

Dynamics of *in Vivo* Release of Molt-Inhibiting Hormone and Crustacean Hyperglycemic Hormone in the Shore Crab, *Carcinus maenas*

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Very little is known regarding the release patterns or circulating titers of neuropeptides in crustaceans, in particular those concerned with regulation of molting hormone (ecdysteroid) synthesis, molt-inhibiting hormone (MIH), and crustacean hyperglycemic hormone (CHH), which is also an adaptive hormone, centrally important in carbohydrate metabolism. Furthermore, the currently accepted model of molt control is founded on an untested hypothesis suggesting that molting can proceed only after decline in MIH titer. Accordingly, we measured simultaneous circulating neuropeptide profiles for both MIH and CHH by RIA of purified hemolymph during the molt cycle at fine temporal scale during day/night cycles and seasonally. For CHH we additionally determined release patterns after physiologically relevant stress. Results show that both hormones are released exclusively and episodically, rather than continuously, with nota-

bly short half-lives in circulation, suggesting dynamic and short-lived variations in levels of both hormones. During the molt cycle, there are no overt changes in MIH titer, except a massive and unprecedented increase in MIH during late premolt, just before ecdysis. The function of this hormone surge is unknown. Treatment with various stressors (hypoxia, temperature shock) showed that CHH release occurs extremely rapidly, within minutes of stress. Release of CHH after stressful episodes during premolt (when gut endocrine cells synthesize large quantities of CHH) is exclusively from the sinus gland: CHH from the gut is never involved in the stress response. The results show a hitherto unsuspected dynamism in release of MIH and CHH and suggest that currently accepted models of molt control must be reconsidered. (*Endocrinology* 146: 5545–5551, 2005)

IN THE PAST FEW years, a large number of structurally related neuropeptides, generically known as members of the crustacean hyperglycemic hormone (CHH) peptide family have been identified from the X-organ sinus gland neurosecretory system of crustaceans using microsequencing and cDNA cloning strategies (1–3), and this list is being expanded continually. The CHH family peptides can be classified into two groups, depending on the presence, in the unprocessed peptide of a precursor-related peptide (type I, or CHH type) or lack of this precursor and a glycine residue at position 12 of the mature peptide, the insertion of which aligns peptide sequences in all members in this group (and if a gap is inserted at a corresponding position for CHH peptides, aligns all members), and which immediately highlights all members of this group as type II or molt-inhibiting hormone (MIH)/gonad-inhibiting hormone type (1).

Whereas the names of these hormones obviously reflect their first discovered biological activity (or their identity to peptides with well-established roles), it has become ever more apparent that many of them have multiple roles or probably that the physiologically relevant ones have yet to be established. For the best-known prototype hormone, CHH, apart from its defining role in regulation of carbohydrate metabolism (4), related activity as a secretagogue (5)

and in lipid mobilization (6) has been shown. Furthermore, roles in molt control (7, 8) and iono-osmoregulation (9–11) have been described. Involvement in reproduction (inhibition of protein and vitellogenin synthesis) has also been suggested (12). Further complexity is added when other members of the CHH family are considered. For example, whereas a distinctive (MIH type) mandibular organ-inhibiting hormone, which represses *in vitro* juvenoid (methyl farnesoate) synthesis by the mandibular organs, exists in *Cancer pagurus* and other cancrids (13 and own unpublished observations) for other decapod crustaceans, CHH appears to fulfill this role (14, 15). Similarly (as alluded to earlier) in several crustaceans in which distinctive MIH-type peptides appear to be absent, CHH-type peptides are the functional MIHs (8, 16, 17).

Given this background, it is clear that we know very little regarding the physiologically relevant roles of most of the CHH group peptides. Whereas appropriate bioassays are useful in pointing to possible functions, we reasoned that these could be more credibly defined by *in vivo* measurement of circulating hormone titers. For example, the current model of molt control in crustaceans is completely based on the supposition that MIH is released only during intermolt, in which its action is to repress ecdysteroid synthesis by the Y-organs. Reduction in MIH release could thus permit increased ecdysteroid synthesis, leading to permissive entry to premolt and subsequent molting. Similarly, whereas it is known that CHH release occurs after stressful episodes in some crustaceans (18–20) and from gut endocrine cells immediately before ecdysis in *Carcinus maenas* (10), we know

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Abbreviations: CHH, Crustacean hyperglycemic hormone; DA, dopamine; MIH, molt-inhibiting hormone; SG, sinus gland.

