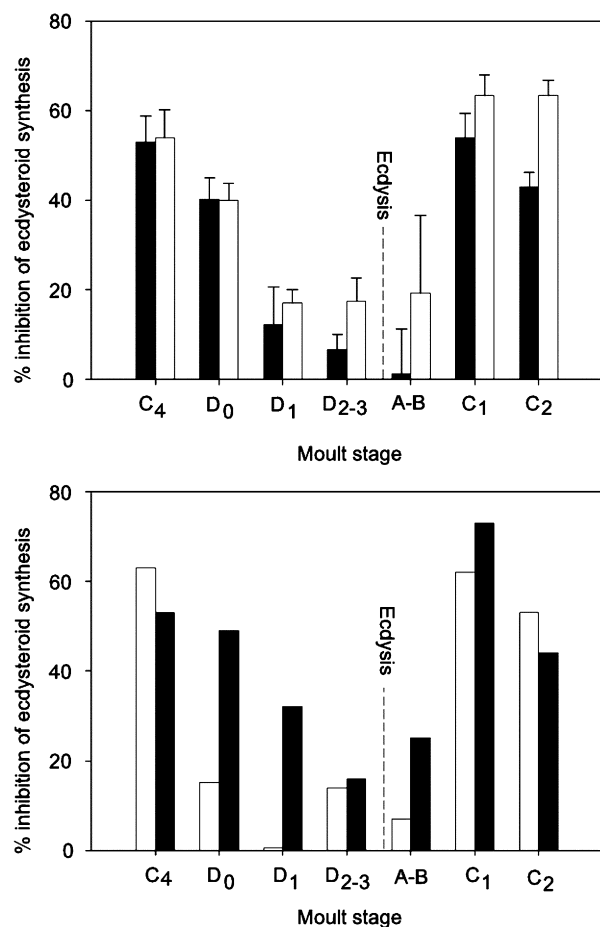


Fig. 1. Effects of MIH and CHH upon ecdysteroid synthesis by YO *in vitro*. Upper graph shows the moult stage dependent inhibition of ecdysteroid synthesis (mean \pm 1 SEM) following incubation in 5 nM MIH (solid bars) or 50 nM CHH (open bars) between five and 10 pairs of YO were used at each moult stage. Lower graph shows moult stage dependent inhibition by 5 nM MIH of identified ecdysteroids from corresponding pooled material after HPLC. Filled bars, 25-deoxyecdysone; open bars, ecdysone.

(receptor number), little variation was seen for YO membrane preparations during at all stages of the moult cycle, excepting those from YO membrane preparations saturated with MIH taken from postmoult crabs (stage B); where B_{\max} increased seven- to 15-fold from 1 to 2×10^{-10} M·mg⁻¹ protein in intermoult and premoult, to 15×10^{-10} M·mg⁻¹ protein in postmoult (stage B). This observation was not an artefact of individual experiments, as these were always run with intermoult membranes as positive controls. Furthermore, in experiments using CHH as the saturating ligand, a parallel increase in receptor number during postmoult was not observed. Thus, during postmoult, a significant recruitment of receptors for MIH was suggested.

Levels of MIH and CHH in the sinus gland during the moult cycle

As individual SG from single crabs always contained almost identical profiles and quantities of peptides (preliminary

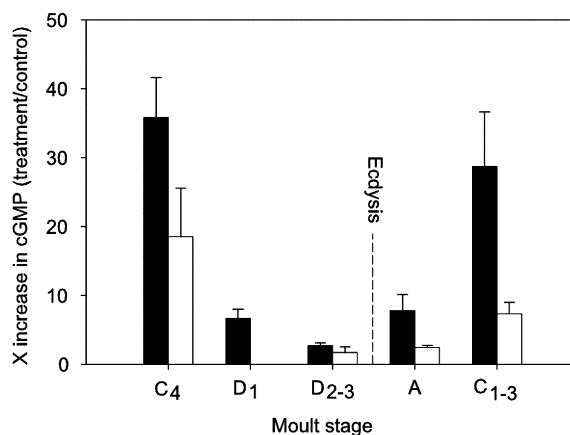


Fig. 2. Effects of MIH and CHH upon accumulation of cGMP in YO, following 30-min incubations with either 5 nM MIH (solid bars) or 50 nM CHH (open bars) (mean \pm 1 SEM). All incubations were supplemented with 500 μ M 3-isobutylmethylxanthine to minimize phosphodiesterase-mediated hydrolysis of accumulating cGMP. Five pairs of YO were used at each moult stage. Values are displayed as fold (x) increases in cGMP levels, as there are considerable variations in initial levels of cGMP between individuals, but in unstimulated YO, levels of cGMP were similar in each YO of individual crabs.

