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Identification and developmental expression of mRNAs encoding crustacean cardioactive peptide (CCAP) in decapod crustaceans

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Summary

Full-length cDNAs encoding crustacean cardioactive peptide (CCAP) were isolated from several decapod (brachyuran and astacuran) crustaceans: the blue crab *Callinectes sapidus*, green shore crab *Carcinus maenas*, European lobster *Homarus gamarus* and calico crayfish *Orconectes immunis*. The cDNAs encode open reading frames of 143 (brachyurans) and 139–140 (astacurans) amino acids. Apart from the predicted signal peptides (30–32 amino acids), the conceptually translated precursor codes for a single copy of CCAP and four other peptides that are extremely similar in terms of amino acid sequence within these species, but which clearly show divergence into brachyuran and astacuran groups. Expression patterns of CCAP mRNA and peptide were determined during embryonic development in *Carcinus* using quantitative RT–PCR and immunohistochemistry with whole-mount confocal microscopy, and showed that significant mRNA expression (at 50% embryonic development) preceded detectable levels of CCAP in the developing central nervous system (CNS; at 70% development). Subsequent CCAP gene expression dramatically increased during the late stages of embryogenesis (80–100%), coincident with developing immunopositive structures. In adult crabs, CCAP gene expression was detected exclusively in the eyestalk, brain and in particular the thoracic ganglia, in accord with the predominance of CCAP-containing cells in this tissue. Measurement of expression patterns of CCAP mRNA in *Carcinus* and *Callinectes* thoracic ganglia throughout the moult cycle revealed only modest changes, indicating that previously observed increases in CCAP peptide levels during premoult were not transcriptionally coupled. Severe hypoxic conditions resulted in rapid downregulation of CCAP transcription in the eyestalk, but not the thoracic ganglia in *Callinectes*, and thermal challenge did not change CCAP mRNA levels. These results offer the first tantalising glimpses of involvement of CCAP in environmental adaptation to extreme, yet biologically relevant stressors, and perhaps suggest that the CCAP-containing neurones in the eyestalk might be involved in adaptation to environmental stressors.

Key words: crustacean cardioactive peptide, mRNA sequence, development, expression, decapod crustacean.

Introduction

The cyclic nonapeptide crustacean cardioactive peptide (CCAP) was first identified in the green shore crab *Carcinus maenas* almost 20 years ago (Stangier et al., 1987). It produces potent chronotropic effects on semi-isolated heart preparations, in contrast to proctolin, which exhibits mainly inotropic effects (Stangier, 1991). Additionally, CCAP is involved in modulation of stomatogastric ganglion motor patterns in crustaceans (Weimann et al., 1997; Richards and Marder, 2000). CCAP has been identified in many arthropods, revealing highly conserved neural networks (Dircksen, 1998), and related molecules may exist in other phyla, such as urechoid worms and molluscs (Ikeda et al., 1991; Minakata et al., 1993; Muneoka et al., 1994; Hernadi and Agricola, 2000; Vehovsky et al., 2005).

The role of CCAP in insects has been an area of particular interest since CCAP has several identified functions apart from its first defined biological activity – cardioacceleration (Tublitz and Truman, 1985a; Tublitz and Truman, 1985b; Tublitz and Evans, 1986; Cheung et al., 1992). These include modulation of hindgut activity involved in gut emptying during metamorphosis (Tublitz et al., 1992), increasing blood circulation during wing inflation (Tublitz and Truman, 1985b), modulation of oviduct contractions in *Manduca sexta* (Marshall and Reynolds, 1998) and *Locusta migratoria* (Donini et al., 2001; Donini and Lange, 2002) and a secretagogue action in induction of release of adipokinetic hormone from the corpora cardiaca in *L. migratoria* (Veelaert et al., 1997). However, one of the most significant actions of CCAP in insects concerns its role in the proximal triggering of ecdysis (Gammie and Truman, 1997). Specifically, targeted ablation of

CCAP-expressing neurones in *Drosophila* leads to severe defects in execution of pupal ecdysis and abnormal gating of circadian eclosion rhythms (Park et al., 2003), but intriguingly only some disruption to larval ecdysis behaviour (Clark et al., 2004). CCAP knockouts are also defective in wing expansion and cuticle tanning following eclosion (Dewey et al., 2004).

Although evidence is now accumulating to suggest that CCAP is a critical neurohormone during insect ecdysis, much less is known for crustaceans, which are genetically intractable. In crayfish and crabs a very dramatic increase in CCAP synthesis and release during moulting has been observed over a precise temporal scale (Phlippen et al., 2000), which may point to fundamental, and probably analogous, roles of this peptide to that in insects during ecdysis. One way in which we might address the role of CCAP in crustacean moulting and development in the near future, given the intractability of crustaceans to contemporary molecular approaches, would be to use knock-down techniques such as RNA interference (RNAi). Although genomic and cDNA sequences for CCAP are known in some insects, for example *Drosophila* (Park et al., 2003), *Manduca sexta* (Loi et al., 2001) and *Periplaneta americana* (Sakai et al., 2004), no comparable information is available for crustaceans. Thus, to begin to address this issue we have cloned and sequenced full-length cDNAs from a variety of brachyuran and astacuran decapods. We have used quantitative molecular approaches (quantitative reverse transcription–polymerase chain reaction, qRT–PCR) to measure CCAP gene expression during development and the moult cycle. Secondly, we have coupled a description of developmental expression of CCAP peptide in the embryonic CNS to that of the cognate mRNA to further elucidate the role of CCAP in development in crustaceans. Finally, we have measured CCAP gene expression following exposure to environmentally relevant stressors (severe hypoxia, thermal stress) that might be proposed to relate to cardiac output.

Materials and methods

Animals, experiments, tissue collection and RNA extraction

Specimens of juvenile blue crabs, *Callinectes sapidus* (Rathbun) (carapace width 45–80 mm) were reared in the Aquaculture Research Center (ARC), Center of Marine Biotechnology (COMB, Baltimore, MD, USA). They were maintained in individual compartments (0.033 m^3), in artificial seawater (15‰, 22°C) under ambient photoperiod, and were fed daily with chopped squid. Adult *C. sapidus* were obtained from the Chesapeake Bay (MD, USA) by local fishermen. Green shore crabs, *Carcinus maenas* (Linnaeus) and lobsters, *Homarus gammarus* (Linnaeus) were collected using baited traps (Anglesey, UK), and were maintained in a recirculating seawater system, under ambient conditions of photoperiod and temperature, with *ad libitum* feeding of fish and squid. Specimens of Calico crayfish, *Orconectes immunis* (Hagen), were collected from lakes in Jones Falls, Baltimore MD, USA.

Nervous systems (eyestalk, brain and thoracic ganglia) were dissected from ice-anaesthetised, moult-staged animals and immediately placed in RNAlater (Ambion, Austin, TX, USA), (4°C overnight) and stored at –80°C. Additionally, batches of 100 developmentally staged embryos (Chung and Webster, 2004) were taken from ovigerous *Carcinus maenas*. Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA, USA), and samples were subsequently treated with 2 i.u. DNase I (37°C, 1 h) followed by clean up on DNA-free (Ambion). For embryo samples, mRNA was subsequently isolated using Dynabeads (Dynal, Oslo, Norway), and stored at –80°C in 10 mmol l^{-1} Tris (10 embryo equivalents μl^{-1}).