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little regarding day-to-day variation in and release patterns of CHH family peptides, which are clearly necessary to understand the physiological significance of these hormones. To address these questions, we used highly sensitive RIAs for both CHH and MIH to investigate diel patterns of release, the effects of stressors on release of CHH, and influence of the molt cycle on MIH titers.

Materials and Methods

Animals

Specimens of *Carc. maenas* were collected by baited traps around low water level in the Menai Strait (Wales, UK). After capture they were immediately selected (males, 45–55 mm carapace width, intermolt stage C₄) and placed in a recirculating seawater system (10–12 per 50 liters) under conditions of ambient temperature and photoperiod. Great care was taken to ensure crabs were unstressed during this period. Tubes were added for refuge so that each crab could avoid interaction with others. Crabs were left to acclimate for 72 h before experimentation and fed once (fish and mussel) during this period. For some experiments premolt crabs were used (molt staging according to Ref. 21).

RIA

RIAs for CHH and MIH were performed as described and validated previously (MIH: Ref. 22; CHH: Ref. 23). Standards were triplicated and unknowns duplicated. The MIH assay was made much more sensitive by adding 10 μ l/tube of 1/2 \times crustacean saline, which increased the slope of the dose-response curve and the minimum detectable dose. Detection limits were less than 0.5 fmol/tube for MIH and CHH. ED₅₀ values were 11.97 \pm 1.60 (n = 14) and 6.52 \pm 0.74 (n = 14) fmol/tube for MIH and CHH, respectively. Data were analyzed using WIACALC (Wallac, Turku, Finland). Any values lower than the most dilute standard were scored as zero. All standards originated from single batches of peptides, which had previously been quantified by amino acid analysis [gas-phase hydrolysis at 150 C in 6 M HCl for 60 min, followed by *o*-phthaldialdehyde precolumn derivatization and HPLC as previously described (24)].

Hemolymph samples were taken from the base of the walking legs and immediately frozen in liquid nitrogen. Because unpurified hemolymph samples could not be used in these assays, at volumes compatible with detection without interference, all samples were purified using Sep-Pak C₁₈ (Waters, Milford, MA) 360-mg cartridges, which all originated from a single batch. After gentle thawing on ice, hemolymph samples were centrifuged (14,000 \times g, 5 min) and applied (up to 2.2 ml) to conditioned cartridges attached to a 20-place vacuum manifold. Samples were gravity loaded, then washed with 10 ml water, and eluted (1 ml/min) with 3 ml 40% (CHH) or 60% (MIH) isopropanol. Samples were immediately dried by vacuum centrifugation (40 C). Recoveries were approximately 70%.

Manipulations and stresses

Hemolymph samples (up to 3.5 ml) were taken from crabs kept under ambient conditions of temperature and photoperiod every 2 h for 24 h during winter (December to February) and summer (June to August). Ten to 12 crabs were sampled at each time, discarding these after hemolymph removal. During nighttime, samples were taken using a dim red head torch to avoid disturbance.

Temperature stress experiments were performed on groups of up to 10 animals. Crabs were taken from water at ambient temperature (15 C) and immersed for 5 min in water at 30 C before return to ambient water. This procedure was repeated twice more at hourly intervals. Before and after each heat shock, small hemolymph samples (250 μ l) were taken for CHH RIA and glucose estimation (performed as detailed in Ref. 19). Further temperature stress experiments were performed on groups of five crabs using 5 C increments or decrements for 5-min exposures. Hemolymph samples were taken for CHH estimations as described.

Hypoxia stress experiments were performed in tightly sealed containers using water deoxygenated by continuous nitrogen sparging

(which reduced O₂ levels to less than 2.5% saturation). Controls were continuously aerated. Hemolymph samples were taken at hourly intervals for 3 h. Crabs that had been eyestalk ablated 48 h before experimentation served as controls and also to determine whether known extra eyestalk sources of CHH, such as from gut endocrine cells, could be involved in CHH release after hypoxic stress. Hemolymph samples were assayed for CHH and glucose, as detailed above. Assays for circulating glucose and lactate were performed as previously described (19).

To estimate half-life of CHH and MIH, small quantities (2.5 kBq) of iodinated peptides were injected, followed by estimation of hemolymph radioactivity 2 min after injection (to allow the bolus to perfuse), followed by sampling at T = 5, 10, 15, 30 min. These assays gave identical results to those previously described, in which native peptides were injected, followed by estimation of undegraded peptide by RIA (19, 23, 25) and thus gave reasonably accurate estimations of half-life.