experiments using pairs of SG from 10 crabs in which CHH and MIH levels were individually measured and analyzed by ANOVA showed highly significant pairing in peptide contents between sinus glands from individuals, thus demonstrating that each SG from individuals contained identical quantities of peptides), pairs of SG were chromatographed and quantified by HPLC. Results are shown on Table 3 as pmol peptide per SG, for postmoult (A, B), intermoult (C₂, C₄) and late premoult (D₂) crabs. For MIH, levels were around 50 pmol per SG for much of the moult cycle, but decreased during premoult and early postmoult (36–38 pmol). By comparison, levels of CHH were about six- to 11-fold higher. SG from animals sampled in stage B contained more CHH (490 pmol per SG) than at any other time in the moult cycle. As quantities of CHH were quite variable at different stages, ratios of CHH/MIH were calculated for pairs of sinus glands. This analysis showed that the only consistent trend was that of moderate, statistically insignificant reduction in MIH content of the SG, relative to CHH, during late premoult.

Expression patterns of MIH and CHH in the X-organ during the moult cycle

Expression patterns of mRNA levels of both MIH and CHH were measured using 'real-time' RT-PCR, using homologous quantified transcripts to measure copy number. An example of some of the data (for CHH) obtained is shown on Fig. 3, and the results are summarized on Fig. 4. For all samples, melt-curve analyses were performed: gDNA contamination was not observed at the level of abundance of transcripts present – both MIH and CHH mRNAs are extremely abundant in the XO. For all analyses, primer-dimer formation (as evidenced in melt curve analyses) was never an issue below 35 cycles of amplification, thus SYBR green detection of amplicons was

Table 2. Receptor binding characteristics of YO membranes at various stages of the moult cycle. Means and standard errors are shown for preparations from independent batches of approximately 100 YO, where two or three preparations were made. For YO from early pre-moult crabs, using MIH as ligand, only one preparation was made. For all assays, duplicate tubes were measured. Membrane preparations from intermoult animals were run in parallel as quality controls, to ensure acceptable binding characteristics for each experiment. CHH, crustacean hyperglycaemic hormone; MIH, moult-inhibiting hormone.

Moult stage	MIH		CHH	
	K_d ($\times 10^{-10}$)	B_{max} ($\times 10^{-9}$)	K_d ($\times 10^{-10}$)	B_{max} ($\times 10^{-9}$)
C ₄	3.9 \pm 0.3	1.1 \pm 0.2	27.5 \pm 11.1	0.4 \pm 0.06
D ₀	8.1	0.9	8.3 \pm 4.3	0.3 \pm 0.09
D ₂₋₃	10.3 \pm 1.9	2.1 \pm 0.03	13.7 \pm 1.2	0.2 \pm 0.01
B	18.7 \pm 10.0	15.3 \pm 4.7	13.7 \pm 0.9	0.2 \pm 0.1

Table 3. Levels of MIH and CHH (measured by HPLC) in sinus glands of *Carcinus* during several stages of the moult cycle. Means \pm 1 SEM are shown. * Values that were significantly greater (Welch's *t*-test) than those of intermoult (C₄) crabs. CHH, crustacean hyperglycaemic hormone; MIH, moult-inhibiting hormone.

