

Expression and release patterns of neuropeptides during embryonic development and hatching of the green shore crab, *Carcinus maenas*

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Summary

Crustacean ecdysis is controlled by at least three neuropeptides: moult-inhibiting hormone (MIH), which represses ecdysteroid synthesis; crustacean hyperglycaemic hormone (CHH), which not only influences ecdysteroid synthesis but also water uptake during moulting; and crustacean cardioactive peptide (CCAP), which is involved in stereotyped ecdysis behaviour. During embryonic development, moulting takes place in the egg, but there is little information regarding developmental expression of these neuropeptides during this period or during hatching – an event that is analogous to eclosion in insects. To address this problem, we determined expression profiles of MIH and CHH mRNA by quantitative RT-PCR, together with developmental peptide expression studies [confocal immunocytochemistry (ICC) and radioimmunoassay (RIA)]. Likely homologous events relating to neuropeptide surges of both CHH and CCAP were seen during larval hatching, when compared to the adult moult, and cell-specific copy concentration of both MIH and CHH mRNAs was identical to that of the adult during late embryonic development. We measured parallel mRNA and peptide expression of two neuropeptides (red pigment-concentrating hormone RPCH) and pigment-

dispersing hormone (PDH) during development, as these have roles as neuromodulators and as classical neurohormonal roles. For MIH and CHH, gene expression was in accordance with peptide expression, but novel sites of CHH expression were found (abdominal peripheral neurones), the expression and release patterns of which may be related to larval eclosion and water uptake necessary for eggshell rupture and hatching. For RPCH and PDH, gene transcription and peptide expression were not in accordance. A significant contribution of maternally derived (non-translated) PDH mRNA to the embryo was seen, and for RPCH, high-level mRNA and peptide expression during late embryogenesis is related to a long ignored, but potentially important release site – the enigmatic post-commissural organs – which are the most prominent structures expressing RPCH during late embryogenesis.

Key words: *Carcinus maenas*, Embryogenesis, Embryonic ecdysis, Quantitative RT-PCR, Crustacean hyperglycaemic hormone, Moulting-inhibiting hormone, Pigment-dispersing hormone, Red pigment-concentrating hormone







Introduction

Embryogenesis in most marine decapod crustaceans, except Euphausiacea and Dendrobranchiata is epimeric in that the naupliar stage is rapidly superseded by a period of extensive embryonic morphogenesis, widely known as a 'metanauplius' stage, irrespective of the developmental phenotype exhibited at hatching (Goudeau and Lachaise, 1983). With regard to embryonic moulting, for crabs (*Carcinus maenas*) progression from the nauplius to metanauplius is recognised by the addition of a fourth egg envelope, in addition to two distinctive fertilisation envelopes. Subsequent metanaupliar development is associated with development of a loosely fitting cuticle, which is rapidly shed just after hatching (the prezoaea), and a cuticle belonging to the first free-swimming planktonic stage (the zoea) (Goudeau and Becker, 1982; Goudeau and Lachaise, 1980a; Goudeau and Lachaise, 1980b). Thus, during development, the embryo essentially completes three moult

cycles, one concerned with early events, which occur without exuviation, and a second, during which considerable morpho- and neurogenesis occur, as illustrated by eye development which has long been used to stage ontogenic processes during this period (Perkins et al., 1972). The third moult, and the only true ecdysis, occurs just after hatching (the prezoaeal moult). Subsequent important refinements have been used to completely temporally define the metanaupliar moult cycle in developing lobster embryos (Helluy and Beltz, 1991).

An important issue concerns the hormonal control of embryonic moulting. The fertilised egg of crustaceans contains large quantities of maternally derived ecdysteroids and corresponding polar metabolites, which decline during naupliar development (Goudeau and Lachaise, 1983; Lachaise et al., 1981; Okazaki and Chang, 1991; Wilder et al., 1990). These are presumed to be important in naupliar development. However, during metanaupliar development, dramatic

Table 1. Developmental stages of embryogenesis in *Carcinus maenas*.

Stage	Description	Diameter μm (<i>n</i>)	Duration (days) at 18°C	% development
Yolk 	Initially only yolk visible. Later, the embryo can be seen as an inconspicuous peripheral arc, without obvious internal structure.	–	Up to 90	
Embryogenesis 	Limb buds become visible (arrow), the embryo begins rapid development.	338±15 (12)	9.3±0.8	>25-30
Eye anlage+heart beat 	Visual pigment appears as a faint red arc (arrow). At this stage, no heart beat is seen.	360±13 (7)	5.2±0.1	>50
Eye anlage+heart beat 	Eye pigment seen as red oval (arrow), regular heart beat is established. Finally, small punctate red chromatophores develop.	393±11 (7)	5.5±0.2	>70
Developing eye 	About half the yolk sac remains (white arrow), melanophore development is complete by the end of this stage (arrow). Eye screening pigment (black).	419±11 (9)	5.3±0.2	>85
Full development 	Yolk sac consumed, but many large lipid globules present (arrow). White (reflective) pigment in eye. Embryo mobile. Both heart rate, limb and abdominal movements increase before hatch, the egg finally becomes elliptical.	445±4.3 (8)	4.4±0.2	>100

Mean duration of each stage at 18°C was estimated from regular observation of egg strings from 7-12 crabs, and from embryos cultured in vitro.

increases in ecdysteroid levels are seen which are believed to originate via de novo ecdysteroid synthesis by the developing Y-organ. The embryonic Y-organ has only been observed in palaemonid shrimps during the period just prior to eye development (Le Roux, 1983) (A. Le Roux, Thèse de Doctorat d'Etat, Université de Rennes, 1989).

In adult crustaceans, ecdysteroid synthesis by the Y-organ is negatively regulated by moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) (Böcking et al., 2002; Webster, 1998). Metanaupliar development is characterised by rapid neurogenesis, including development of the eyestalk neurosecretory complex. We were therefore interested in determining the development of the anatomy of neurosecretory neurones expressing MIH and CHH during embryonic development, since very little is known about these events during embryogenesis (Charmantier and Charmantier-Daures, 1998), except that an X-organ is present in the metanauplius of *Homarus gammarus* (Rotllant et al., 1995) and that MIH neurones are evident in the first zoeal stage of *Carcinus* (Webster and Dirksen, 1991). Additionally, since hatching is entirely dependent on water uptake necessary to rupture the eggshell (Saigusa and Terajima, 2000), we were interested in the occurrence of peripheral sources of CHH during development. In adult *Carcinus maenas*, moulting

involves a rapid uptake of water just prior to ecdysis, which is necessary for rupture of the old exoskeleton, and swelling to the subsequent postmoult dimensions. This phenomenon involves the ephemeral release of CHH from gut endocrine cells (Chung et al., 1999), and crustacean cardioactive peptide (CCAP) which is involved in stereotyped moulting behaviour in crustaceans (Phlippen et al., 2000) in ways that are reminiscent of insect eclosion (Gammie and Truman, 1997; Gammie and Truman, 1999). Thus, we reasoned that hatching (eclosion) in crustacean embryos is analogous to an adult moult, and that anatomical and physiological correlates might be common to both. Secondly, it has been recognised that many neuromorphological correlates exemplifying those of the adult are already in position at the time of hatching (Harzsch and Dawirs, 1993). In view of the considerable amount of information that now exists regarding the development of the embryonic and larval nervous system of decapods (Harzsch et al., 1998; Harzsch, 2003), we were also interested in contrasting the embryonic development of peptidergic systems involved in moulting (MIH, CHH) with those of the classical eyestalk neurohormones and neuromodulators – namely red pigment-concentrating hormone (RPCH) and pigment-dispersing hormone (PDH). The approach used here was firstly to relate embryonic stages where neuropeptide gene expression

could first be measured, using quantitative RT-PCR, and secondly to correlate these results with microanatomical analyses of peptide expression during development, particularly with regard to the identification of embryonic neurones. Finally, correlations were made regarding quantitative expression of peptides during hatching, to compare these with homologous events during adult ecdysis.

