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# RESEARCH ARTICLE

# Identification of the elusive peptidergic diuretic hormone in the blood-feeding bug *Rhodnius prolixus*: a CRF-related peptide

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## SUMMARY

**Probing of a host and ingestion of a blood-meal in a fifth instar *Rhodnius prolixus* results in a cascade of tightly integrated events. The huge blood-meal is pumped into the anterior midgut during feeding, then modified by diuresis and stored until it is digested. While serotonin is known to be a diuretic hormone in *R. prolixus*, a peptidergic factor(s) was also known to play a role in diuresis. In the present study we employed molecular techniques and mass spectrometry to determine the sequence of a native CRF-like peptide from *R. prolixus* (Rhopr DH). In addition, we confirmed the distribution and localization of Rhopr DH using *in situ* hybridization and immunohistochemistry, and demonstrated its potent biological activity on both the anterior midgut and Malpighian tubules.**

Key words: *Rhodnius*, diuresis, neurohormone.

## INTRODUCTION

The blood-feeding hemipteran *Rhodnius prolixus* ingests a large blood meal, which is followed by a period of rapid diuresis. The ingested blood is taken into the anterior midgut, modified by the removal of water and salt to reduce the volume of the meal, and stored until it is subsequently digested in the posterior midgut (Maddrell, 1976). The water and salt are moved across the epithelium of the anterior midgut into the haemolymph (Farmer et al., 1981; Barrett, 1982; Te Brugge et al., 2009) and then across the Malpighian tubules. The urine travels down the Malpighian tubules, is modified by re-absorption of KCl, and empties into the hindgut (see Orchard, 2009). Rapid elimination of urine usually commences within 2–3 min of feeding and lasts for the next 3 h, during which time the insect may lose 40% of the mass of the meal. *In vivo*, this rate is 400–700 $nl\,min^{-1}$ for the first 2–3 h (Maddrell, 1964a; Maddrell, 1964b).

Diuresis in *R. prolixus* has been extensively described in the pioneering work of Maddrell (Maddrell, 1963; Maddrell, 1964a; Maddrell, 1964b; Maddrell, 1966; Maddrell, 1976; Maddrell, 1981; Maddrell et al., 1969; Maddrell et al., 1971; Maddrell et al., 1991; Maddrell et al., 1993). In seminal publications, Maddrell demonstrated that the post-prandial diuresis was under hormonal control (Maddrell, 1966) and that diuretic activity was present in extracts of the brain, mesothoracic ganglionic mass (MTGM) and abdominal nerves (Maddrell, 1964). Extracts of specific individual cells in the MTGM, the posterior-lateral neurosecretory cells (NSCs), were found to contain potent diuretic activity which was diminished after feeding (Berlind and Maddrell, 1979). The posterior lateral NSCs send projections out to the abdominal nerves, and their neurohaemal sites on the abdominal nerves (Maddrell, 1966) are a major site of release for diuretic hormones (DHs). The DHs stimulate a post-feeding increase in the rate of ion and fluid movement across both the anterior midgut and the Malpighian tubules (Maddrell, 1966; Farmer et al., 1981; Te Brugge et al., 2002; Te Brugge et al., 2009). Maddrell and colleagues (Maddrell et al., 1991; Maddrell et al., 1993) demonstrated that diuresis in *R. prolixus* is under the control of serotonin (5HT) and a peptidergic DH, but the chemical identity of the peptidergic DH present in the posterior-lateral NSCs of the MTGM has remained elusive (Aston and White, 1974; Aston, 1979; Hughes, 1979).

In other insects, members of the corticotropin releasing factor (CRF) family of peptides have been shown to have diuretic activity. CRF, urotensin-I, urocortin and sauvagine form a family of neuropeptides that are found in nematodes, arthropods, molluscs, tunicates and the chordates (Lovejoy and Barsyte-Lovejoy, 2010; Lovejoy and Balment, 1999). The first insect CRF-like diuretic peptide was isolated from *Manduca sexta* by Kataoka and colleagues (Kataoka et al., 1989). Subsequently, CRF-like peptides have been sequenced or predicted (using bioinformatic and molecular techniques) from many insects (Cabrero et al., 2002; Christie, 2008; Li et al., 2008; Roller et al., 2008). The insect CRF-like peptides are 30–54 residues in length and fall into two groups, long (sequences greater than 40 residues) and short (sequences less than 40 residues), which are thought to be paralogues (Blackburn et al., 1991; Coast, 1998; Coast et al., 2001; Lovejoy and Jahan, 2006).