Moult stage	Number	pmol substance per SG		Ratio CHH/MIH
		MIH	CHH	
A	4	38 \pm 4	284 \pm 39	7.5 : 1
B	4	53 \pm 2	489 \pm 27*	9.2 : 1
C ₂	4	55 \pm 10	378 \pm 45	6.9 : 1
C ₄	4	48 \pm 4	270 \pm 24	5.6 : 1
D ₂	4	36 \pm 7	402 \pm 25*	11.1 : 1

eminently suitable to quantify these abundantly expressed transcripts. Typically MIH copy number was around $2\text{--}3 \times 10^7$ copies per XO equivalent, for CHH, levels were around 1.5×10^9 copies per XO equivalent, i.e. 50-fold greater than MIH. When non-normalized results were analyzed, no difference in expression of transcripts was seen, when intermoult or pre-moult animals were compared. However, when the results were normalized against a control gene (arginine kinase, AK), copy number appeared to increase during pre-moult. However, this observation was artefactual: transcript number of the control gene declines about twofold to threefold during pre-moult. Although we also used a second control gene (β -actin) in an attempt to overcome this inadequacy, this was useless for eyestalk neural tissues. However when normalized against total RNA, an acceptable correlation between MIH and CHH copy number ratios for intermoult and MT was obtained (Fig. 4), which was better than that for AK normalized data. Notwithstanding this, it was apparent that the relationship between MIH and CHH copy number was lost in pre-moult MT, where a wide scatter was evident.

Discussion

In this study we have attempted to further elucidate possible control mechanisms in moulting of a crab model,

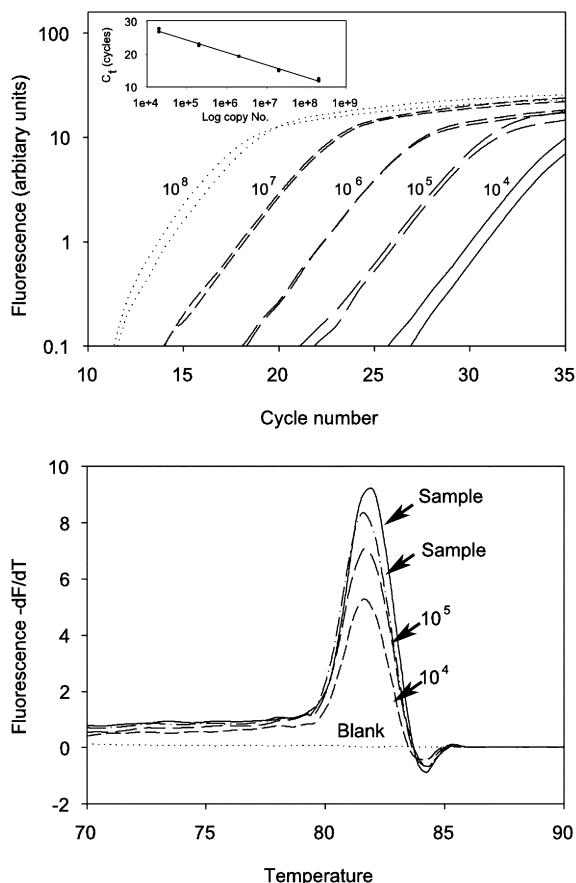


Fig. 3. Examples of quantitative RT-PCR data output. The upper figure shows amplification curves for $2 \times 10^8\text{--}10^4$ copies of CHH RNA standards in duplicate, inset standard curve derived from this data (slope = -3.8 , equivalent to 83% PCR efficiency; Ct, crossing threshold). Lower figure shows melt curve analysis of two standards (2×10^5 and 2×10^4 copies and two unknown medulla terminalis RNA samples). Blank (no template control) shows no product or primer-dimer formation.

C. maenas, by determining the inhibitory action of MIH and CHH on the target tissue, receptor binding, a second messenger pathway, MIH and CHH peptide and transcript levels in the XO with reference to the moult stage of the crustacean.