Materials and methods

Quantitative RT-PCR

Batches of 100 staged embryos were taken from ovigerous *Carcinus maenas* (maintained at 18°C, 12-14 hours light:12-10 hours dark), immediately transferred to RNAlater (Ambion) (4°C overnight) and stored at -80°C. Total RNA was extracted using TRIzol (Invitrogen). Genomic DNA was removed by incubation in 2U DNase I (37°C, 1 hour) followed by clean up on DNA-free (Ambion). mRNA was immediately isolated using Dynabeads (Dyna), and stored at -80°C in 10 mM TRIS (10 embryo equivalents/μl); 40 embryo equivalents of mRNA were reverse transcribed with AMV reverse transcriptase and random primers (Roche Molecular Biochemicals). Standard quantified cRNA for MIH, CHH, PDH and RPCH, were prepared as detailed in Chung and Webster (Chung and Webster, 2003). To account for changes in RT efficiency, batches of both embryo mRNA and cRNA standard dilutions were reverse transcribed simultaneously. For the standard samples, all four standard cRNAs were added to each reverse transcription reaction, adjusting all to give final copy numbers of 10⁷-10³ copies/sample. Primer sequences used to prepare MIH and CHH standards were as detailed in Chung and Webster (Chung and Webster, 2003). The following primer pairs were used to prepare templates for cRNA synthesis. PDH (accession code L08635) forward: 5' ATCGTAACCATGCTGGTGGT, reverse: 5' CGTTCATCACCTTGGGAAGT (product size 196 bp). RPCH (accession code S65357) forward: 5' TAGAACCGAGTGACGCTTT, reverse: 5' TGACAGCTGAGACAGGGATG (product size 254 bp). Neuropeptide mRNA quantification was performed by 'real-time' quantitative RT-PCR using a Roche Light Cycler and DNA Master kits (Roche Diagnostics), with SYBR green detection. Primers used were designed to span intron II for MIH and CHH, enabling possible gDNA contamination to be detected by melt-curve analysis, and also to give short (100-140 bp) products with minimal primer-dimer formation. Sequences of the primers used are given in Chung and Webster (Chung and Webster, 2003). For PDH and RPCH (where information on exon-intron boundaries was unavailable), the following primer pairs for quantitative PCR were used. PDH forward: 5' AAGAACGTGAGATGGTGGCT, reverse: 5' CGTTCATCACCTTGGGAAGT (product size 134 bp). RPCH: forward: 5' TCAGCTTTAACTTCCCCTG, reverse: 5' CACAGAATGGTGGAGAGCTG (product size 100 bp). For quantitative PCR, 10 μl volumes were used in the capillaries. Mg²⁺ concentration was 3 mM, primer concentration 500 nM. Standards were duplicated. Embryo samples were single. To detect interassay drift, a previously quantified embryo sample was included. PCR conditions were: initial denaturation 95°C 30 seconds, 20°C per second; annealing 55°C 10 seconds, 20°C per second; extension 72°C 13 seconds, 2°C per second; denaturation 95°C 0 seconds, 20°C per second, 40 cycles. Melt curve data acquisition was from 65 to 95°C, 0.1°C per second.

Immunohistochemistry

Staged embryos were either taken from ovigerous females, or for experiments involving temporally timed staging (i.e. days after eye anlage formation, or days before hatch), groups of embryos attached to egg strings were cultured at 18°C in 24-well tissue culture plates in sterile seawater, which was changed daily. Under these conditions, development was identical to corresponding events in the mother crab (at 18°C), and even under prolonged periods of in vitro culture (~3

weeks), hatching times were the same. This allowed us to precisely stage embryo samples and estimate the duration of each stage (Table 1). Groups of embryos were microscopically dissected in fixative to remove the eggshell (essential to allow adequate fixation and antibody penetration). For CHH, MIH, and PDH, Stefanini's fixative was used (Stefanini et al., 1967). For CCAP and RPCH, embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) containing 1% 1-ethyl 3' (3-dimethyl aminopropyl)-carbodiimide. All fixations were for 24 hours at 4°C. Embryos were subsequently washed for 24 hours in 0.1 M PB containing 0.1% Triton X-100, 0.05% sodium azide (PTX). Antisera raised against *Carcinus* MIH and *Uca* PDH have previously been described (Dirksen et al., 1987; Dirksen et al., 1988) and were used at 1:1000 in PTX for up to 4 days at 4°C. RPCH antiserum was raised in rabbits using an N-terminal acetylthioacetyl RPCH analogue (J. Riehm, Pensacola) coupled to bovine thyroglobulin via maleiamidocaproyl-*N*-hydroxysuccinimide (H. Dirksen, Stockholm, Sweden). Milligram quantities of conjugate were emulsified in Freund's adjuvant and injected at multiple subcutaneous sites into two New Zealand white rabbits at 4-weekly intervals, followed by terminal exsanguination after 12 weeks. Principles of laboratory animal care and specific national laws were followed. This antibody showed high specificity to RPCH and was used at 1:500 for whole mounts. Detection of primary antiserum was by goat anti-rabbit FITC (1:50, 1-2 days RT). For double immunolabelling experiments, the second primary antibody used (CHH) was directly coupled to Cy3, by incubating the purified IgG (Protein A Sepharose) with excess dye in 0.1 M bicarbonate buffer, pH 9.2 (1 hour, RT), followed by Sephadex G-25 chromatography and concentration by ultrafiltration (Amicon PM10). Optimum working dilutions were determined by experiment. Incubations in the Cy3-labelled antibodies were performed for 24 hours at RT. Embryos were mounted in 50% glycerol/PTX. Preparations were analysed by confocal microscopy using a Zeiss LSM 510 instrument. For stacked projection image analysis, proprietary instrument software was used. In general, between 25 and 40 serial 1.5 μm images were collected. Images were manipulated using Adobe Photoshop 7.0 and Coreldraw 8.0 software.