For experiments on *Callinectes* involving anoxic and thermal stress, crabs were either exposed to reduced O_2 (0.5%) for 1 h, by continuous nitrogen sparging (monitored using a dissolved oxygen meter; YSI, Yellow Springs, OH, USA), or given thermal challenge for 2 h (from 22°C to 4°C or from 22°C to 29°C); controls remained at 22°C. All experiments were performed in 15‰ seawater. Following these experiments, eyestalk and thoracic ganglia were rapidly dissected from ice-anaesthetised crabs, and immediately frozen on dry ice, prior to storage (–80°C). Subsequent RNA extractions were as detailed above.

cDNA synthesis and rapid amplification of cDNA ends

1–2 μg samples of total RNA were reverse transcribed using AMV-RT (Promega, Madison, WI, USA) or Superscript III (Invitrogen) (42°C, 1 h). For 3′ rapid amplification of cDNA ends (RACE) cDNA, reactions were primed with Gene Racer 3′ oligo(dT) adaptor primer (Invitrogen). RNA was subsequently removed by incubation (37°C, 1 h) with 2 i.u. RNase H. For 5′ RACE cDNA, 1 μg RNA was ligated to a 5′ RACE adapter primer (Invitrogen) according to the manufacturer's instructions, and reverse transcribed using random primers.

3′ RACE was performed using PCR with nested primers. Reverse primers were as supplied by the manufacturer (Invitrogen) and forward degenerate nested primers (dF1, dF2, dF3; Table 1) were designed from the sequence of CCAP, and C-terminal amidation and cleavage sites.

3′ RACE PCR of Carcinus and Homarus cDNAs

For the first PCR, conditions were: 1 μl 10 mmol l^{-1} dNTPs, 5 μl 10 amplification buffer, 2 μl 50 mmol l^{-1} $MgSO_4$, 4.25 μl 3′ Gene Racer primer, 1 μl dF2 (100 μmol l^{-1}), 0.5 μl (1.25 units) Platinum *Pfx* DNA polymerase (Invitrogen), 2 μl cDNA, and water to 50 μl final volume. Touchdown PCR conditions were: 1 cycle of 94°C 2 min; 5 cycles of 94°C 30 s, 72°C 1 min; 5 cycles of 94°C 30 s, 70°C 1 min; followed by 25 cycles of 94°C 30 s, 55°C 1 min, 68°C 1 min; and a final extension at 68°C for 10 min. Following this PCR, a second nested PCR was performed using 1 μl of the first PCR reaction as template, with 1.25 μl of the nested 3′ RACE primer, 1.25 μl dF3 (100 μmol l^{-1}) and 22.5 μl Megamix Blue (Helena Biosciences, Sunderland, UK). Conditions were: 1 cycle of 94°C 4 min, followed by 35 cycles of 94°C 30 s, 55°C 1 min, 72°C 1 min and final extension for 10 min at 72°C. PCR products were electrophoresed on agarose gels, and bands excised and extracted (Ultrafree-DA, Millipore, Bedford, MA, USA).

Table 1. *Primer sequences used for crustacean cardioactive peptide sequence identification and quantitative PCR*

Primer	Sequence
(A) Crustacean cardioactive peptide (CCAP)	
Degenerate primers	
dF1	AARMGICCNTTYTGYAAYGC
dF2	CCITTYTGYAAYGCNTTYAC
dF3	TGYAAYGCITTYACNGGITG
Carcinus maenas GSP	
CCAP5′R1	GCCAGAAGCAAGTCCTTCTAGCTCAGGG
CCAP5′R2	CTCAGGGTCAGACCGCTTCTTACCAC
CCAP1F	GTTGGGACGTACATGGCTGGTG
CCAP1R	GTCGGCTGTGTTTTCTGGTCTTCA
CCAP2F	AGCCTTCTCCTTCTGGTGTTT
CCAP2R	CATCTTGCTTTGGAGTTGTTC
Callinectes sapidus GSP	
CCAP5′R1	GCAAGGCCCTCCAGCTCAGGATCGGA
CCAP5′R2	CTCAGGATCGGATCGTTTCTTGCCGC
CCAPLF	ATCCTCCTTCTGGCATTCCT
CCAPLR	CCGCTATGTCTCCTGCTGTT
CCAPSF	AAACACTGAAGCAGGGATCG
CCAPSR	AGCTCTGAGCCAGAAGCAAG
Homarus gammarus GSP	
CCAP5′R1	GAGGCCAAGCCTTCCATGCTGGGGTCTG
CCAP5′R2	CTTCCATGCTGGGGTCTGACCTCTT
Orconectes immunis GSP	
CCAP5′R1	GAAGCCACGCCCTCCAGGCCGGGGTCTG
CCAP5′R2	CCTCCAGGCCGGGGTCTGACCGCTT
(B) Arginine kinase (AK)	
Carcinus maenas AK (accession no. AF167313)	
AKSF	AAACGGTCACCCTCCTTGA
AKLF	AAAGGTTTCCTCCACCCTGT
AKLR	ACTTCCTCGAGCTTGTCACG
Callinectes sapidus AK (accession no. Q9NH49)	
AKLF	GACCCCATCATCGAGGACTA
AKLR	CCACACCAGGAAGGTCTTGT
AKSF	ACCACAAGGGTTTCAAGCAG
AKSR	GGTGGAGGAAACCTTGGAC

GSP, gene-specific primers.

5′ RACE PCR of Carcinus and Homarus cDNAs

Nested PCR was performed using the Gene Racer forward 5′ primers and R1 (outer) and R2 (inner) reverse gene specific primers (Table 1), which were designed from sequence information from 3′ RACE. Reagent composition for the first PCR was similar to that described for the first 3′ RACE PCR (using *Pfx* polymerase), except that the 1 μl of R1 (10 μmol l^{-1}) was used. PCR conditions were: 4 min 94°C, followed by 30 cycles of 94°C 30 s, 55°C, 1 min, 68°C 1 min, final extension 68°C 10 min. Following this PCR, 1 μl of product was re-amplified with the nested Gene Racer forward primer (1.25 μl) and R2 (10 μmol l^{-1}), 1.25 μl, using Megamix Blue. PCR conditions were 1 cycle of 94°C, 4 min, followed by 30 cycles of 94°C 30 s, 58°C 1 min, 72°C 1 min and a final extension at 72°C for 10 min. PCR products were electrophoresed and bands extracted, as above.

3′ RACE of Callinectes and Orconectes cDNAs

cDNA for 3′ RACE was synthesised using AMV and a Gene Racer 3′ oligo(dT) adapter primer (Invitrogen) with 1 μg total RNA from either *Callinectes* or *Orconectes*. 20–50 ng of cDNA was firstly amplified as detailed for *Carcinus* but using forward primer dF1 (100 μmol l^{-1}). Touchdown PCR conditions were: 1 cycle of 94°C 3 min, 3 cycles of 94°C 30 s, 57°C 30 s, 68°C 1 min, 3 cycles of 94°C 30 s, 55°C 30 s, 68°C 1 min, 3 cycles of 94°C, 53°C 30 s, 68°C 1 min, 25 cycles of 94°C 30 s, 58°C 30 s, 68°C 1 min, and a final extension at 68°C for 10 min. Following this, a second nested PCR was performed using 1 μl of the first PCR as template, essentially as described for *Carcinus*, but using primers dF2 or dF3. PCR conditions were: 1 cycle of 94°C 3 min followed by 30 cycles of 94°C 30 s, 58°C 30 s, 72°C 1 min and a final extension at 72°C for 7 min. PCR products were electrophoresed on agarose gels, and bands were extracted with a DNA extraction kit (Qiagen, Valencia, CA, USA).