Results

Normal crabs

Hormone levels of crabs that were presumably unstressed (after adaptation to laboratory conditions, ambient temperature, and photoperiodic conditions) were taken at monthly intervals over 24-h periods, sampling at least 10 animals at each time point (2 h). For each blood sample from subsequently molt-staged animals, levels of CHH and MIH were determined. In total, more than 1000 hemolymph samples were processed. Initial analysis of individual experiments revealed that there was, as expected, a high level of stochasticity. However, when all results (intermolt crabs) were collated (Fig. 1), elevated levels of CHH or MIH were rather infrequently observed. Nevertheless, few instances of simultaneously high levels of both CHH and MIH were observed. Comparison of elevated levels of hormones (MIH > 5 fmol/ml, CHH > 5 fmol per 100 μ l) between night and daytime showed that during daytime, only 28 (5.1%) samples showed elevated MIH levels, compared with 55 (11.9%) during nighttime. For comparison, elevated CHH levels were similar during the day (13.7%) and night (14.7%).

Experiments designed to estimate the half-lives of MIH and CHH showed that both are cleared from the hemolymph quite dynamically. For both peptides, half-lives are in the order of 5–10 min (Fig. 1, inset). Thus, if these hormones are released in an episodic manner, the possibility of obtaining hemolymph samples close to a release event is small. However, assuming that the elevated hormone levels (defined earlier) arise from episodic release, all CHH or MIH hormone levels constrained within these parameters may be considered to be those arising from release events that have occurred within two half-lives (10–20 min) of release.

Analysis of changes in seasonal levels of CHH and MIH for intermolt animals (Fig. 2) showed that during winter, CHH levels were significantly lower (*ca.* 25 fmol/ml) than those of animals sampled in the summer (50–55 fmol/ml) and that there were no significant differences between samples collected during day or nighttime. For MIH, during the winter, levels were significantly higher during nighttime (2.8 fmol/ml) than during daytime (2.1 fmol/ml).

Analysis of MIH and CHH titers according to molt stage showed that during intermolt (C₄) and early premolt (D₀–D₁), despite reasonable sample sizes, there were no significant changes in hormone titer (Table 1). However, during late premolt (despite the difficulties in taking adequate hemolymph samples at this time), there were unprecedented