The first insect CRF-like peptide demonstrated to be a true DH was from *Locusta migratoria*, *Locusta* DH (Locmi DH) (Patel et al., 1995), where it stimulates Malpighian tubule secretion (Coast et al., 1993), is present in NSCs of the brain and abdominal ganglia (Patel et al., 1994) and is released into the haemolymph after feeding (Patel et al., 1995; Audsley et al., 1997). In *R. prolixus* we have shown that the medial NSCs of the brain and the posterior lateral NSCs of the MTGM are CRF-like immunoreactive, as are their associated neurohaemal sites (Te Brugge et al., 1999). In addition, insect CRF-like peptides are potent stimulators of anterior midgut

absorption and Malpighian tubule secretion in *R. prolixus* (Te Brugge et al., 1999; Te Brugge et al., 2002; Te Brugge et al., 2009). Previous attempts to purify and sequence the native DH from *R. prolixus* using classical purification and isolation techniques were not successful (Aston and White, 1974; Aston, 1979; Hughes, 1979) (D.A.S., V.T.B. and I.O., unpublished).

In the present study we employed molecular techniques and mass spectrometry, facilitated by the database sequences of the *R. prolixus* genome project, to determine the sequence of a native CRF-like peptide from *R. prolixus*, and report here the sequence of the Rhopr DH. In addition, we confirm the distribution and localization of Rhopr DH using *in situ* hybridization and immunohistochemistry, and demonstrate its potent biological activity on both the anterior midgut and Malpighian tubules.

## MATERIALS AND METHODS

### Insects

Fifth instar *R. prolixus*, Stål 1859, were taken from a long-standing colony maintained at 25°C under high humidity. The insects were unfed for 5–7 weeks and had previously been fed on defibrinated rabbit's blood (Cedarlane Laboratories, Burlington, ON, Canada) as fourth instars.

### Peptides and chemicals

The synthetic CRF-like peptides from the termite *Zootermopsis nevadensis* (Zoone DH) and cockroach *Diploptera punctata* (Dippu DH) were used. The putative native CRF-like peptide Rhopr DH was synthesized by Genscript (Piscataway, NJ, USA). Serotonin and all other chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise noted. The anti-CRF antiserum was generated in rabbit against the C-terminal sequence residues 29–46 of Locmi DH and was the generous gift of Dr Geoff Coast.

### Primers and sequencing

A *R. prolixus* central nervous system (CNS) cDNA library (Paluzzi et al., 2008) was used as a template for 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR. Gene-specific primers were designed based on the partial sequence predicted from BLAST results of the *R. prolixus* Trace archives (NCBI). The primers used for 3′ RACE were as follows: CRF FOR1, GATGATGAAGTAAGCTGG-AGAGGA; CRF FOR2, CCCAGGGTCCATCATTGTC; and CRF FOR3, AGGCGTCGCATGAAGGA. These 3′ RACE primers were used with the plasmid reverse primer (pDNR-LIB REV1, with the sequence GCCAAACGAATGGTCTAGAAAG) in a nested PCR approach. Similarly, the 5′ RACE primers were designed as follows: CRF REV1, ATACCTGTTTGTATGTCGTTTGCC; CRF REV2, CTGACTCTTGAGGCATCTTGTTC; and CRF REV3, CGA-CGCCTGGCAATCTC. These 5′ RACE primers were used in combination with the plasmid forward primer (pDNR-LIB FOR1, with the sequence GTGGATAACCGTATTACCGCC) in a nested PCR approach. Conditions for the 5′ and 3′ RACE PCR were as follows: 3 min initial denaturation at 95°C, 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 61°C, extension for 1 min at 72°C, and a final extension for 10 min at 72°C. The amplified fragments were run and visualized on a 1.4% agarose gel stained with ethidium bromide. The purified products were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA). Sequencing was carried out at the Centre for Applied Genomics at the Hospital for Sick Children (MaRS Centre, Toronto, ON, Canada), and sequences were confirmed from at least three independent clones to ensure base accuracy.