During pre-moult, YO became unresponsive to the inhibitory effects of both MIH and CHH, but during late postmoult (C₁), competence was restored. This effect was rather more marked for MIH (5 nM) than CHH, and was seen for both major secretory products of the YO, ecdysone and 25-deoxyecdysone. Inhibition of 3-dehydroecdysone synthesis was not measured, but this is a minor secretory product of *Carcinus* YO [11]. Loss of sensitivity of YO to the inhibitory influence of crude SG extracts during pre-moult has been noted for the shrimp *P. vannamei* [9] but in this species, a CHH-like peptide fulfils a role as an MIH [9,17]. The reduction of biological activity was also noted when the influence of MIH and CHH upon GMP levels in YO was considered. For *Carcinus*, cGMP seems to be a particularly important part of the MIH signal

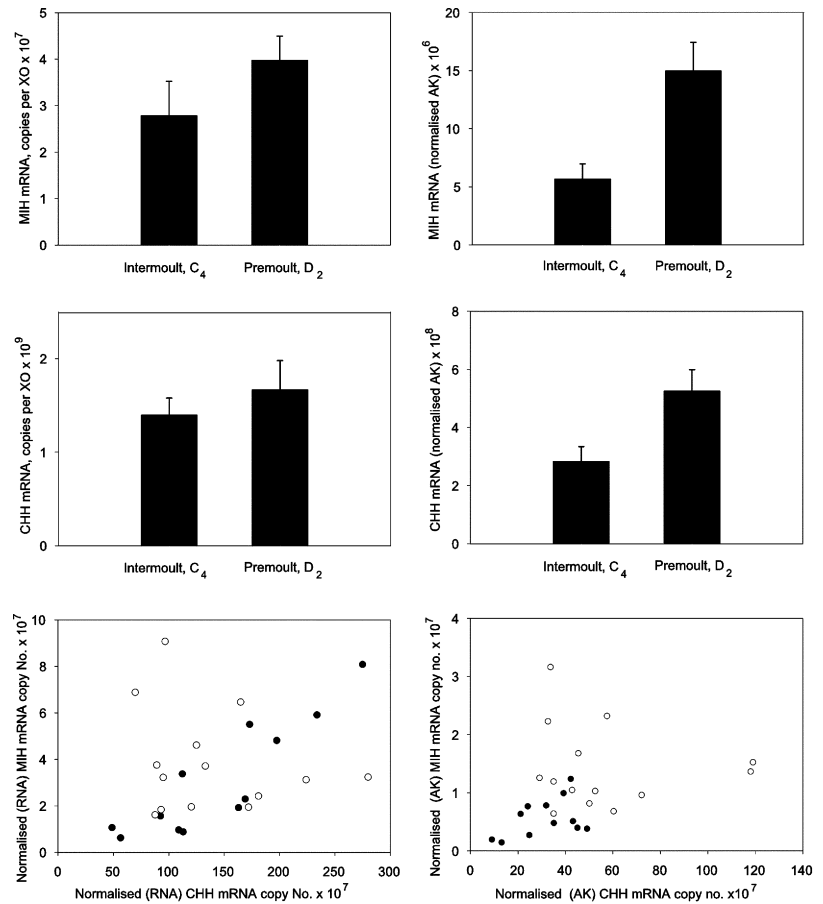


Fig. 4. Summary of results from 'real-time' quantitative RT-PCR experiments. Left histograms show non-normalized steady-state copy numbers of MIH and CHH transcripts per XO from intermoult, C₄ ($n = 12$) and late premoult, D₂ ($n = 14$) samples. Right histograms show the same data normalized against the control gene, AK. The bottom row of scattergraphs shows the relationship between MIH and CHH copy number per XO, normalized against total RNA, or AK. Solid symbols, intermoult; open symbols, premoult.

transduction mechanism [18,19]. The results for both MIH and, to a lesser extent CHH clearly show that during premoult, increases in cGMP levels following a 30-min incubation in peptide were dramatically blunted during premoult, and that competence to respond to peptide was restored in late postmoult.

With regard to downstream events, it has been shown that in *Carcinus*, protein kinase G is activated during early premoult by 8Br-cGMP, and that in this species, protein kinase A seems to be unimportant in signal transduction [19]. However, there is still much uncertainty about the physiologically relevant processes involved in MIH signal transduction [20,21]. Nevertheless, as the second messenger and bioassay results are congruent, and in view of earlier results showing that 8Br-cGMP mimics the effect of MIH [18], it is tempting to suggest that they are causally related. It would be interesting to see if exogenous application of membrane permeant cGMP analogues would salvage inhibition of ecdysteroid synthesis by the late premoult YO.