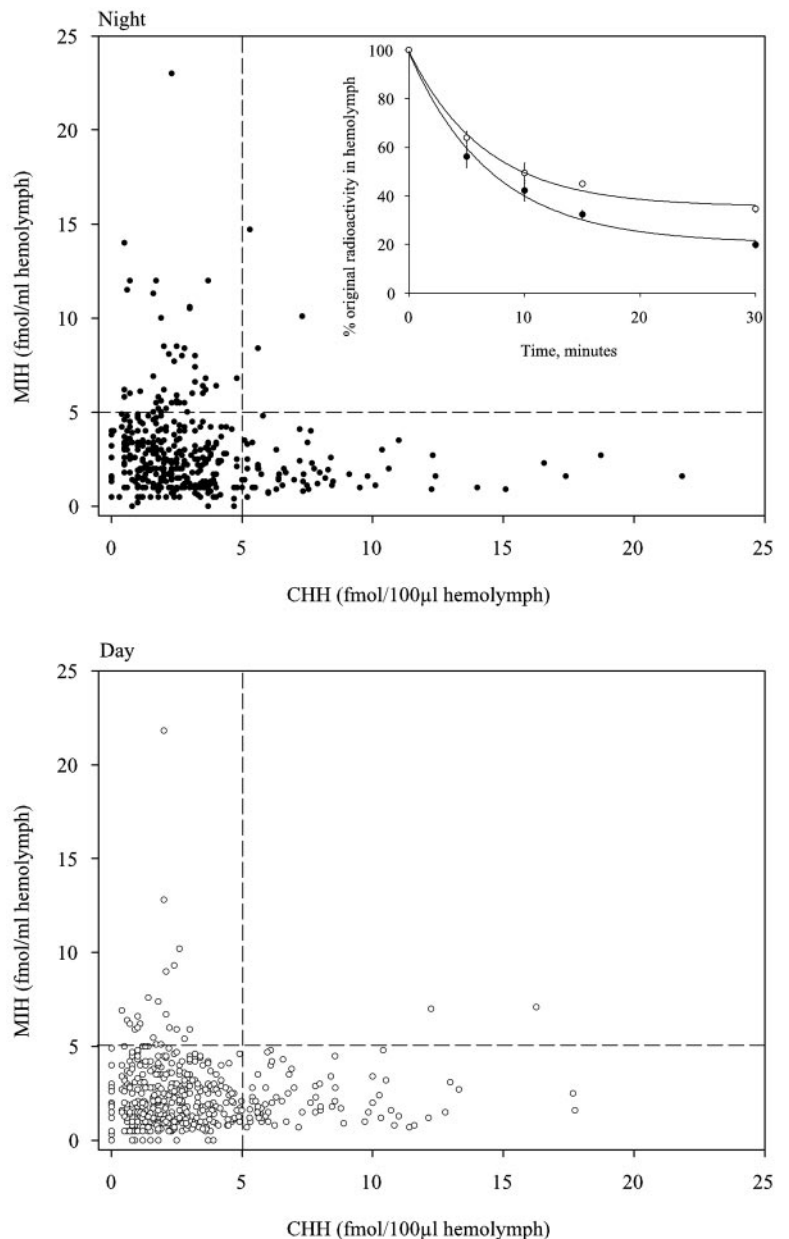


FIG. 1. Simultaneous hemolymph titers of CHH and MIH taken from individual crabs during night (filled circles, $n = 462$) and day (open circles, $n = 547$). Only data in which both MIH and CHH hormone measurement were obtained from individual intermolt crabs are shown. Inset shows kinetics of removal of CHH (filled circles, $n = 6$) and MIH (open circles, $n = 8$) from hemolymph, estimated by measurement of residual radioactivity after injection of 2.5 kBq of ^{125}I -labeled peptides. Bars, ± 1 SEM. Curves of best fit were generated using a negative exponential algorithm ($r^2 = 0.993$). Dashed lines show approximate levels of peptide estimated to occur after two half-lives of the highest titers observed (ca. 20 fmol/ml MIH, 20 fmol per 100 μl CHH), assuming episodic release of peptides.

changes in levels of MIH. In animals sampled during late premolt (D_{3-4}) MIH levels were enormously elevated, to the extent that sinus gland levels of stored MIH were significantly depleted (Fig. 3).

Stressed crabs

For crabs exposed to thermal stress, CHH release was immediately apparent, within 5 min of exposure (Fig. 4). An interesting feature was that both hyper- and hypothermic stress elicited somewhat similar hormone release profiles. For hyperthermic stress, crabs were also exposed to brief periods (5 min) of repeated thermal shock and showed that each exposure to high temperature resulted in hormone release and accumulated hyperglycemia (Fig. 5).

Hypoxia was a potent elicitor of CHH release. In both intermolt and premolt animals, 2-h periods of extreme hyp-

oxia ($<5\%$ O_2 saturation) elicited quite dramatic increases in CHH levels (Fig. 6).

Field crabs

Because one of the accepted roles of CHH concerns its action as an adaptive hormone, we were interested in determining relevant changes in hormone levels in the intertidal environment. *In situ* measurement of instantaneous hormone titers and correlative glucose and lactate levels were measured from crabs at different times during the tidal cycle from a site around midtide level (Table 2). The results indicated that for crabs exposed to long periods of emersion, CHH levels were somewhat elevated, but paradoxically, glucose levels were somewhat lower in these animals, compared with others in which emersion times were shorter. During emersion, blood lactate levels remained unchanged.

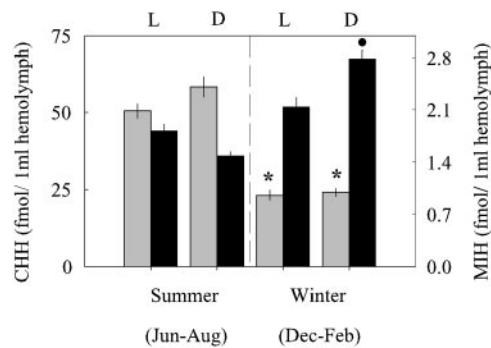


FIG. 2. Seasonal differences in CHH (gray bars) and MIH (black bars) hemolymph levels. Samples were taken during summer (June to August) and winter (December to February) and divided into daytime (L) or nighttime (D) samples ($n = 122\text{--}209$ for each sample). Bars, ± 1 SEM. Winter CHH levels were significantly lower than summer levels (*, $P < 0.001$). Nighttime MIH levels were elevated during winter, compared with daytime (•, $P < 0.05$). Kruskal-Wallis ANOVA and Dunn's multiple comparisons used for statistical analysis.