Radioimmunoassays

Batches of 100 staged embryos were homogenised in PB, centrifuged, applied to Sep-Pak C₁₈ cartridges (Waters), eluted with 40% isopropanol and dried by vacuum centrifugation. Following reconstitution in PB, aliquots were assayed for CHH by RIA using ¹²⁵I-CHH radioligand (Webster, 1996). For the CCAP radioimmunoassay (RIA), ¹²⁵I-Y₂CCAP was prepared using chloramine T iodination (Bolton, 1989). Final antibody dilution was (1:12,000) for both assays. For pericardial organ (PO)-CHH estimations, PO-CHH was similarly radioiodinated, and in this case the corresponding unlabelled ligand was used as competitor, using the existing sinus gland (SG)-CHH antibody (final dilution 1:10000). In both cases, subsequent RIA procedures were as previously described (Webster, 1996). To identify immunoreactive fractions, HPLC-RIA for all three neuropeptides was performed on a batch of 5000 embryos (1-2 days before hatching) which were purified on Sep-Pak cartridges (10), pooled, dried, reconstituted in 2 M acetic acid, and separated on a μBondapak Phenyl (Waters) column (3.9×300 mm), using a linear gradient of trifluoroacetic acid (TFA): acetonitrile (solvent A, 0.11% TFA, B, 0.1% TFA in 60% acetonitrile, 30-80% B over 50 minutes, 1 ml/minute, detection at 210 nm). Fractions (1 minute) were dried and assayed.

Estimation of water uptake during embryonic eclosion using [³H] water

Batches of 20 embryos, which were staged as 'imminent hatch' (within 1 hour) were incubated in 10 μl crustacean saline (with/without Ca²⁺) containing 1.85 MBq [³H] water for 1 hour. At the end of the incubation period, embryos were rapidly and extensively

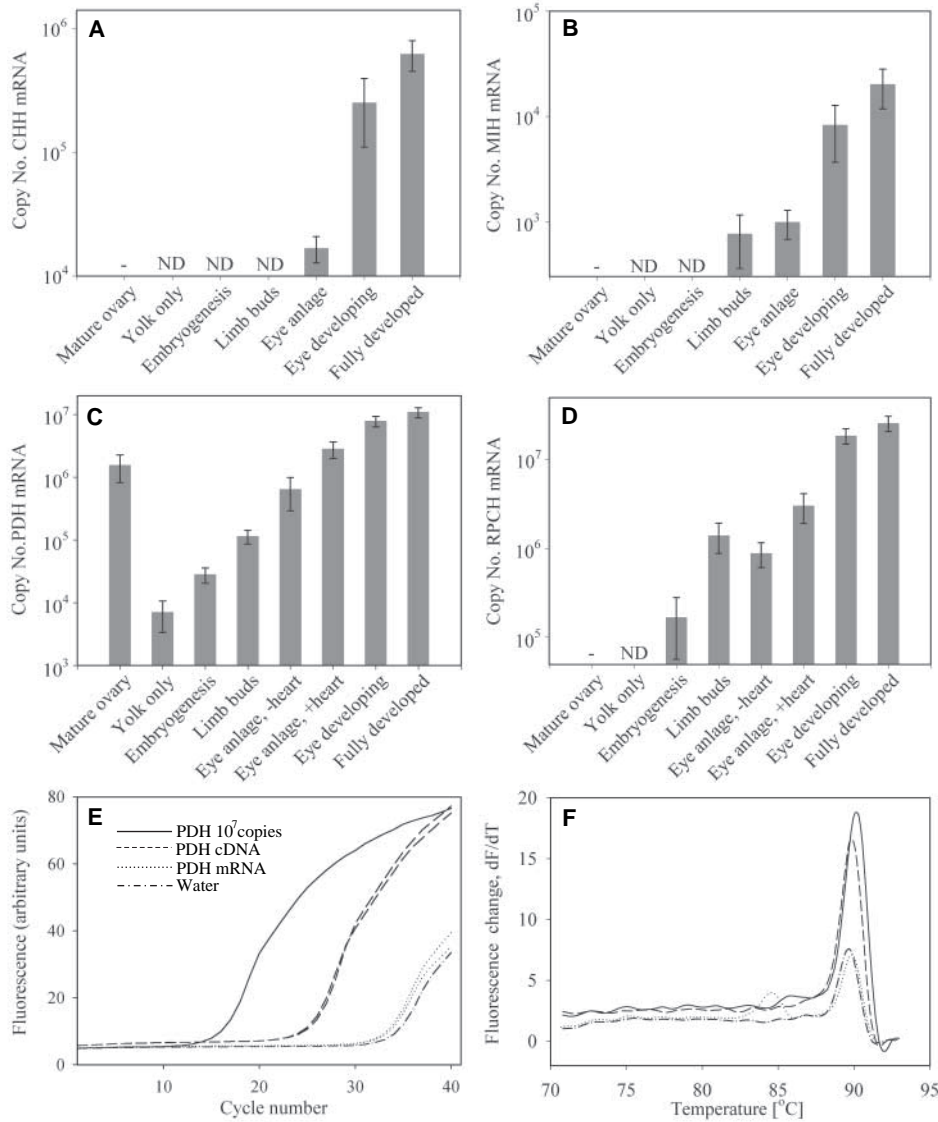


Fig. 1. Expression of neuropeptide mRNAs during embryonic development. (A) CHH, (B) MIH, (C) PDH, (D) RPCH; $n=3-11$ independent measurements. Error bars are ± 1 s.e.m.; y-axes are logarithmic. ND, no specific product detected, - not sampled. (E) and (F) amplification and melt curves from standard copy-quantified PDH (10^7) copies/capillary in comparison with cDNA and mRNA extracted from unfertilised eggs (two egg equivalents/capillary). For definition of developmental stages see Table 1.

washed with crustacean saline, briefly blotted, transferred into tubes containing scintillation fluid, and counted. Conversion of dpm to nanolitres (nl) gave a measure of the net water uptake/embryo.

Results

Expression of neuropeptide genes during embryonic development

Steady-state mRNA levels, measured by quantitative PCR for CHH, MIH, PDH and RPCH are shown in Fig. 1. For CHH, low-level expression was first observed during eye anlage formation [1.7×10^4 copies/embryo (CPE)]. Expression increased dramatically during eye formation, and at full development (6.2×10^5 CPE) had increased by more than 30-

fold. For MIH, the situation was somewhat similar, except that transcript levels were more than an order of magnitude lower. While low-level expression was occasionally measured during limb formation, at eye anlage formation, all embryos showed MIH expression (1.3×10^3 CPE). At full development, mean levels were 2.3×10^4 CPE – a 20-fold increase in expression. Since these peptides appear to only function as neurohormones, it was interesting to compare them with the peptides of PDH and RPCH that have proven additional neurotransmitter/modulator roles in the adult central nervous system (CNS). For PDH, levels increased about 500-fold during embryogenesis, from $2-3 \times 10^4$ CPE to more than 10^7 CPE) at full development. Interestingly, only PDH transcripts were expressed at significant levels in mature unfertilised oocytes ($\sim 10^6$ copies/oocyte, $n=5$). This was not due to gDNA contamination, since mRNA samples did not show appreciable amplification of specific product until approximately 35 cycles, which was similar to that of water-only controls (Fig. 1E). Product specificity was shown by melt-curve analysis (Fig. 1F), which shows that standard and cDNA amplicons have a melt temperature of 90°C . The small peak seen for PDH mRNA at this temperature could be due to the intrinsic low-level Taq-RT activity, gDNA or carry-over contamination. The very small peak at 85°C is primer-dimer/non-specific amplification. Thus the mRNA seen in the oocytes is maternally derived. For RPCH, mRNA was first detected during early embryogenesis (1.7×10^5 CPE), but in this case a significant increase was seen during limb bud and eye anlage formation. Levels in fully developed embryos were 2.5×10^7 CPE, an increase of about 150-fold during embryogenesis. For RPCH and PDH, transcripts were much more abundant (10- to 100-fold) than for MIH and CHH during comparable stages of later embryonic development, and significant expression of PDH and RPCH mRNA occurred before eye anlage formation.