5′ RACE of Callinectes and Orconectes cDNAs

The same PCR conditions as described above were employed for 5′ RACE; nested reverse gene-specific primers (Table 1) were used with nested forward 5′ RACE Gene Racer primers (Invitrogen).

Cloning and sequencing of PCR products

Purified PCR products were ligated into a pCR 4-TOPO vector (Invitrogen), transformed (TOP-10F′, Invitrogen) according to the manufacturer's instructions, and plasmid DNA from positive clones containing inserts of correct size were purified and sequenced.

Quantitative RT–PCR, Carcinus

RNA samples (0.1–1 μg) from adult tissues and mRNA from embryos (40 embryo equivalents) were reverse transcribed with AMV–RT and random primers. For cRNA standards, a PCR product from amplification of 430 bp CCAP sequence (using primer pairs CCAP1F, CCAP1R; Table 1) was ligated to T7 promoter adapters (Lig'n Scribe, Ambion) and run-off transcripts prepared and purified as previously described (Chung and Webster, 2003). RNA was quantified using Ribogreen (Molecular Probes, Eugene, OR, USA) using yeast tRNA as standard, diluted in 1× TE and stored in silanised tubes at concentrations of 10^{11} copies per μl. For estimation of CCAP copy numbers, samples were reverse transcribed simultaneously with a standard series of cRNA samples (10^{9}–10^{3} copies per reaction) and cDNAs amplified on a Roche Light Cycler using DNA Master kits (Roche Diagnostics, Mannheim, Germany), with SYBR Green detection. 10 μl reaction volumes were used in the capillaries, adjusting reagent volumes accordingly. Mg^{2+} concentration was 3 mmol l^{-1}, primer concentration 500 nmol l^{-1} (using primer pairs CCAP2F, 2R; Table 1; 210 bp product). Standards were duplicated, embryo samples were single. To detect interassay drift, each carousel used contained a previously quantified sample. PCR conditions were as described previously (Chung and Webster, 2004). To normalise samples from adult CNS,

arginine kinase (AK) expression was quantified in parallel with CCAP. Production of cRNA standards for AK was as previously described (Chung and Webster, 2003). Primer sequences used are shown on Table 1.

To verify that different quantitative PCR instruments gave comparable results, quantitative PCR was also performed on cDNA from adult *Carcinus* CNS using an Applied Biosystems 7700 instrument, using Sensimix (dT) reagents (Quantace, Watford, UK) with SYBR Green detection. 25 μl reaction volumes were used in 96-well plates. Mg^{2+} was 1.5 mmol l^{-1}, primer concentration 200 nmol l^{-1}, using the primer pairs shown above. PCR conditions were: initial denaturation 95°C 10 min, 40 cycles of 95°C 15 s, 60°C 60 s.

Quantitative RT-PCR, Callinectes

Total RNA extracted from the CNS of *Callinectes* was reverse transcribed (1 μg, AMV, random primers), and cDNA used to prepare quantified run-off transcripts essentially as detailed above. Quantitative PCR was performed on an Applied Biosystems 7700 instrument, using a proprietary SYBR Green kit (Applied Biosystems, Foster City, CA, USA) using the same reagent concentrations as described above, and the primer pairs shown in Table 1. PCR conditions were: initial incubation 50°C 4 min, denaturation 95°C 10 min, 40 cycles of 95°C 15 s, 60°C 1 min.

Immunohistochemistry

Embryos were taken from ovigerous *Carcinus*, staged as previously described (Chung and Webster, 2004) fixed (24 h, 4°C) in 2% paraformaldehyde, 15% aqueous saturated picric acid in 0.1 mol l^{-1} sodium phosphate buffer, pH 7.3 (Stefanini et al., 1967). To aid fixation and (essentially) to allow antibody penetration, batches of 50–100 embryos were carefully microscopically dissected within 1 h of fixation to remove the entire egg shell. Embryos were then washed extensively (48 h) in 0.1 mol l^{-1} sodium phosphate buffer containing 0.1% Triton X-100, 0.05% sodium azide (PTX). CCAP antisera were produced in rabbits using a mixture of both 1-ethyl-3, 3′-dimethyl-aminopropyl-carbodiimide (EDC) and glutaraldehyde-conjugated thyroglobulin-CCAP, prepared as previously described (Dircksen and Keller, 1988) by Proteintech Group Inc. (Chicago, USA). The resulting antiserum showed excellent specificity to CCAP, and gave strong immunostaining (1:1000 dilution) in whole-mount tissues using Stefanini's fixative. Embryos were incubated in primary antisera in PTX for 4 days at 4°C, extensively washed in PTX (2 days, 4°C), and incubated in secondary antiserum, 1:50 goat-anti-rabbit fluorescein isothiocyanate conjugate for 2 days (4°C), followed by extensive washing. Embryos were mounted in Vectashield (Vector Labs., Burlinghame, CA, USA) and preparations examined by confocal microscopy using a Zeiss LSM 510 instrument. Proprietary software was used for stacked projection analysis. Between 20 and 32 consecutive (1.5 μm distance) images were collected for each projection. Images were manipulated using Adobe PhotoShop 7.0 and CorelDraw 8.0 software.

Results

Characterisation of cDNAs encoding CCAP precursors

Using fully degenerate primers encoding the sequence of CCAP and putative dibasic cleavage sites, we used a strategy of nested 3′ RACE to obtain sequence information to subsequently design gene-specific primers (GSPs) for RNA ligation-mediated nested 5′ RACE, using cDNA derived from total RNA extracted from tissues known to express CCAP (thoracic ganglia) from model brachyuran and astacuran crustaceans to elucidate the full-length cDNA in the crabs *Callinectes sapidus*, *Carcinus maenas*, and lobster and crayfish *Homarus gammarus* and *Orconectes immunis.* Sequences have been submitted to EMBL/GenBank databases: accession nos. *C. sapidus*, DQ225100; *C. maenas*, DQ225101; *H. gammarus*, DQ221502; *O. immunis*, DQ225103.

In all instances, cDNAs encoded short 5′ untranslated regions (UTRs), followed by open reading frames (ORFs) encoding a (conceptually translated) conventional 30–32 residue signal peptide. In all cases, except *Orconectes immunis*, the 3′ UTR included a putative polyadenylation site (AATAAA). In the case of *Orconectes* and *Callinectes*, it was notable that the 3′ UTRs were rather shorter than for *Carcinus and Homarus*. Analysis of the putative signal peptides within the ORFs, using Signal P3.0 (www.cbs.dtu.dk/services/SignalP), indicated that all were probably cleaved from the signal at the AG boundary (positions 32–33; Fig. 1). The precursor peptide contains four putative dibasic (37–38, 49–50), tribasic (61–63) and tetrabasic (115–119) cleavage sites, which could result in the genesis of five peptides, including that encoding CCAP. However, none of these, excepting that encoding CCAP contain amidation (GK, K, R) signals. Comparing all four sequences (Fig. 1), it is readily apparent that all are similar, and encode a putative tetrapeptide (CCAP AP1), a decapeptide (CCAP AP2), CCAP, a 51-mer (CCAP AP3), and a 23–25-mer (CCAP AP4). In particular, it is notable that CCAP AP3 contains a number of identical domains, and that the precursor peptides of brachyuran and astacurans are identifiable in terms of sequence identity.