To investigate the initial stages of signal transduction, i.e. receptor binding, we determined receptor number (B_{\max}) and affinity (K_d) of binding sites in membrane preparations of YO obtained from crabs at various stages of the moult cycle. Results showed quite clearly that in general there were no obvious changes in receptor binding characteristics over most of the moult cycle. There was certainly no evidence for reduction of receptor number during premoult, which might account for the loss of response to MIH and CHH during

premoult. However, a large increase in MIH receptor density compared to all other moult stages was observed for postmoult (B) YO membranes. This was not due to stochastic variations in binding kinetics, as YO membranes from C₄ animals were always run in parallel, to compare other experiments, and similar increases in B_{\max} for CHH were not seen during postmoult. The significance of this observation is unclear, although it is tempting to suggest that this reflected a recruitment of new MIH receptors to the YO plasma membrane at this time, which is interesting in that this is nearly coincident with the resumption of competence of the YO at stage C₁. Thus, it seems that selectivity, with regard to YO responses to MIH or CHH during the moult cycle is not at the receptor level.

In view of these observations, we also investigated levels of MIH and CHH peptides in the sinus gland and levels of transcripts in XO neurones during the moult cycle, to see whether significant events, such as up- or down-regulation of transcription or peptide synthesis were moult cycle related. Whilst it would have been preferable to measure levels of newly synthesized peptide in XO cells by a sensitive method, such as RIA, dissection of perikarya, without inclusion of part the XO-SG tract (which contains large quantities of peptide) proved impossible. Thus, our option was restricted to measurement of levels of stored peptide in the SG.

It is now well established that the release of peptides from neurosecretory neurones is preferentially restricted to newly

synthesized products from neurosecretory terminals, for example in locusts [22,23], molluscs [24], mammals [25], crabs [26] and shrimps [27]. Thus, any changes in transcription, not withstanding translational control mechanisms, might indicate periods within the moult cycle when peptides are released, as available evidence suggests that aged peptides are not released. As low titres of ecdysteroids typical of intermoult, contrast with peak titres during late premoult (D_2), our approach was to compare steady state transcript levels in the XO, and peptide levels in the SG during these significant stages of the moult cycle, as these are of fundamental importance with regard to the currently accepted model of moult control. Our results show that with regard to non-normalized steady-state transcription of MIH and CHH, that both mRNAs appear to be expressed constitutively, at very high levels (MIH $2-4 \times 10^7$; CHH 1.5×10^9 copies per XO). Average ratios (CHH/MIH) of transcript number were: 50, intermoult, 42; premoult. *Carcinus* XO contain between 28 and 36 MIH and 62–65 CHH immunoreactive perikarya [28], thus steady-state transcript numbers are around $5-10 \times 10^5$ copies per cell for MIH and $2-2.4 \times 10^7$ copies per cell for CHH, i.e. there are about 25–50 times more transcripts in CHH neurones than those expressing MIH. For comparison, analysis of data from [29], where steady state mRNA levels for CHH (by ribonuclease protection assays) in *Orconectes limosus*, were measured, show that CHH-A and CHH A* copy numbers per XO are about $7 \pm 1.4 \times 10^5$ and $4.6 \pm 0.5 \times 10^6$, i.e. about 500 times lower than *Carcinus* CHH mRNA levels.

To account for possible differences in RT efficiency, and tissue size, we used AK as an 'invariant' reference control gene, as it is expressed at relatively constant, but not highly abundant levels by many tissues of *Carcinus* [30,31] (data not shown). There is still much controversy regarding the use (or misuse) of invariantly expressed housekeeping control genes in quantitative PCR [32–34]. Whilst we had little option but to use, a widely (moderately) expressed, but generally invariant gene in our study of a 'nonmodel' organism, where few housekeeping gene sequences are available, we were aware of this problem. When results were normalized against AK, it appeared that both MIH and CHH transcript numbers were upregulated during premoult. This was entirely artefactual: during late premoult, transcription of AK is downregulated by twofold in eyestalk neural tissues. The best fit for normalized data involved one using total RNA, which has recently been suggested as an eminently suitable alternative method [34]. For intermoult animals, a reasonable correlation between copy number of both MIH and CHH could be observed using this transformation, which was much better than that obtained after normalization with AK (Fig. 3). However, correlations were not observed in premoult animals, whichever normalization was used. Despite these caveats, our results contrast vividly with those obtained for *C. sapidus* where Northern blot analysis (using a lobster β -actin probe to normalize the data) indicated that MIH mRNA was dramatically (five- to 10-fold) downregulated during premoult [7]. However, in the penaeid shrimp, *P. japonicus*, MIH (SGP-IV) mRNA levels are not downregulated at this time [6]. In view of the very much more sensitive, quantitative and reproducible technique used here, we are