Neuronal expression patterns of neuropeptides during embryonic development

To put the neuropeptide gene expression information in context with translated peptide and neuronal development, we investigated this using whole-mount immunohistochemistry and confocal microscopy of embryos during development, and

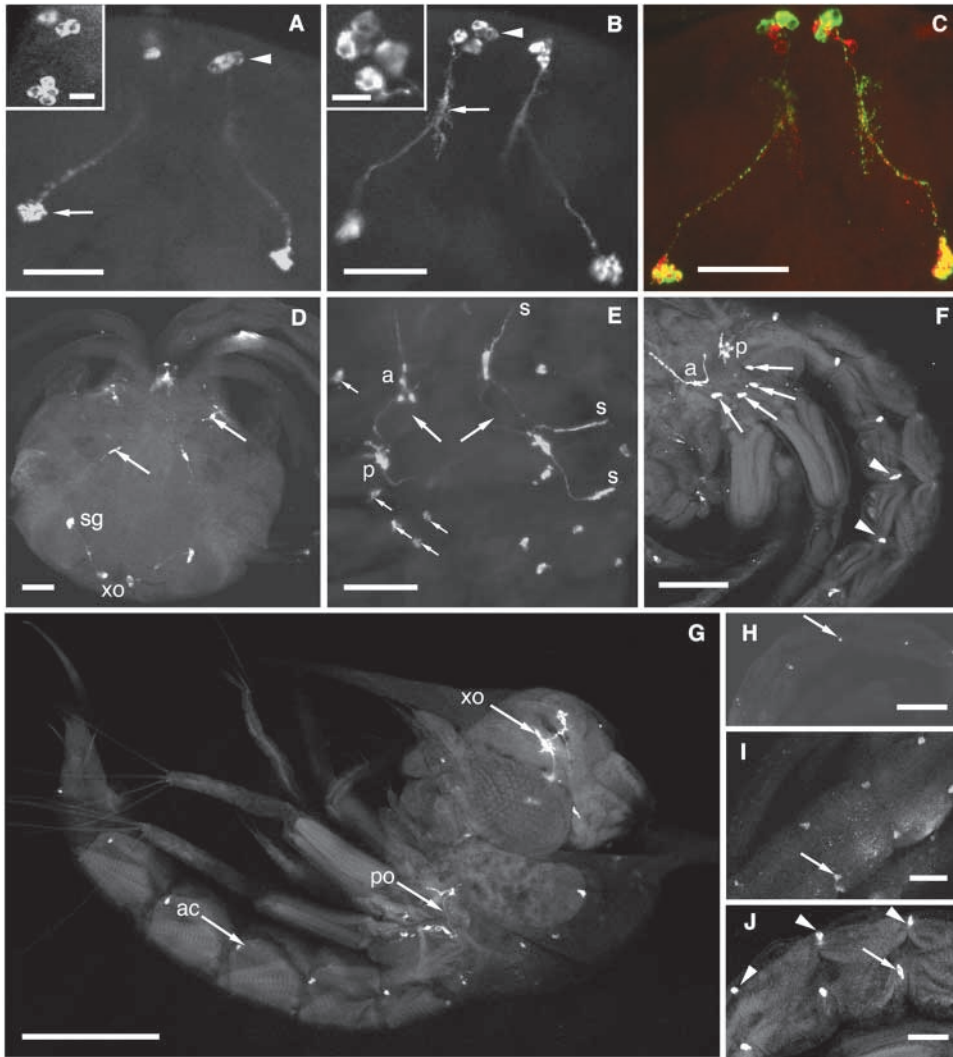


Fig. 2. Developmental profiles of CHH- and MIH-expressing neurones during embryonic development. All confocal images are single sections, with the exception of D, which is a stacked and flattened projection image. (A) MIH-IR neurones at mid-eye development. Arrowhead, perikarya; arrow, sinus gland. Inset, higher magnification showing groups of four perikarya in each side of the developing eye. (B) CHH-IR neurones at mid-eye development. Note perikarya (arrowhead) and collaterals (arrow). Inset shows higher magnification of four perikarya. (C) Stacked ($40 \times 1.5 \mu\text{m}$), dual immunolabelled projection image of MIH (green, FITC) and CHH (red, Cy3) X-organ perikarya, axon tracts and sinus gland. Note that both peptides are not colocalised, but that the merged view through the sinus gland shows some areas of false colocalisation (yellow) due to processing of the image. (D) Expression of CHH in the pericardial organs at mid-eye development. Immunoreactive structures in the anterior and posterior bars of the pericardial organs are arrowed; sg, sinus gland, xo, X-organ. (E) Detail of the CHH-IR structures in the PO and adjacent areas during late eye development just prior to hatching. Note anastomoses between anterior and posterior bars (arrows; a,p), and five pairs of peripheral IR perikarya (left, small arrows). (F) View of posterior thorax and abdomen of a newly emerged zoea. Anterior and

posterior bars of the pericardial organs are shown (a,p). Arrows show four small ventral peripheral thoracic perikarya (arrows) and segmentally iterated lateral abdominal perikarya (arrowheads) in lateral positions close to the anterior insertions of the abdominal flexor muscles.

(G) Overview of CHH-IR structures in a newly emerged zoea. Arrows show the X-organ (xo), pericardial organs (po), and abdominal cells (ac). (H-K) Development of CHH-IR cells in the abdomen. (H) 4 days before hatching, (I) 2 days before hatching, (J) at hatching. Arrowheads in J show dorsal cells, which develop at this time, and appear to be close to the posterior insertions of the abdominal extensor muscles. Scale bars: 200 μm (A), 50 μm (B-F), 100 μm (G), 200 μm (H), 50 μm (I-K). Insets (B,C): 10 μm .

the embryonic staging system as described (Table 1), but in some instances subdivisions based on time (after eye anlage formation or to hatching) for cultured embryos were used.