Expression of CCAP mRNA and peptide during embryogenesis

Quantitative RT-PCR was performed on mRNA extracted from *Carcinus* embryos at key stages of embryonic development, as previously described (Chung and Webster, 2004). The results showed that prior to development of eye tissues CCAP expression was minimal, amounting for less than 1–2000 copies per embryo. Such expression was deemed to be intrinsic, rather than due to genomic DNA contamination, or from limited reverse transcriptase activity of Taq, since RT controls showed no amplification of gene-specific products, and amplification and melt curve analysis suggested low level expression (data not shown). However, highly significant increases in expression were observed at the beginning of eye formation (eye smear, 50% development) as shown in Fig. 2. A further approx. threefold increase was seen when eye formation was established (~70% development). During later

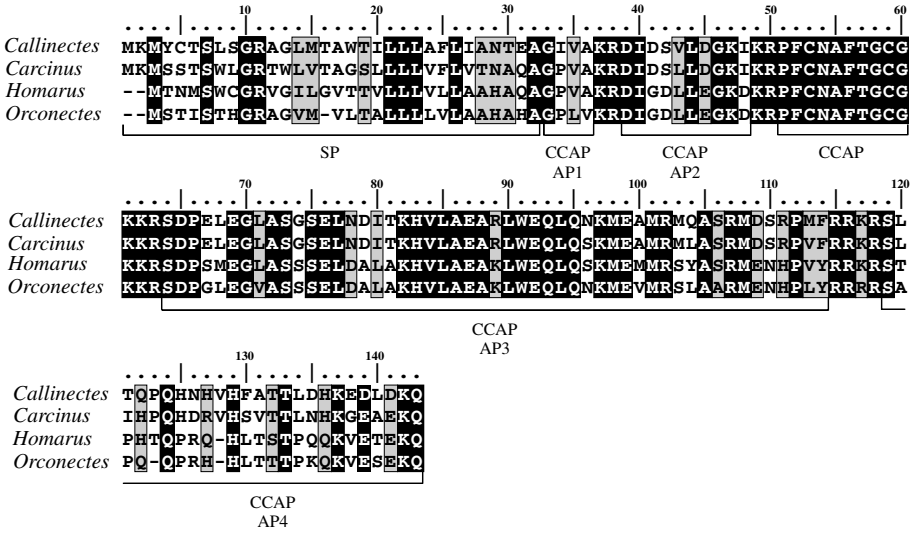

Fig. 1. Alignments and comparisons of deduced amino acid sequences of CCAP precursor peptides from *Callinectes sapidus*, *Carcinus maenas*, *Homarus gammarus* and *Orconectes immunis*. Identical and similar amino acids are in black and grey shaded boxes, respectively. SP, signal peptide; CCAP AP1, 2, crustacean cardioactive peptide associated peptides 1, 2; CCAP, crustacean cardioactive peptide, CCAP AP3, 4, crustacean cardioactive peptide associated peptides 3, 4. Gaps (–) have been introduced to maximise sequence identities.

stages of development, increased CCAP expression continued. Just prior to hatching, CCAP mRNA expression appeared to increase, but levels were not significantly different from those of the preceding stage (85–90% development).

Expression profiles of CCAP and corresponding neurogenesis were monitored by whole-mount confocal microscopy of embryos using CCAP immunohistochemistry. Expression of CCAP peptide was first observed at 70% development, when small, rather inconspicuous immunoreactive structures could be observed on the dorsal side of the embryo, corresponding to the position of the heart (Fig. 3A). Ventral views of the embryos at this time revealed bilaterally symmetrical immunopositive structures (Fig. 3B). CCAP immunopositive neurones became clearly visible at 80% embryonic development, when axons in the developing thoracic ganglion and two segmental nerves became prominent (Fig. 3C). Subsequent stages in embryogenesis (85–100%) (Fig. 3D,E) showed accumulation of CCAP immunoreactivity (IR) in the segmental nerves and, in particular, in structures reminiscent of the anterior ramifications (AR) in the adult CNS. For fully developed embryos, some details of neuroanatomy of the thoracic ganglion (TG) could be established, in particular the projection of contralateral axonal projections and varicosities at four or five fairly defined positions in the developing TG (Fig. 3D,F), but the positions of corresponding perikarya were very difficult to establish. In one preparation, some outlines of presumptive perikarya were just observable (Fig. 3F). Despite extensive investigation of many embryos, only a few preparations revealed faintly staining perikarya, where three pairs of small cells (<10 μm diameter) were visible in stacked confocal images (Fig. 3G).

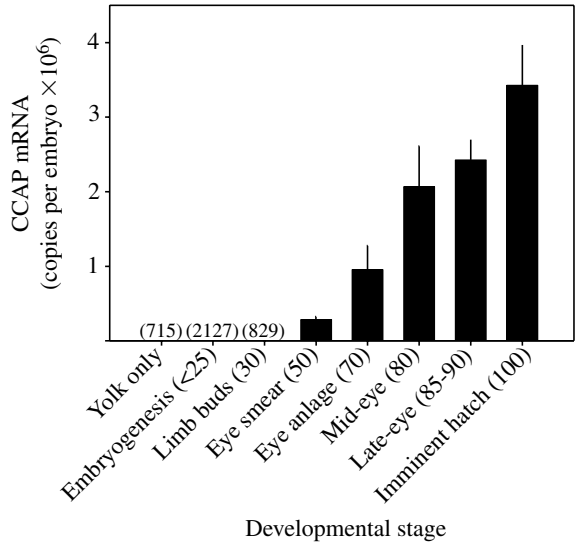

Fig. 2. Expression of crustacean cardioactive peptide (CCAP) mRNA during embryonic development of *Carcinus maenas*. Three to 11 independent measurements of different embryo batches were made at each developmental stage. Error bars indicate +1 s.e.m. Developmental stages were defined according to (Chung and Webster, 2004); additionally, the percentage development is indicated in brackets. During early developmental stages (yolk-limb buds), very low, but significant numbers of transcripts were observed, which are shown in brackets.

Lateral views of embryos at 85–90% development showed a single axon within a segmental nerve rising dorsally to arborisations [presumably the developing pericardial organs (PO) adjacent to the heart], and a single anterior segmental nerve projection (Fig. 3H). These may correlate to the profiles seen in Fig. 3C–E. Control preparations where antisera were preabsorbed with nanomolar quantities of CCAP completely abolished immunostaining (results not shown).