Discussion

In the present study, we measured instantaneous circulating levels of both CHH and MIH from large numbers of crabs during intermolt and early and late premolt to define temporal release patterns of these peptides and defined release patterns in normal and stressed animals to gain further information on mechanisms of molt control and regulation of energy mobilization. Measurement of both hormones from more than 1000 crabs during 24-h cycles initially suggested stochastic release patterns. Bearing in mind the very short-half-lives that we measured for both peptides, it is certain that both are released in an episodic fashion, as indicated by the low numbers of animals exhibiting much higher hormone titers than the abundant group in which hormone levels are basal. Furthermore, elevated titers of these hormones cannot be continuous because this would depend on extremely dynamic synthesis and release: for MIH, maintenance of 10–20 fmol/ml titers would necessitate synthesis/release of 50–100 fmol of MIH every 5–10 min (for a crab with a hemolymph volume of 5ml), *i.e.* 0.6–1.2 pmol/h. Whereas rates of MIH secretion from the sinus gland (SG) have not been measured, high K^+ evoked release of CHH from *Cardisoma carnifex* X-organ-SG *in vitro* of about $0.02\% \text{ min}^{-1}$ of the total CHH content of a single SG has been observed (26). Because *Carcinus* SG contain 30–50 pmol MIH (27), assuming that similar rates of MIH release could be sustained over 1 h, then up to 1.2% of the total content of the MIH in both SG could be released per hour (0.7–1.2 pmol). Because levels of CHH in the hemolymph are about 10 times higher than MIH,

TABLE 1. Comparison of hemolymph titers of CHH and MIH during intermolt (C_4) and early premolt (D_{0-1})

	CHH (fmol/ml) C_4	CHH (fmol/ml) D_{0-1}	MIH (fmol/ml) C_4	MIH (fmol/ml) D_{0-1}
Mean	21.5	24.8	2.9	3.1
SEM	1.0	1.3	0.1	0.17
n	313	147	324	166
Significance		NS		NS

Values were taken from 24-h experiments during spring (February through April). NS, No significant differences (Kruskal-Wallis ANOVA and Dunn's multiple comparison).

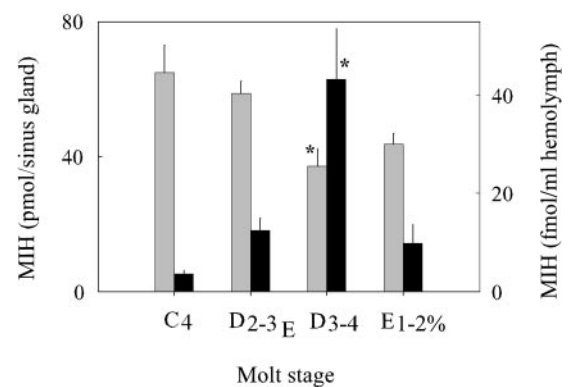


FIG. 3. Changes in circulating levels of MIH in the hemolymph (solid bars) and MIH levels in the SG (gray bars) during intermolt and late premolt ($n = 4\text{--}15$ animals at each stage, bars, ± 1 SEM). Large asterisk indicates highly significant increases ($P < 0.001$, Kruskal-Wallis ANOVA and Dunn's multiple comparisons) in circulating MIH, small asterisk, significant decrease ($P < 0.05$, Student's *t* test) in SG MIH content during late premolt, compared with intermolt.

which correlate well with ratios in the SG, the same type of calculation can be applied for CHH, with similar results. However, sustained patterns of release would undoubtedly be untenable; rapid depletion of SG peptide would occur. Turnover rates of SG peptides are probably in the order of months (28), and there is firm evidence that in crustaceans (29, 30), as in other organisms (31–33), that immediate release pools of neuropeptides preferentially contain freshly synthesized rather than aged hormone. Thus, episodic rather than sustained release of CHH and MIH seems likely.