MIH and CHH

Immunopositive perikarya located in the developing X-organ were first detected at eye anlage formation. By mid-eye development, the complete eyestalk neurosecretory system could be seen (Fig. 2A-C). For each peptide, four antero-dorsal perikarya (6-8 μm) projected axon tracts posteroventrally to the sinus gland (20 μm). Along the axon tract, branching collaterals were observed. To eliminate the possibility (in view of the identical numbers of CHH and MIH neurones) of colocalisation of both peptides at this time, we performed double labelling immunohistochemistry (Fig. 2C), which conclusively demonstrated the absence of

colocalisation. During subsequent development, no further changes were seen for MIH, but for CHH the situation was different: At mid-eye development, about 3-4 days after establishment of regular heartbeat, CHH immunoreactivity (IR) was seen in the developing pericardial organs (PO) (Fig. 2D). By full development, fine detail could be seen including IR structures corresponding to the segmental nerves and anastomoses between anterior and posterior bars. Although generally discrete intrinsic perikarya could not clearly be distinguished in the anterior and posterior bars, some preparations (Fig. 2E) suggested that there were 3-4 cells in the anterior bar. At this time, 4-5 pairs of CHH-expressing cells could be observed immediately ventral to the PO (Fig. 2E,F) and further novel CHH-IR cells were seen (Fig. 2F). At the posterior midline of each abdominal segment, seven pairs of tiny (6-7 μm) serially iterated cells could be seen. The position

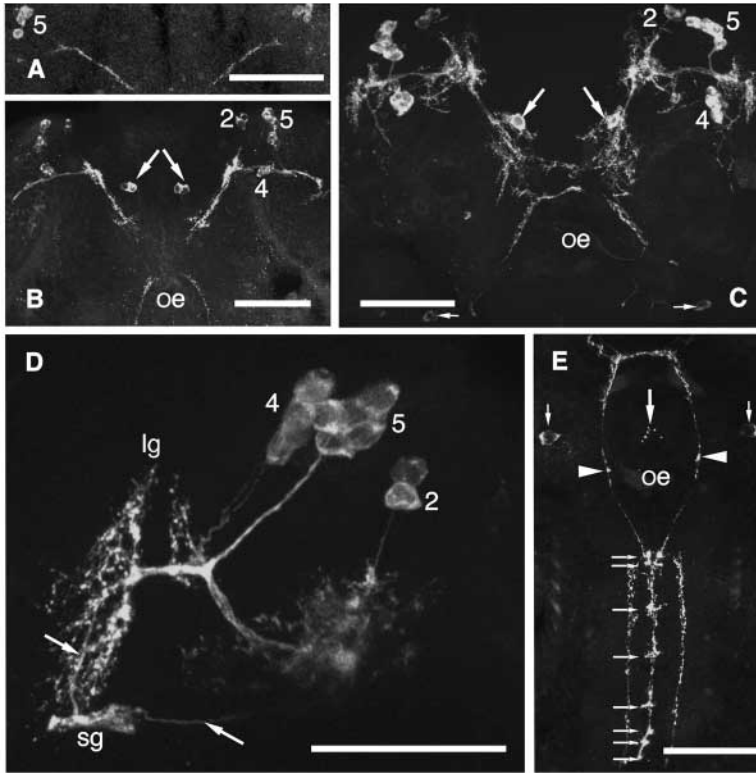


Fig. 3. Developmental profiles of neurones immunoreactive to PDH during embryonic development. All images are stacked flattened projection images (20–40 1 μ m slices) (A) PDH-IR neurones 1–2 days after eye anlage formation, five pairs of perikarya can be seen. (B) PDH-IR neurones 2–3 days after eye anlage formation. Note two pairs of anterior-median brain neurones and groups of 2, 4 and 5 perikarya in the eye neuropil. (C) PDH-IR neurones 7–8 days after eye anlage formation. Note that only one pair of anterior-median protocerebral neurones is now evident (arrows) and that single immunoreactive neurones appear in the posterior region of the tritocerebrum (small arrows). (D) PDH-IR neurones in the eye neuropil of a fully developed embryo. Note axons entering and leaving the sinus gland (sg, arrows), and development of a prominent plexus in the lamina ganglionaris (lg). Perikarya numbers (11) remain unchanged. (E) PDH-IR structures 7–8 days after eye anlage formation. Perikarya marked with small arrows correspond to those in C. Note the immunoreactive neurones possibly projecting to the stomatogastric ganglion (arrow), which originate from a small IR structure in the circumoesophageal connectives (arrowheads) Small arrows point to arborising (possibly segmentally iterated) dendrites in the thorax. Abbreviations: lg, lamina ganglionaris; oe, oesophagus; sg, sinus gland. Scale bars: 50 μ m.

of these cells appeared to correspond closely with the position of the abdominal flexor muscle insertions. These cells were first observed 4 days before hatching (Fig. 2H). Immunolabelling was most intense just before hatching, when a further set of dorsal, paired cells appeared (Fig. 2J), which seemed to be associated with the insertions of the abdominal extensor muscles. The abdominal cells could be observed in the freshly hatched zoea; an overview of all the CHH-IR structures is shown in Fig. 2G. The fate of these cells in subsequent zoeal life could not be followed, since the larval cuticle becomes an overwhelming barrier to antibody penetration within a few hours of hatching at the beginning of zoeal intermoult.

PDH

A group of five (8 μ m) antero-dorsal perikarya, that project axons postero-ventrally towards the midline, were first seen 1–2 days after eye anlage formation (Fig. 3A), i.e. a little later than had been seen for MIH and CHH perikarya of the X-organ. Subsequent neurogenesis was rapid; 2–3 days after eye anlage formation, further groups of two and four perikarya developed in the eye, together with an extensive network of axons directed to the circumoesophageal connectives. At this time, two pairs of ventro-medial perikarya (8 μ m) became prominent in the posterior protocerebrum (Fig. 3B). Within 7–8 days of eye anlage formation, the three groups of PDH perikarya in the developing eye became strongly immunopositive, and plexuses in the lamina ganglionaris and close to the X-organ were visible, as well as large numbers of branching dendrites in the deutocerebrum. At this time only one pair of ventro-medial perikarya was visible, but a further pair of ventral perikarya were seen in the tritocerebrum (Fig.

3C). At complete development, PDH-IR structures in the eye were reminiscent of those seen in the adult, particularly with regard to the position of the plexuses, and a prominent axon which enters and leaves the sinus gland, presumably directed to the optic nerve (Fig. 3D). At this time, thoracic PDH-IR structures became prominent throughout the thorax (Fig. 3E). Up to eight segmentally iterated regions of arborisations corresponding to the developing thoracic ganglia could be seen. Additionally, fine fibres were seen, which may correspond to the position of the stomatogastric nerves, projecting from a thickening in the circum-oesophageal connectives. Prior to hatching, branches of the PDH immunopositive axons, terminating in the last abdominal segment were seen in the abdominal ventral nerve cord (results not shown), but in no instances were perikarya observed.