Expression of CCAP mRNA in adult CNS

Conventional PCR using primer pair CCAP F1, R1 (430 bp product) showed that CCAP was only expressed in eyestalk, brain and thoracic ganglia, and was undetectable in non-neural tissues (Fig. 4 inset). qRT–PCR results showed that although thoracic ganglia expressed over 100-fold more CCAP than other neural tissues, the eyestalk tissues expressed significantly more CCAP mRNA than brain tissues (Fig. 4). With regard to copy number per tissue, for adult *Carcinus* (carapace width

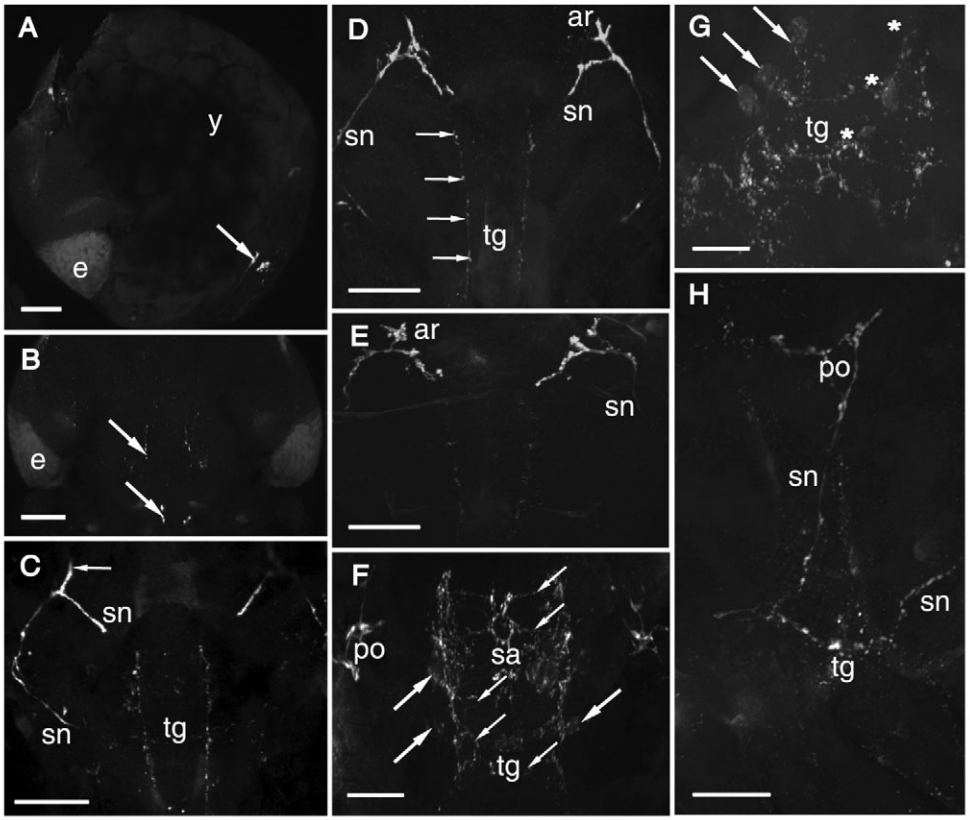

Fig. 3. Developmental profiles of crustacean cardioactive peptide (CCAP)-expressing neural structures during embryonic development of *Carcinus maenas*. All confocal images have been stacked (30–32 slices; distance between slices: 0.8–2 μm) and flattened. (A) Lateral view of embryo at 70% development. Arrow points to the first immunoreactive (IR) structures seen on the dorsal side of the embryo corresponding to the position of the heart, which by this stage of development, has a regular beat (y, yolk sac; e, eye) (B) Ventral view of embryo at 70% development, showing bilaterally symmetrical IR structures, probably corresponding to segmental nerves (upper arrow) and projections adjacent to the heart (lower arrow). (C) Dorsal view of embryo at 80% development. Small arrow points to anteriorly projecting IR structure which is probably the developing anterior ramification. At this time IR structures corresponding to the segmental nerves (sn) and thoracic ganglion (tg) become visible. (D) Dorsal view of embryo at 85% development. Small arrows point to a series of prominent varicosities in the axons forming part of the developing thoracic ganglion. At this time the anterior ramification (ar) becomes digitate. (E) Dorsal view of embryo at 100% development showing IR structures associated with the segmental nerves adjacent to the anterior ramifications. (F) Dorsal view of IR structures associated with the thoracic ganglion at 100% development. Contralaterally projecting axons (small arrows) can be observed and weakly IR structures probably corresponding to perikarya (large arrows) can be seen. The developing pericardial organs (po) become prominent at this stage. (G) Dorsolateral view of thoracic ganglion at 100% development. This image has been rotated to show three pairs of weakly IR perikarya (arrows, asterisks). (H) Lateral view of embryo at 85–90% development, showing a single IR axon within a segmental nerve (sn) arising from the thoracic ganglion (tg), and a second, anteriorly projecting axon within a segmental nerve. The ring-like structure of the developing pericardial organ (po) is prominent in this orientation. Scale bars, 50 μm.

45–55 mm) these were [$\times10^6$, mean ± s.e.m. (*N*)]: eyestalk 3.50±0.4 (14), cerebral ganglia 1.03±0.35 (8), thoracic ganglia 527±103.3 (8). For adult *Callinectes* (which were much larger; carapace width ~160–180 mm), copy numbers were about tenfold higher [$\times10^7$, mean ± s.e.m. (*N*)]: eyestalk 4.6±1.3 (6), cerebral ganglia 4.4±1.5 (6), thoracic ganglia 681±20.6 (6). Intriguingly, results from one set of simultaneous isolations from both males and females indicated that in female eyestalks CCAP mRNA expression was significantly greater [26.7±7.3 (6) (P<0.05)] compared to males [4.6±1.3 (6)].

Quantitative expression patterns of instantaneous levels of CCAP mRNA in thoracic ganglia were determined by qRT-PCR throughout the moult cycle in three ways: without normalisation, normalisation to total RNA, and to the invariant housekeeping gene, arginine kinase (AK) (Fig. 5). RT reactions were simultaneously performed on all samples to avoid inter-assay drift. For *Carcinus*, mean levels of un-normalised CCAP copy numbers were between 0.9×10^9 and 1.6×10^9 per thoracic ganglion, when normalised against total RNA they were between 60×10^6 and 117×10^6 copies per μg RNA, and when normalised to AK they were between 19.3 and 51 (10^3 copies CCAP/10^6 copies AK). None of these changes were statistically significant (one-way ANOVA, and Dunn's multiple comparison tests). For *Callinectes*, copy number of CCAP per thoracic ganglion increased from 1.85×10^8 to 3.7×10^8 during postmoult to premoult, normalised patterns

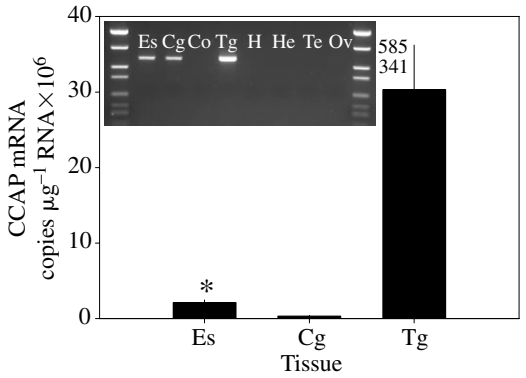

Fig. 4. Expression of crustacean cardioactive peptide (CCAP) mRNA in *Carcinus maenas* tissues. Inset panel shows 430 bp PCR product (using CCAP F1, R1 gene-specific primers) amplified from cDNA derived from reverse transcription of 100 ng total RNA from eyestalk (Es), cerebral ganglion (Cg), circum-oesophageal commissure (Co), thoracic ganglion (Tg), heart (H), hepatopancreas (He), testis (Te) and ovary (Ov) tissues. Numbers to right of panel are sizes of ladder markers (bp). Histogram shows levels of CCAP mRNA from quantitative RT–PCR expressed as copies per μg RNA for Es (N=14), Cg (N=8) and Tg (N=8). Error bars indicate +1 s.e.m. Eyestalk tissues contain significantly more CCAP mRNA than cerebral ganglia (*asterisk, P<0.05, Dunn's multiple comparison).