confident that premoult *Carcinus* do not exhibit downregulation of either MIH or CHH during premoult, and that both peptides are constitutively transcribed at high levels. Furthermore, as the MIH transcript number is not downregulated during premoult, feedback inhibition of MIH transcription by ecdysteroids during the time of maximal titre (D_2) is not likely. However, on the basis that premoult eyestalk neural tissues express high levels of ecdysteroid receptor (EcR) in *Uca pugnator* [35] and the observation that high concentrations of injected ecdysteroid reduced secretable MIH-like activity from *Cancer antennarius* eyestalk ganglia, negative feedback loops have been suggested [36].

With regard to release patterns of MIH and CHH during the moult cycle, little can as yet be said. Our experiments (data not shown) indicate that MIH is episodically and, to a certain extent, stochastically released in intermoult animals, and has a short half-life of between 5 and 10 min. As peaks in circulating MIH levels occur sporadically (maximum titre 10–20 pM), recording release events for MIH throughout the moult cycle remains a formidable technical challenge. As we reasoned that moult cycle related release events for CHH and MIH might be sufficient to lead to depletion or accumulation of peptides in the SG, we quantified steady-state levels of peptides in the SG during the moult cycle. When levels of MIH in premoult SG were compared to those in intermoult, there was evidence for a small but insignificant reduction in MIH content during late premoult. This result contrasts vividly with those obtained for the crayfish *P. clarkii* [8], where SG MIH levels doubled during early premoult and fell to levels below intermoult values during late premoult. It was interesting to note that whilst steady-stage transcript ratios in the X-organ were about 25–50 : 1 CHH/MIH, ratios of peptides in the SG were always at least fivefold lower (6–11 : 1 CHH/MIH). Whilst this may suggest differential translation rates, it may also reflect higher rates of secretion of CHH than MIH, in accord with its role as an adaptive hormone. CHH is released during times of stress, hypoxia, or nocturnal activity [37–41], which are pervasive phenomena in the life histories of crustaceans. Additionally, when circulating levels of both MIH and CHH are simultaneously measured in *Carcinus* haemolymph, CHH titres are always at least 10-fold higher than those of MIH (own unpublished observations), in keeping with the hypothesis that CHH secretion is a dynamic process. Taking these observations into account, and given that CHH is about 10-fold less effective than MIH in repressing ecdysteroid synthesis [4], it now seems possible that CHH titres may be sufficient to inhibit ecdysteroid synthesis *in vivo*, at an equivalent level to that of MIH. As there is some evidence for synergistic action of both peptides on the YO [42], and with regard to the results reported here, a truly functional role for CHH in moult control in *Carcinus* seems feasible.

The results obtained in this study suggest that the accepted model of moult control in crustaceans may need revision. For *Carcinus*, it seems likely that MIH and CHH transcription and translation are somewhat invariant during the moult cycle, certainly there are no overt differences in mRNA levels in the XO or stored peptide contents of the SG when intermoult and premoult conditions are compared. However, there are clear differences in the potencies

of both peptides upon inhibition of ecdysteroid synthesis, and in term of the magnitude of second messenger (cGMP) responses, which are moult cycle dependent. Rather surprisingly, there were no changes in receptor affinity or number during the moult cycle (excepting the possible recruitment of MIH receptors to the organ during post-moult); it seems that YO MIH and CHH receptors from both intermoult and premoult crabs retain competence to bind their respective ligands. Thus, we suggest that that an important mechanism involved in control of the moult cycle relates to intracellular signalling pathways within the YO. Further studies in other species are now needed to comprehensively define these, both in intermoult and premoult crustaceans.

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