RPCH

Immunopositive neurones were first observed 5–6 days after eye anlage formation, which may be significant in that this corresponded to the time of first appearance of red chromatophores. At this time three perikarya (8 μ m) were seen in each X-organ, and the sinus gland and associated tract were strongly immunopositive (Fig. 4A). However, as other neurones began to express RPCH, the eyestalk perikarya showed surprisingly little RPCH-IR. At 6–7 days after eye anlage development, three pairs of perikarya in the posterior region of the protocerebrum became strongly immunopositive to RPCH (Fig. 4B). These cells project descending axons to the circum-oesophageal connectives, where they form ipsi- and contralateral projections. These neurones then send processes across the post-oesophageal commissure, and direct branches dorsally to prominent three-branched neurohaemal structures

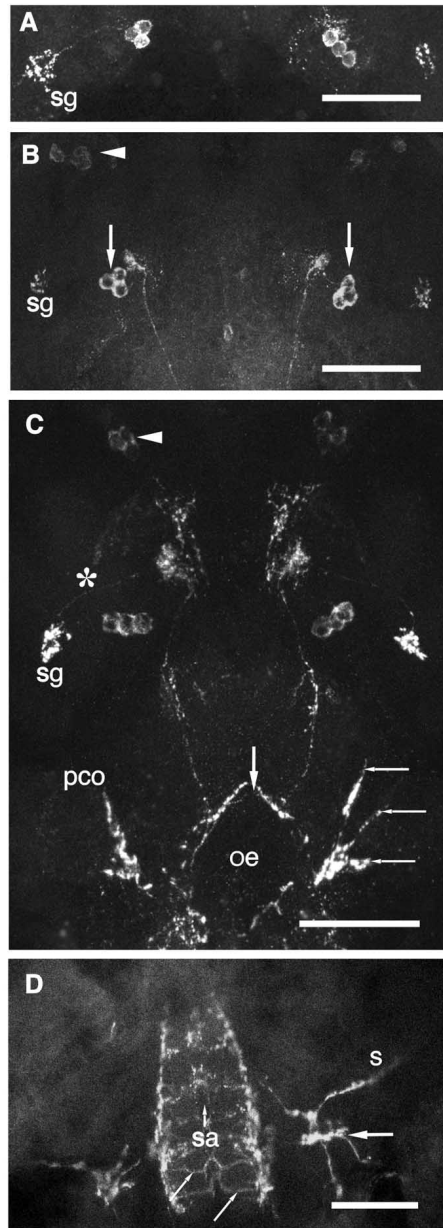


Fig. 4. Developmental profiles of neurons immunoreactive to RPCH and CCAP during embryonic development. (A) RPCH-IR neurons (X-organ) and immunopositive endings within the sinus gland (sg) in the eye, 5-6 days after eye anlage formation. (B) RPCH-IR structures in the brain and eye 6-7 days after eye anlage formation. Note that immunoreactivity in the X-organ (arrowheads) is weak, but that three pairs of prominent cells in the protocerebrum (arrows) now give rise to descending axons. (C) RPCH-IR structures in an embryo 2-3 days before hatching. Note descending axons, some (if not all) of which project contralaterally, anterior to the oesophagus (oe, arrow), and the prominence of, and connections to the putative post-commissural organs (pco), which dorsally project three digitate extensions (small arrows). (D) CCAP-IR structures associated with the thoracic ganglion and pericardial organs during mid-eye development. Large arrow, the posterior bar of the pericardial organ; small arrows, contralateral projections of axons reminiscent of those seen in the adult. Perikarya were never observed in these preparations. Abbreviations: oe, oesophagus; pco, post-commissural organs; s, segmental nerve; sa, sternal artery; sg, sinus gland. Scale bars: 50 μm .

which reach full development about 2-3 days before hatching (Fig. 4C).

CCAP

Immunopositive structures were only observed during mid-eye development. Despite many attempts, using a variety of fixatives, adequate maintenance of antigenicity and antibody penetration was problematical. While perikarya were never observed, neuronal structures in the thoracic ganglion, and posterior bar of the PO were seen, and the morphology of these, including contralaterally projecting neurones, was reminiscent of adult morphology (Fig. 4D).

Quantification of CHH and CCAP content of embryos during development

We measured whole-embryo levels of both peptides by RIA, during complete embryonic development, to see whether events in embryos resembled those seen during adult moulting. CHH was first detected during eye anlage development, and remained low (0.2 fmol/embryo) until 5 days before hatching when there was a steady increase to about 2 fmol/embryo, 1 day before hatching. During hatching, levels increased dramatically, to over 6 fmol/embryo, and immediately declined to less than 2 fmol/embryo within 1 hour of hatching (Fig. 5A). CCAP was first detected 15 days before hatching, at late eye anlage stage – it is significant to note that this stage was marked by the appearance of a regular heartbeat (82 ± 20 beats/minute, $n=15$). Peak levels of CCAP (4 fmol/embryo) were seen during hatching; these declined to intermediate levels within 1 day post-hatching (Fig. 5A). HPLC-RIA analysis of a sample of fully developed embryos (2 days before hatching), showed the presence of immunoreactive material corresponding to elution times of CCAP, XO-CHH, and the earlier eluting PO-CHH (Fig. 5B).

Water uptake during hatching

Water uptake prior to hatching was measured by estimation of tritiated water uptake, since rupture of the eggshell, which must involve significant water uptake, is critical to the appropriate emergence of the prezoa. Since CHH stimulates a very large uptake of water during adult ecdysis, we were interested in measuring this phenomenon during larval hatching and correlating it to CHH release dynamics. Embryos incubated in normal crustacean saline took up 7.2 ± 1.9 nl (mean \pm s.e.m.; $n=5$) [^3H] water/hour, just before eggshell rupture. At this time, CHH levels were at their highest (6 ± 0.75 fmol/embryo ($n=8$)) just before hatching, but declined to 1.7 ± 0.3 fmol/embryo ($n=6$) at the completion of larval eclosion (Fig. 5A). For embryos incubated in Ca^{2+} -free saline, water uptake was markedly diminished (1.9 ± 0.5 nl, $n=6$), and the decrease in CHH at completion of eclosion was not as marked (3.7 ± 0.8 fmol/embryo, $n=7$) as that of embryos incubated in normal saline.