(per μg RNA) from 9.8×10^6 to 17.3×10^6 and for AK normalised samples, from 7.6 to 13.2 (10^3 copies CCAP/10^6 copies AK). Nevertheless, these increases were not significant (P>0.05).

Expression of CCAP mRNA in eyestalk and thoracic ganglia following hypoxic and temperature stress

CCAP expression in eyestalk and thoracic ganglia was determined following severe hypoxic stress (1 h, <0.5% dissolved O_2). The results are summarised in Fig. 6. Although no significant changes in CCAP or AK expression were seen in the thoracic ganglia, hypoxia induced a significant downregulation in CCAP expression in eyestalk ganglia, which seemed to be specific, in that AK expression levels, although very variable, were not reduced to the same extent as those seen for CCAP. With regard to temperature stress, where crabs were subjected to severe hypothermal episodes or hyperthermic stress, to mimic yearly extremes in the Chesapeake Bay, CCAP copy numbers were rather lower in those exposed to hypothermic stress (22–24°C) than in those animals exposed to hyperthermic stress (22–29°C). Nevertheless, these changes were statistically insignificant, and were not mirrored by changes in expression patterns of AK in eyestalk tissues (Table 2).

Discussion

cDNAs encoding CCAP in crustaceans

The sequence data determined from this study show for the first time the mRNA and conceptually translated peptide sequences of the CCAP precursor in several crustaceans. For the crab (brachyuran) examples, full-length cDNAs encode open reading frames of 143 amino acids, and for the lobster and crayfish (astacuran) 139–140 amino acids. In all cases, the translated precursor consists of a signal peptide, followed by five putative peptides: a tetrapeptide (CCAP AP1), a decapeptide CCAP AP2, the nonapeptide CCAP, a 51-mer-CCAP AP3, and a 24–25-mer, CCAP AP4. With regard to other insect CCAP precursors identified to date, it is readily apparent that all encode a variety of peptides (Fig. 7). Apart from the region coding CCAP, there is very little similarity in the proposed peptide sequences, excepting that of *Periplaneta*, for which there is significant identity with CCAP AP3, where, gapped alignment shows that 15/51 amino acids are identical, and a further 18/51 are similar. Although putative neuropeptides that are flanked by conventional dibasic, tribasic or tetrabasic amino acids can be considered to be candidates for release following proteolytic processing, and thus might have biological activities, our preliminary studies (matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; MALDI-TOF MS) has yet to show that putative neuropeptides AP1–4 are authentic and releasable constituents of the neuropeptide inventory of the pericardial organs (PO). Nevertheless, much more detailed studies of the peptidome of *Cancer borealis* PO (Fu et al., 2005a) have clearly identified CCAP, and quite a number of peptides (39), yet molecular ions similar to those predicted in our studies were not determined in this study. Nevertheless, direct profiling by MALDI-TOF MS of PO homogenates in *Cancer borealis* have identified CCAP, and also a number of as yet unidentified peptides of mass ranges similar to those predicted in the present study, in particular, those similar to CCAP AP3 and 4 (Li et al., 2003). However, should these putative peptides be authentic neurohormones, their functions are of course unknown. *Carcinus* sinus gland (SG) and PO express three crustacean hyperglycaemic hormone precursor-related peptide (CPRP) variants (Dircksen et al., 2001), and others (*Cancer productus*) may express up to four CPRPs, and possibly truncated variants (Fu et al., 2005b). To date no biological activity has been ascribed to these peptides, and for CPRP, in view of its notably long half-life in circulation (Wilcockson et al., 2002), a hormonal role seems unlikely. Additionally, for the CCAP precursor, it should be noted that there are few similarities in sequence of the putative peptides identified so far when insects and crustaceans are compared, and none of these bear C-terminal amidation sites characteristic of the majority of secreted neuropeptides. Thus it seems possible that the CCAP-associated peptides may have no functionally relevant role as neuropeptides. It would be interesting to determine the cellular expression and distribution patterns of the CCAP-associated peptides in the PO and to determine (co) release patterns from the PO by raising specific antisera for ICC and radioimmunoassay studies.

Expression patterns of CCAP mRNA and peptide during embryonic development

Using qRT-PCR, expression of CCAP mRNA copy number was measured throughout embryonic development and compared with protein expression using whole-mount confocal

microscopy with antisera raised against CCAP. Although it would be expected that identification of significant quantities of transcripts would precede those identified by immunohistochemistry, an unexpected finding was that significant CCAP gene expression occurred many days before peptide expression could be seen in the developing PO. For example, significant CCAP gene expression was observed at around 50% development (eye smear) (Chung and Webster, 2004), yet the first expression of CCAP, as detected by immunochemical localisation, was observed a little later, at 70% embryonic development, which corresponded to a lag of around 5 days (Chung and Webster, 2004). Nevertheless, it could be argued that measurement of CCAP mRNA expression is inherently more sensitive than detection of peptide by ICC. However, we have previously observed correspondence in detection of gene expression and peptide for moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) at the eye anlage stage (50% development) of *Carcinus* embryos (Chung and Webster, 2004), suggesting that detection limits using mRNA quantification and immunohistochemistry may be similar in some instances.