Comparison of all results from intermolt crabs from night or day revealed a pattern suggesting that either MIH or CHH was released; high titers of both peptides were very rare. Only two to three hemolymph samples above thresholds of 5 fmol/ml MIH and 5 fmol per 100 μl CHH were observed in more than 1000 samples analyzed. There was also some evidence to suggest that MIH release was greatest during nighttime. It is interesting to note that short (5 min) exposure of the Y-organ to MIH (which is likely to be analogous to single release events of hormone) results in measurable in-

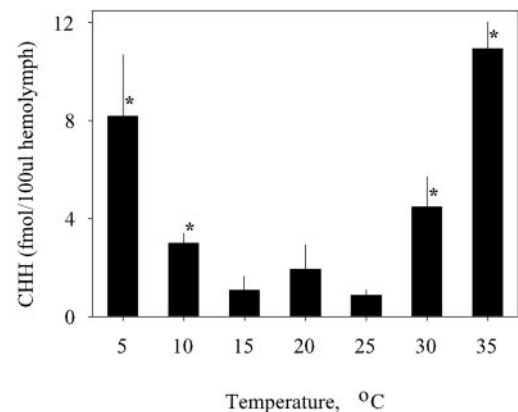


FIG. 4. Effect of temperature change on CHH release. Crabs ($n = 5\text{--}8$) were acclimated at 15 C and then thermally stressed (5 min) at 5–35 C. Bars, ± 1 SEM. Hemolymph samples were taken before and immediately after thermal stress. Asterisks indicate significant increases ($P < 0.05$, Student's matched-pair *t* test) in CHH levels.

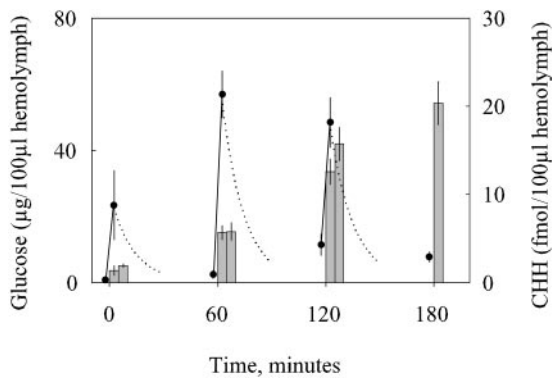


FIG. 5. Effects of repeated thermal stress on CHH levels. Crabs (n = 10) acclimated at 15 C were given hourly thermal stress for 5 min, 30 C. Gray bars show hemolymph glucose levels, filled circles, hemolymph CHH levels (offset for clarity). Bars, ± 1 SEM. Dotted lines show approximations of presumptive decline in hemolymph CHH levels, given an estimated hormone half-time of 5 min (see Fig. 1, inset).

hibition of ecdysteroid synthesis (34) and that after MIH exposure the Y-organ remains repressed for long periods of time (35, 36). Because CHH potentiates the action of MIH in a greater than additive manner (37), it is possible that seasonal changes in day length might alter MIH or CHH release patterns, in ways that could subsequently affect ecdysteroidogenesis by the Y-organ.

Because release of both hormones is episodic, it should be stressed that any analysis of hormone titers in which results are pooled will reduce apparent changes because in the majority of animals, hormone levels were basal. Thus, any responses will be difficult to detect. However, in view of the unprecedented numbers of samples taken in this study, some analysis seemed worthwhile. For seasonal changes (intermolt crabs) in CHH and MIH levels, CHH levels during winter were about half that in summer, which likely reflects reduced locomotor activity and metabolic rate at this time. It was interesting to note that during the winter, MIH levels were significantly elevated during the night.

The widely accepted view of the hormonal control of molt-

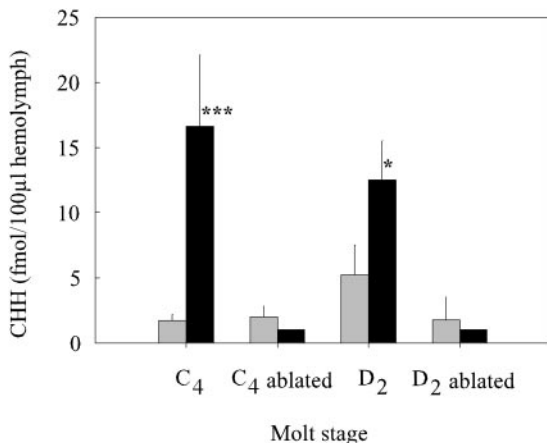


FIG. 6. Effect of hypoxia (2 h) on hemolymph CHH levels in intact and eyestalk ablated intermolt and premolt crabs (n = 6–8 at each stage). Gray bars, t = 0; black bars, t = 2 h. Bars, ± 1 SEM. For intact intermolt crabs, hypoxia resulted in greatly elevated CHH levels (P > 0.001); this was also seen in premolt animals (P < 0.05, Student's matched-pair t test).