Discussion

The present study has focussed upon the expression of MIH, CHH and CCAP during embryonic development, given their roles as important neurohormones regulating ecdysteroid synthesis and eclosion behaviour in the adult, since we consider embryonic eclosion to be essentially similar to the

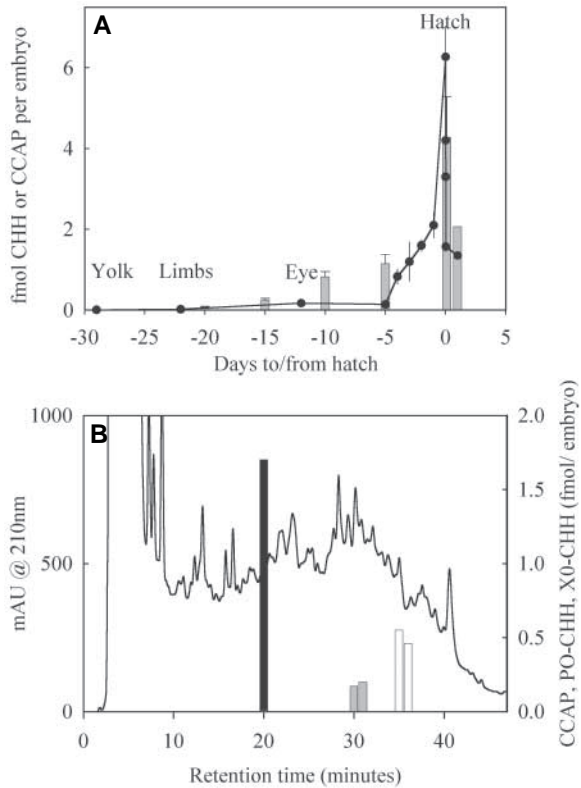


Fig. 5. Quantification of CHH and CCAP levels during embryonic development, measured by RIA. (A) Developmental profiles for both neurohormones, bars CCAP; line, CHH; $n=5-13$ for CCAP, $5-23$ for CHH. (B) An HPLC-RIA analysis of CHH and CCAP immunoreactive fractions of a Sep-Pak purified sample of 5000 embryos. Filled bar, CCAP; grey bar, PO-CHH; open bar, XO-CHH (see Materials and methods for details of analysis).

adult moult. However, to contrast this situation with other neurohormones that are expressed during embryonic development, we have also included an analysis of RPCH and PDH, since these are also important neuromodulators. In view of the observations on expression of neuropeptides in the stomatogastric ganglion of lobsters, suggesting that some projection neurones do not express a 'peptide phenotype' until late in development (Le Feuvre et al., 2001), we considered our studies, which involve quantitative measurement of specific mRNAs in conjunction with neuroanatomical studies, to be especially relevant: Do transcript phenotypes mirror peptide expression?

For MIH and CHH we observed significant expression of both mRNAs at the beginning of eye anlage formation, which rapidly increased during subsequent development. By analogy to the detailed developmental series described by Helluy and Beltz (1991) for the lobster, *Homarus americanus*, this event could be correlated to a period just following the naupliar moult (12% development); however, for crabs, it seems to be somewhat later, at about 50% development (Okazaki and Chang, 1991), which just precedes the rapid increases in ecdysteroid titre indicative of premoult, and which would also correspond with a developmental stage of about 50% in the lobster. This difference is probably due to the extended embryogenesis in the former species. The only work that has

mentioned the development of the Y-organ in embryonic crustaceans (Le Roux, 1983) (A. Le Roux, Thèse de Doctorat d'Etat, Université de Rennes, 1989) indicates that it forms just before eye development, i.e. during metanaupliar intermoult. For *Carcinus*, both MIH and CHH mRNAs are clearly expressed at the beginning of eye anlage formation, an observation that was consistent with immunohistochemical studies. During mid-eye development, four pairs of anteriodorsal perikarya showing immunoreactivity to MIH or CHH were observed in the eyes, projecting neurones to a posteroventral SG. While in the adult it is well known that both neuropeptides do not colocalise (Dirksen et al., 1988), in view of the very similar morphologies exhibited by both sets of neurones, we further investigated this by double immunolabelling (Fig. 1C). These experiments showed conclusively that, despite the identical neuronal number and similar position (in the developing X-organ), these peptides never colocalise. In our previous studies on larval expression of MIH, we observed that only four neurones (in each eyestalk) express MIH throughout larval development (Webster and Dirksen, 1991). The present study also confirms this during embryonic development. In the adult, 28-36 eyestalk neurones express MIH in comparison to 62-65 for CHH (Dirksen et al., 1988). In adult *Carcinus*, steady-state expression of CHH mRNA is about 2.3×10^7 and for MIH $0.5-1.3 \times 10^6$ copies/cell, i.e. a ratio of 20-40 CHH:1 MIH (Chung and Webster, 2003). Since both CHH and MIH perikarya have diameters of $\sim 70 \mu\text{m}$ in the adult, and $8 \mu\text{m}$ in the embryo, adult cell volumes are about 180 pl, and for embryos, 27 pl. Thus in adults, copy number/pl are about 1.3×10^5 for CHH and $3-3.4 \times 10^3$ for MIH. For embryos at mid-eye development, respective copy numbers/pl are $0.9-1.4 \times 10^5$ for CHH, and 4.4×10^3 for MIH, which are in accordance. Thus, in the embryo, transcriptional processes are dynamically similar to those of the adult, and steady-state ratios of CHH and MIH transcripts are similar.

During later embryonic development, extra-eyestalk sources of CHH-like peptides become significant: during late eye development, 3-5 days before hatching, CHH-IR neurones became prominent in the pericardial organs (PO), and associated peripheral neurones, and later in a set of serially iterated pairs of lateral and dorsal cells in each segment of the abdomen. It should be noted that with the primers used for quantitative RT-PCR, only the prototypical CHH (XO-CHH) was measured. The PO-CHH splice variant, which is expressed by intrinsic cells in the PO of the adult (Dirksen et al., 2001) was not amplified. Nevertheless, the antiserum used for ICC could detect both translated products. We addressed this problem regarding expression of the two CHH isoforms by measuring CHH by RIA during the days prior to eclosion. This assay only measures XO-CHH. However, by using quantified PO-CHH, and ^{125}I radiolabel in conjunction with the XO-CHH antiserum, we could detect the PO-CHH splice variant in addition to XO-CHH in HPLC-separated peptide fractions from embryos at 3-5 days prior to hatching (Fig. 5B). As in the adult, both splice variants are expressed, and the PO-CHH is possibly expressed primarily by the intrinsic neurones in the PO. For XO-CHH there is a gradual increase in CHH levels in late embryos, culminating with a dramatic increase prior to eclosion, and an equally impressive decline within an hour of this event, which corresponds with shedding of the prezoal cuticle. These events exactly mirror those in the adult crab,

where premoult is associated with a dramatic release of CHH from gut endocrine cells (Chung et al., 1999). Since gut CHH endocrine cells were absent in *Carcinus* embryos, we suggest that in the embryo, the embryonic abdominal serially iterated cells may be involved in the CHH surge seen in adult moulting. However, although CHH-IR declines precipitously during postmoult, we were unable to record diminution in immunoreactivity of these cells following zoeal moulting, since the cuticle becomes completely impermeable to antibody penetration at this time. Since rupture of the eggshell during hatching must involve significant water uptake (Saigusa and Terajima, 2000), we measured net water influx in the immediate period prior to hatching, using tritiated water. During the hour before hatching, net uptake was around 7-8 nl (approximately 15-20% of the embryo body volume). Since this was correlated with the peak in whole-body CHH titre, we tried to manipulate CHH release, to see whether this would affect water uptake, in an attempt to establish a causal relationship between water uptake and CHH release. In the adult, CHH release from premoult (D₃₋₄) hindgut in vitro is dramatically diminished in nominally calcium-free conditions (J.S.C. and S.G.W., unpublished). In the embryo incubated in Ca²⁺-free medium, water uptake was reduced compared to normal controls, and CHH release was also impaired.