In embryos of the lobster *Homarus americanus*, clearly

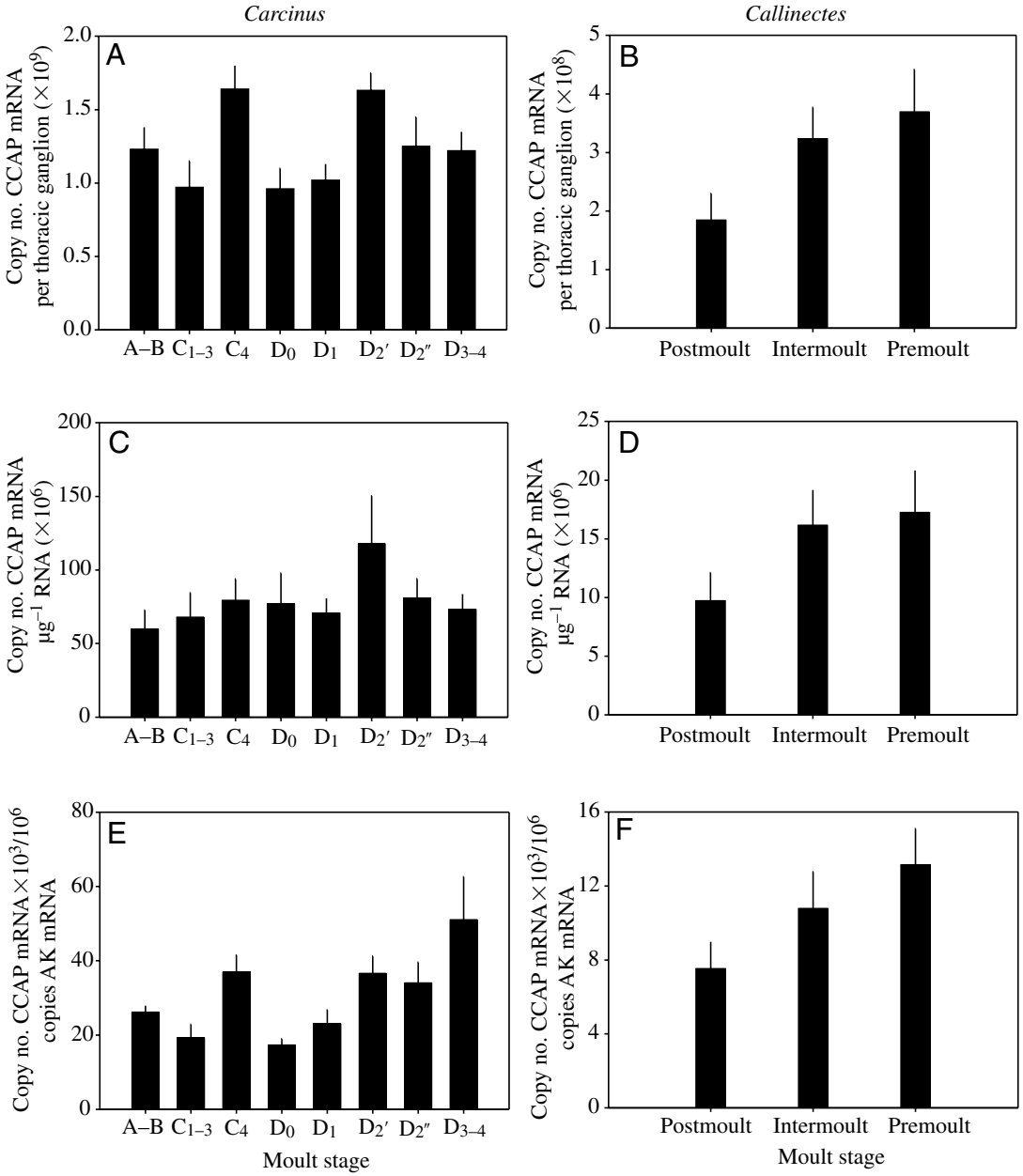

Fig. 5. Transcription profiles for crustacean cardioactive peptide (CCAP) mRNA during the moult cycle of *Carcinus* (A,C,E) and *Callinectes* (B,D,F). Data are shown as un-normalized (copy no. per thoracic ganglion; A,B), normalized against total RNA (copy no. μg^{-1} RNA $\times 10^6$; C,D), and normalized against a housekeeping gene, arginine kinase (AK; copy no. CCAP mRNA $\times 10^3/10^6$ copies of AK mRNA; E,F). For *Carcinus*, *N*=5–11 independent samples were taken at each moult stage. For *Callinectes*, *N*=8–10. Error bars indicate +1 s.e.m.

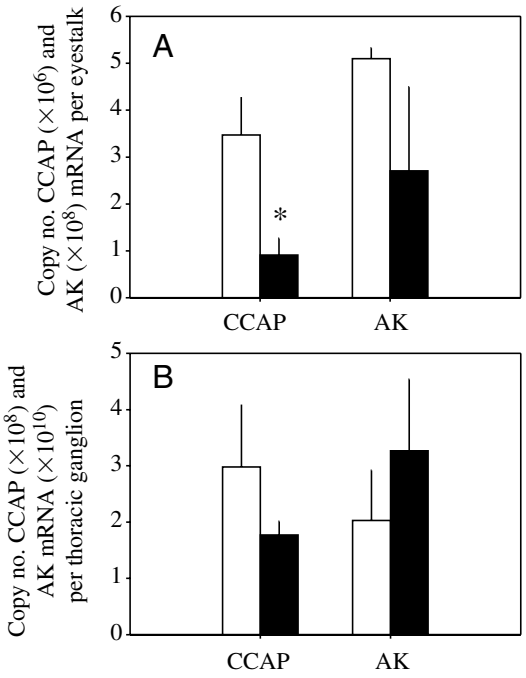

Fig. 6. The effect of 1 h exposure of *Callinectes* to severe hypoxia (0.5% O_2) upon crustacean cardioactive peptide (CCAP) and arginine kinase (AK) expression in eyestalk tissues (A) and the thoracic ganglion (B), expressed as copy no. per eyestalk and per ganglion, respectively. Open bars indicate expression levels at t=0, filled bars at t=1 h (N=6). Error bars indicate +1 s.e.m. Asterisk indicates significant (P<0.05, Students t-test) reduction of mean CCAP levels after exposure to hypoxic conditions.

defined immunoreactivity corresponding to CCAP (and a variety of other neuropeptides) in a well developed PO have been observed at about 50% development (Pulver and Marder, 2002), somewhat earlier that recorded in the present study. However, the lobster has a prolonged embryonic life and hatches at a relatively advanced developmental stage, compared to crabs. In the lobster, gross anatomy of the PO, segmental nerves 1–5 and dorsal nerves 1–3 are easily visible at 50% development, thus a direct comparison based on timed criteria of development may

Table 2. *Effect of temperature change on expression levels of crustacean cardioactive peptide and arginine kinase mRNA in juvenile Callinectes sapidus*

	Copy no. per eyestalk	
Treatment	CCAP (×10^5)	AK (×10^7)
22 to 4°C	4.83±0.95	10.7±3.49
22 to 22°C	6.15±0.82	8.04±1.92
22 to 29°C	9.74±1.25	16.0±2.78

CCAP, crustacean cardioactive peptide; AK, Arginine kinase.

Crabs were exposed to hypothermal stress (22°C to 4°C), hyperthermal stress (22°C to 29°C), or kept at 22°C, for 2 h, followed by rapid extraction and purification of eyestalk tissues. Values are means ± 1 s.e.m. (N=6).

