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, Moult-inhibiting hormone, Pigment-dispersing hormone, Red pigment-concentrating hormone

Introduction

Embryogenesis in most marine decapod crustaceans, except Euphausia 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 prezoea), 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 prezoeal 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
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 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 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 (Philpen 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 (Dynal), 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 10^7 to 10^10 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' TGGGAAGT (product size 134 bp). RPCH (accession code S65357) forward: 5' TAGAACCCAGGTGAGCTTT, reverse: 5' TACAGCTGAGACCGGATG (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, ensuring possible extension 72°C 13 seconds, 2°C per second; 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°C 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, neurones. Finally, correlations were made regarding quantitative expression of peptides during hatching, to compare these with homologous events during adult ecdysis.

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 Ca^2+) containing 1.85 MBq [3H] water for 1 hour. At the end of the incubation period, embryos were rapidly and extensively

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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 [1.73 \(10^4\) copies/embryo (CPE)]. Expression increased dramatically during eye formation, and at full development (6.23 \(10^5\) 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 (13 \(10^3\) CPE). At full development, mean levels were 23 \(10^4\) 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 \(10^4\) CPE to more than \(10^7\) CPE at full development. Interestingly, only PDH transcripts were expressed at significant levels in mature unfertilised oocytes (~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 (1.73 \(10^5\) CPE), but in this case a significant increase was seen during limb bud and eye anlage formation. Levels in fully developed embryos were 2.53 \(10^7\) 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
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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 posterioventrally 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. 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 (403 1.5 μm), 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). Inserts (B,C): 10 μm.
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 μm) anterio-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 μm) 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 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 ventral 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 circumoesophageal 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 μm) 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.
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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 prezoea. 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±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 Ca²⁺-free saline, water uptake was markedly diminished (1.9±0.5nl, 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. 4. Developmental profiles of neurones immunoreactive to RPCH and CCAP during embryonic development. (A) RPCH-IR neurones (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.
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’État, 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 anterior-dorsal perikarya showing immunoreactivity to MIH or CHH were observed in the eyes, projecting neurones to a posterio-ventral SG. While in the adult it is well known that both neuropeptides do not colocalise (Dircksen et al., 1988), in view of the 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 Dircksen, 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 (Dircksen et al., 1988). In adult Carcinus, steady-state expression of CHH mRNA is about 23.10^7 and for MIH 0.5-1.3.10^6 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 ~70 μm in the adult, and 8 μm in the embryo, adult cell volumes are about 180 pl, and for embryos, 27 pl. Thus in adults, copy number/pl are about 13.10^5 for CHH and 3-3.43.10^3 for MIH. For embryos at mid-eye development, respective copy numbers/pl are 0.9-1.43.10^5 for CHH, and 4.43.10^3 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 (Dircksen 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 premoult, we were unable to record diminution in immunoreactivity of these cells following zoal 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 (D4-5) 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 (Dircksen, 1998; Dircksen 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 (Philpenn 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, prenauplii 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 ecdisis. 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 ecdisis, 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 (Löhö 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 neurons 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) (Morton 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
neurons (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
anterio-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
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