TABLE 2. *In situ* measurements of CHH, glucose, and lactate levels

Time after midwater (h)	CHH (fmol/100 µl)	Glucose (µg/100 µl)	Lactate (µg/100 µl)
1–1.5	3.9 ± 0.9	2.1 ± 0.3	4.7 ± 1.2
2–3	4.8 ± 1.6	2.8 ± 0.7	4.4 ± 1.3
4.5–5	7.3 ± 1.3 ^a	1.5 ± 0.2 ^a	4.2 ± 0.5

Hemolymph samples were taken from approximately 30 crabs from a site around midtide level, from midwater until inundation on the next advancing tide. Samples were immediately frozen in liquid nitrogen.

^a Significant differences (P < 0.05, Student's t) from samples taken 1–1.5 h after midwater. Means ± 1 SEM are shown.

ing has long been linked to the tenet that MIH titers remain high during intermolt and reduce during premolt, thus freeing the Y-organ from inhibition, resulting in increased ecdysteroid synthesis necessary for premolt (37–41). This long-held hypothesis was tested by measurement of MIH levels during intermolt and early and late premolt. Mean MIH and CHH levels in intermolt (C₄) and early premolt (D₀₋₁) were very similar (MIH: 3 fmol/ml; CHH: 20–25 fmol/ml hemolymph), and it was noteworthy that ratios of both peptides were in accord with their concentrations in the sinus gland (1:7 MIH to CHH) (27). For the only other study (the crayfish *Procambarus clarkii*) in which MIH levels have been measured (using a very sensitive time-resolved fluoroimmunoassay), intermolt MIH levels were about 6 fmol/ml but dropped during early premolt to 1.3 fmol/ml. Intriguingly, subsequent pre- and postmolt MIH levels were very similar to those of intermolt (42). When we investigated MIH levels at a fine temporal scale during late premolt and ecdysis (Fig. 3), a remarkable and unprecedented release of MIH was observed about 1 d before ecdysis (D₃₋₄), when MIH reached high levels (40 fmol/ml), declining rapidly before ecdysis. This was reminiscent of the CHH surge in *Carc. maenas*, in which high levels of CHH (up to 2 pmol/ml), which arise by release of CHH from gut endocrine cells, precede ecdysis (10).

The MIH surge seems to be particularly dramatic in that there is a significant reduction in sinus gland MIH content at this time, which indicates a massive exocytotic event. Whereas this correlates well with the low levels of ecdysteroids characteristic of late premolt and might explain the rapid termination of ecdysteroid biosynthesis characteristic of this stage of the molt cycle (43), our studies on *in vitro* ecdysteroid biosynthesis show that the Y-organ becomes refractive to the inhibitory influence of MIH (5 nM) both in terms of inhibition of ecdysteroid synthesis and second messenger (cGMP) signaling during late premolt and early postmolt (D₂₋₃, A-B) (27). Thus, if the *in vitro* situation is physiologically relevant to that *in vivo*, in which peak MIH levels are much lower (40 pM), it seems unlikely that the MIH surge is directly important in terminating ecdysteroid synthesis. Furthermore, the precipitous premolt decline in circulating ecdysteroid levels also occurs in eyestalkless animals (44–46). Unless there is an extraeyestalk source of MIH, which seems very unlikely, it is difficult to reconcile a role of MIH in termination of ecdysteroid synthesis before molting. Whereas our previous studies have shown that the MIH receptor is only expressed by the Y-organ (intermolt), using

classical radioligand binding studies (22), it is possible that other tissues might express MIH receptors during late premolt, which would uncover novel roles for MIH. Identification of the MIH receptor is now timely.

Our studies have shown that not only MIH release is episodic and that basal, low levels of this hormone do not change during the molt cycle but also that levels do not decline during premolt. Thus, these results contradict the long-established hypothesis of molt control, whereby high levels of MIH are proposed to inhibit ecdysteroidogenesis during intermolt, and falling titers of MIH in premolt subsequently directly lead to a freeing of the Y-organ from the inhibitory influence of MIH, resulting in increased ecdysteroidogenesis and circulating ecdysteroid titers. Furthermore, because we have shown that during late premolt, there is a massive and unprecedented release of MIH, which has no known function, it is clear that the current model of molt control via MIH needs a complete reappraisal.