With regard to developmental expression of CCAP transcripts in embryonic crustaceans, nothing can as yet be said, since the CCAP gene, or coding sequences have not been determined in any crustacean (in contrast to insects, i.e. *Manduca* (Loi et al., 2001)). However, we could observe immunopositive structures reminiscent of those seen in the adult thoracic ganglion (TG) during late eye formation (Fig. 4D,F). Although we could never observe serially iterated perikarya in this tissue, as shown in the adult (Dirksen, 1998; Dirksen and Keller, 1988), the overall morphology of immunopositive structures in the thoracic ganglion and PO was clearly reminiscent of this. Furthermore, during eclosion, CCAP levels, measured by RIA exhibit similar patterns to those seen during adult moulting (Phlippen et al., 2000). It is also notable that during eclosion, analogous stereotyped behaviours were observed, such as dramatic increases in heart rate from 260±12, (n=20) beats/minute in completely developed embryos to 335±14 (n=15) beats/minute just before hatching. Just after hatching, prezoae show stereotyped rapid, intense, but intermittent circular swimming episodes. These phenotypes, together with those showing reduction in CCAP content after hatching are suggestive of a large release of CCAP at eclosion, which is analogous to events during adult ecdysis. The results obtained in this study (for CHH and CCAP) are the first concerning the neurohormonal control of embryonic moulting in crustaceans, and are of interest since these events appear to be essentially the same as those seen during adult ecdysis, but just at a very small scale.

Since expression of CHH or MIH occurred quite late in embryonic development, we were also interested in studying the expression patterns of the other 'eyestalk' neuropeptides which are well known to have neuromodulatory roles in addition to those first established by classical endocrinology as 'neurohormonal', i.e. the crustacean pigmentary effector hormones, PDH and RPCH (Rao, 2001). In view of this, it seemed likely that expression might occur earlier in embryogenesis, in comparison to MIH and CHH. The results

here were surprising. For PDH, expression of mRNA was evident throughout embryonic development, and interestingly, maternally derived mRNA in the unfertilised oocyte was significant. The functional significance of this observation is unknown, but may possibly point to circadian clock-driven processes, since PDH has been identified as an important component of the circadian clock output pathways of *Drosophila* (Park et al., 2000; Renn et al., 1999). For RPCH, mRNA expression was first recorded during naupliar development. Perhaps the most surprising observation related to the magnitude of expression – for both mRNAs, steady-state expression levels were 10 to 100-fold greater than for CHH or MIH – an observation that is at odds with the amount of translated product in the adult. The chromatophorotrophins are rather minor constituents of the neuropeptide inventory of *Carcinus* eyestalks or sinus glands. For example, mean total PDH content of the *Carcinus* eyestalk is less than 20 pmol (Löhr et al., 1993), for MIH, SG levels are 36-55 pmol and for CHH 270-490 pmol (Chung and Webster, 2003). The late appearance of translated peptide, despite the earlier presence of significant numbers of transcripts was intriguing: during development, peptides could only be detected in the metanaupliar stage, i.e. during eye formation. For PDH peptide expression, five pairs of perikarya were first observed 1-2 days after eye anlage formation, and following this a rapid development of descending neurones was observed. By late eye development, a complex arrangement of neurones, involving invariant numbers of PDH-expressing neurones (2,5,4; Fig. 3C,D) was seen in the eye. As has previously been mentioned (Mangerich et al., 1987) it was difficult to trace individual axons, and this was also the case here, given the small size of the developing eye (<100 µm). Notwithstanding this, branching arborisations in the lamina ganglionaris were evident, as were prominent axons which entered and left the SG, projecting towards the optic nerve, and T-shaped axons from cell group 5, projecting to both the lamina ganglionaris and the X-organ. This neuroanatomy was reminiscent of the neuronal architecture in the adult (Mangerich et al., 1987). During later development, axons ran throughout the thoracic ventral nerve cord, and eventually the abdomen. With regard to the thoracic projections (Fig. 3E), it was interesting to note that eight clusters of dendrites, which presumably correspond to the developing segmental ganglia were evident. With reference to the detailed description of the development of the post-mandibular and thoracic neuromeres of decapod crustaceans (Harzsch, 2003) there is some correspondence. If the first developing post-oesophageal dendrites correspond to the anterior mandibular ganglion, and the second (where the median nerve arises) is the posterior mandibular ganglion, then subsequent dendritic masses would correspond to maxilla 1, 2 and the first 4 thoracic neuromeres, notwithstanding the absence of immunoreactive structures in thoracic neuromeres 5-8. It should also be noted that at this time, single fibres, presumably projecting along the stomatogastric nerves, arise from the developing ganglion in the circumoesophageal connectives, which suggest that the beginnings of a PDH phenotype of the adult stomatogastric ganglion (STG) (Mortin and Marder, 1991) develops at this time.

For RPCH, significant levels of transcript were first observed during naupliar development, which then increased dramatically. An important correlative event concerned the

appearance of small red chromatophores coincident with eye anlage development, as reported for *Homarus americanus* (Helluy and Beltz, 1991). This correlated with a 5 to 10-fold increase in expression of RPCH mRNA. However, peptide immunoreactivity was first detected 5-6 days after eye anlage formation (Fig. 4A). While initial expression of RPCH was restricted to the XO-SG axis at this time, further development revealed a notable complexity of RPCH immunopositive neurones (Fig. 4B,C). The salient features here concern the appearance of three pairs of perikarya in the posterior protocerebrum 6-7 days before hatching, which project descending axons, contra- and ipsilaterally, around the oesophagus to the post-commissural nerve, and then project antero-dorsal digitate projections which are undoubtedly release sites (Fig. 4C) and which probably correspond to the somewhat enigmatic post-commissural organs (PCO). Although described over 50 years ago (Knowles, 1953; Maynard, 1961) in adult crustaceans, their structure and function has been, rather surprisingly, overlooked. Excepting early observations (Carlisle and Knowles, 1953; Knowles, 1953) suggesting that they are a source of pigment-concentrating hormone activity, there appear to have been no other studies upon this neurohaemal tissue. Given the large amount of RPCH stored in these structures, which seem (from intensity of immunoreactivity) to far exceed that in the SG, the PCOs may have some particular significance in embryonic life.

In summary, the present study has described the fine detail of developmental expression of neurohormones and their transcripts involved in embryonic moulting in a crab model, and contrasts these with expression patterns of the chromatophorophins, which have roles as neurotransmitters as well as classical neurohormones. This study additionally details embryonic expression of novel peptide-producing cells and neurones. Now that normal developmental expression patterns are known, the next appropriate and exciting step must be to use gene-silencing technologies, to knockdown expression of each of these neuropeptides during embryonic development, and observe subsequent phenotypes. Indeed, the small size of crustacean embryos, coupled to their rapid development and the relatively simple anatomy of neuropeptide-expressing neurones, makes this an attractive prospect.

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