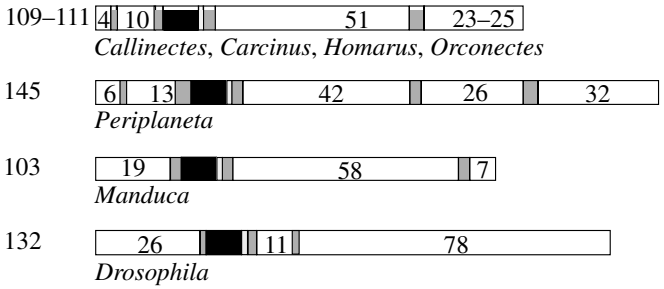

Fig. 7. Scaled schematic of crustacean cardioactive peptide (CCAP) precursor peptide structures of crustaceans (*Callinectes sapidus*, *Carcinus maenas*, *Homarus gammarus*, *Orconectes immunis*) compared with those of insects (*Periplaneta americana*, *Manduca sexta*, *Drosophila melanogaster*). Black bar represents CCAP; pale grey bar, amidation site; dark grey bars, potential dibasic, tribasic and tetrabasic cleavage sites. Numbers on left refer to unprocessed sizes of precursors, those within boxes, to the various peptides that may be potentially generated following cleavage.

not be appropriate. A dramatic increase in expression of CCAP mRNA occurred at mid-eye (80% development). This corresponded to the appropriate phenotype, i.e. at this time the heart starts to beat rapidly, rather that the irregular heart beat seen prior to 70% development (Chung and Webster, 2004). Despite the dramatic increase in CCAP transcript number during embryogenesis (10^3-fold), immunopositive structures whilst developing in complexity, seemed not to mirror transcript number. In particular, the expression of CCAP in three pairs of immunopositive neurones in the TG of late embryos (Fig. 3G) suggested quite low level translation. This observation could not be explained by (for example) poor penetration of the antibody, since the segmental nerves, anterior ramifications and neuronal architecture of the developing thoracic ganglia all gave very intense signals. In the adult a similar situation has been noted with regard to low level translation (Stangier et al., 1988), where low levels of CCAP were observed in the thoracic ganglia (1.4 pmol mg^{-1} protein) compared to much higher levels in the PO (868 pmol mg^{-1} protein).

By comparison with the detailed description of the CCAP-expressing neurones in adult *Carcinus* (Dircksen, 1998), some analysis of CCAP neuroanatomy in embryos can be made. The appearance of strong immunopositive signals in dorsal regions of the segmental nerves at around 80% development (Fig. 3C) allowed identification of the anterior ramifications (Fig. 3D), thus the anterior nerve is segmental nerve 1, which arises from the first thoracic (maxilliped 1) segment [neuromere 7 using criteria adopted by Harzsch (Harzsch, 2003)]. At full development, five pairs of contralaterally projecting axons can be seen in the TG, which probably correspond to projections from large (type-1) CCAP-containing neurones in the adult. However, since these cells contained very small quantities of CCAP, unequivocal identification of only three pairs of cells was possible. Furthermore, the nine pairs of CCAP-expressing neurones corresponding to the fused abdominal ganglion of the adult were not seen in the TG or abdomen of the embryo.

Expression of CCAP mRNA in the nervous system

Expression of CCAP in adult crabs was restricted to the CNS, as has been previously reported from radioimmunoassay studies (Stangier et al., 1988). Although the thoracic ganglia expressed the majority of transcripts for CCAP, it was notable that, for *Carcinus*, transcript number normalised against total RNA, was significantly higher that that of the cerebral ganglia. Although this phenomenon was not seen in *Callinectes*, previous studies have indicated that each eyestalk contains very few, perhaps only one perikaryon, immunopositive for CCAP neurones, in contrast to the cerebral ganglia where at least five pairs of CCAP-immunoreactive perikarya have been observed (Dircksen and Keller, 1988). Radioimmunoassay studies (Stangier et al., 1988) have shown that eyestalks and cerebral ganglia of *Carcinus* contain similar quantities of CCAP (1.4 pmol mg^{-1} protein). Since CCAP has not been identified in the SG (Fu et al., 2005b), it seems that the CCAP immunoreactive structures in the eyestalk, as in the brain, are interneurones.

Expression of CCAP in the nervous system during the moult cycle, and following environmentally relevant stressors

During the moult cycle, no significant changes were seen in CCAP expression in thoracic ganglia. Despite extensive investigations, using normalisation to total RNA, AK and in comparison to un-normalised data (Fig. 5) we could find no significant changes in mRNA transcription during the moult cycle of *Carcinus* or *Callinectes*. Although a general trend could be seen in *Callinectes*, whereby steady state transcription levels were at their lowest during postmoult, and highest during premoult, a feature which would also be discernible by pooling results from *Carcinus* in this manner (given the inevitable errors involved in SYBR green detection of amplicons in quantitative PCR, which cannot determine less than twofold changes in amplicon concentration) it seems more than likely that steady-state transcription of CCAP remains relatively constant throughout the moult cycle. Indeed, the two normalisations performed on the data corroborate this, not withstanding the fact that CCAP-expressing cells in the TG are invariant in number, it seems likely that the first iteration using un-normalised copy number data may be the most biologically relevant. Thus, it seems likely that CCAP expression in the thoracic ganglia is constitutive. Therefore the previously reported surge in CCAP release during ecdysis in crustaceans (Phlippen et al., 2000) cannot be correlated with increased transcription of CCAP during premoult.

Since *Callinectes*, which is a subtidal crustacean, experiences environmental variabilities in the Chesapeake Bay area that are far greater than those experienced by an essentially intertidal crustacean such as *Carcinus*, we determined the steady-state expression of CCAP in *Callinectes*, to determine the possible effects of (extreme) environmental stressors (hypoxia, temperature changes), within the context of recorded variables within Chesapeake Bay. The effects of hypoxia were of particular interest, since, near-shore waters in Chesapeake Bay periodically experience episodes of anoxia or severe hypoxia (<2 mg l^{-1}) during the summer (Breitburg, 1990), which may occasionally be of sufficient severity and duration to cause mass mortalities (Seliger et al., 1985).

Following extreme hypoxic episodes, expression of eyestalk CCAP mRNA was significantly reduced, in contrast to that of thoracic ganglia CCAP expression, which remained unchanged (Fig. 6). This result was interesting (and specific) since AK expression levels remained unchanged in both eyestalk and thoracic ganglia. Nevertheless, following thermal stress, both CCAP and AK expression was unchanged (Table 2). Since the reduction in CCAP expression in eyestalk ganglia prior to extreme hypoxia (copy number: 3.47±0.81 $\times 10^6$) compared to expression after 1 h of hypoxia (copy number: 0.91±0.36 $\times 10^6$) was significant, and in view of our results showing that CCAP expression in the eyestalks is significant in relation to the much lower levels of expression seen in the cerebral ganglia, given that there appear to be many more CCAP-expressing neurons in the latter tissue (Dircksen, 1998), it is possible that CCAP has yet another role in environmental adaptation, which remains to be uncovered. Additionally, the sexual dimorphism in CCAP transcript number in *Callinectes* was intriguing (males: 4.6±1.3 $\times 10^7$; females: 26.7±7.2 $\times 10^7$copies per eyestalk), and might possibly suggest novel roles for this hormone in reproduction. Since CCAP has a role as an adipokinetic hormone release-inducing factor in the locust corpora cardiaca (Veelaert et al., 1997), it is conceivable that this hormone may have modulatory roles in controlling the release of peptides involved in control of vitellogenesis in the eyestalk neurosecretory system of crustaceans, and this deserves further study.

List of abbreviations

AK	arginine kinase
AMV-RT	Avion Myeloblastosis Virus-reverse transcriptase
CCAP	crustacean cardioactive peptide
CPRP	crustacean hyperglycemic hormone precursor-related peptide
ORF	open reading frame
qRT-PCR	quantitative reverse transcription polymerase chain reaction
ICC	immunocytochemistry
MALDI-TOF MS	matrix-assisted laser desorption/ ionization-time-of-flight mass spectrometry
RIA	radioimmunoassay
RT-PCR	reverse transcription-polymerase chain reaction
TE	Tris-EDTA
SG	sinus gland

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