Studies on the response of *Carc. maenas* to stress were exclusively concerned with CHH because in every stressful scenario used (hypoxia, temperature shock, salinity change), increases in MIH levels were never observed. The first reports regarding hypoxia and its influence on CHH were from crayfish (*Orconectes limosus*), in which hypoxic episodes are associated with remarkable increases in CHH titer, consistent with this hormone's presumed adaptive role (18). Emersion stress (which is associated with hypoxia as evidenced by hyperlactemia) also induces CHH release in subtidal crustaceans such as *Can. pagurus* (19) and *Homarus americanus* (20). In the present study, we confirmed this response in *Carc. maenas*, and show that this is entirely due to release of CHH from the eyestalk because eyestalk ablation completely removed hypoxia-induced CHH release in premolt animals. Thus, hypoxia induces release of CHH only from eyestalk neuroendocrine cells. Despite the significant accumulation of CHH in gut endocrine cells during late premolt (10, 47), this material is not released and can be discounted as being of relevance to the CHH-mediated stress response in *Carc. maenas*. With regard to neurotransmitter-mediated signaling mechanisms involved in this CHH release, very little is known. However, it may be pertinent to mention that dopamine (DA) seems to be important. In *Carc. maenas*, DA has been suggested to be involved in CHH release (48), and this has recently been conclusively demonstrated in *Procambarus clarkii* (49). Because prolonged hypoxia increases γ -amino butyric acid levels in the brain of *Carc. maenas* (50), it is possible that it might be involved in the hypoxia-induced CHH release observed in this study. It would thus be interesting to measure correlative profiles of DA, γ -amino butyric acid, and CHH during hypoxic episodes.

Thermal stress is a second and possibly highly important environmental variable, which should lead to CHH release. Elevated temperatures have been shown to cause CHH release in *O. limosus* (18), *H. americanus* (20), and *Can. pagurus* (19), and a similar increase in CHH release after hyperthermic episodes was seen in the current study: We have shown that repeated episodes of thermal shock lead to repeated episodes of CHH release, which, considering the half-life of CHH in the blood, are episodic rather than prolonged. These are correlated with accumulating hyperglycemia (Fig. 5).

Because it has been elegantly shown that CHH neurones in the X-organ of *Can. borealis* hyperpolarize in the presence of physiologically relevant levels of glucose (51), it seems very likely that CHH release involves feedback inhibition and might thus be intrinsically episodic or pulsatile. However, the most environmentally pertinent question might be: are these manipulations environmentally relevant? For an intertidal organism, such as *Carc. maenas*, which exhibits refuge behavior to minimize thermal stress and evaporative loss, hyperthermic stress is probably uncommon. However, hypothermic stress, on inundation with the rising tide, is undoubtedly important in an environmental context: temperate, boreal intertidal crustaceans regularly face twice-daily inundation in which seawater temperature is considerably different, and frequently lower, than that of the exposed intertidal environment. When hypothermic stress was applied, significant increases in CHH release were observed after 5 min exposure to subambient (5–10 C) temperatures. This response was asymmetrical: ca.15 C increases in temperature were needed to elicit the same magnitude of response after hyperthermic stress. This response was not observed in *H. americanus* (20) or *Can. pagurus* (19), which is understandable because these species are cold water stenothermal crustaceans.

In this context, it is interesting to note that hypothermic shock in the tropical palaemonid prawn *Macrobrachium rosenbergii* also leads to hyperglycemia (52). Thus, it is tempting to suggest that thermally elicited CHH release is entirely related to life history strategy. Relating to this, hyper/hyposaline stress results in release of CHH in *H. americanus* (20), as befits a stenohaline organism; in *Carc. maenas*, which is a model euryhaline organism, we failed to measure episodic CHH release, even at salinities approaching its maximal environmental limits (3 parts per thousand seawater). Given the evidence from laboratory-based experiments, suggesting that CHH is an adaptive hormone, it was of interest to measure CHH titers in the field. After prolonged air exposure (low water), CHH levels increased. These changes did not occur as a result of increased lactate levels, which were invariant (suggesting that the crabs did not undergo hypoxia during low water). Whereas it was impracticable to measure hormone titers or glucose levels during inundation, it is tempting to suggest that increasing CHH levels during the advancing tide will result in high water hyperglycemia. Thus, in an adaptive context, increasing levels of CHH might increase glucose before extensive foraging and feeding behavior over high water.

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