### Expression localization using FISH

Localization of the cell-specific expression of Rhopr DH mRNA was accomplished using FISH (fluorescence *in situ* hybridization) conducted as previously described (Paluzzi et al., 2008) with some minor modifications. Digoxigenin (DIG)-labelled RNA was synthesized from a linearized recombinant plasmid DNA containing a 502 bp Rhopr DH cDNA fragment by *in vitro* transcription using the DIG RNA labelling kit SP6/T7 (Roche Applied Science, Mannheim, Germany). Once DIG-labelled RNA synthesis was complete, template DNA was removed with deoxyribonuclease I. The probe was stored at –20°C. Tissues were dissected under phosphate-buffered saline (PBS) prepared in nuclease-free water and then transferred to a tube containing freshly prepared 4% paraformaldehyde solution (40% paraformaldehyde:PBS, 1:9) and incubated for 30 min at room temperature. After this primary fixation, tissues were washed 5 times with PBS and 0.1% Tween 20 (PBST) and subsequently incubated in 1% $H_2O_2$ in PBS for 10 min at room temperature. The $H_2O_2$ was removed and replaced with 4% Triton X-100 in PBST for 45 min at room temperature. The Triton solution was removed and tissues were washed 5 times in PBST for 5 min each to terminate digestion. The tissues were then incubated again at room temperature in the 4% paraformaldehyde solution. After this second fixation, the fix was removed and the tissues were washed 5 times with PBST for 2 min each. The tissues were then rinsed in a 1:1 mixture of PBST-RNA hybridization solution (50% formamide, 5× saline sodium citrate, 100 μg ml$^{-1}$ heparin, 100 μg ml$^{-1}$ sonicated salmon sperm DNA, in 0.1% Tween 20, sterilized through a 0.2 μm filter, and stored in aliquots at –20°C), which was then replaced by 100% RNA hybridization solution. The tissues were stored in hybridization solution at –20°C from overnight to several days. Prehybridization was performed using an aliquot (300 μl per sample) of hybridization solution that had been boiled at 100°C for 5 min and then cooled on ice for a minimum of 5 min. Tissues were incubated with prehybridization solution at 56°C for a minimum of 1.5–2 h. At the end of the pre-hybridization, the solution was removed and replaced with hybridization solution prepared using an aliquot of fresh hybridization buffer with labelled antisense probe (or sense probe for controls) at a concentration between 0.5 and 1 ng μl$^{-1}$. This solution was incubated at 80°C for 5 min to denature the probe, and then cooled on ice for at least 5 min before use. Hybridization was carried out overnight (16–18 h) at 56°C; the hybridization solution containing probe was then removed and tissues were rinsed twice with fresh pre-warmed (56°C) hybridization buffer, and incubated at 56°C for 10 min. The samples were subsequently washed with pre-warmed 3:1, 1:1 and 1:3 (v:v) mixtures of hybridization buffer:PBST for 10 min each. The samples were then washed 3 times with pre-warmed PBST and acclimatized to room temperature. PBST was removed from samples and replaced with 1% block powder (Molecular Probes, Eugene, OR, USA) in PBST (PBTB) and incubated at room temperature with constant mixing for 15 min. After this initial block, tissue samples were incubated with biotin–SP-conjugated IgG fraction monoclonal mouse antidigoxin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at a dilution of 1:400 for 2 h with constant mixing and protected from light. After this primary antibody incubation, tissues were washed for 1 h with six changes of PBTB. Tissues were then transferred to a 1:100 dilution of horseradish peroxidase–streptavidin stock solution (Molecular Probes) in PBTB and incubated for 1 h with constant mixing and protected from light. Tissue samples were then washed for 1 h with six changes of PBTB followed by two brief washes in PBST and three 5 min washes in PBS. The PBS was

removed from the tissues and replaced with tyramide working solution (Alexa Fluor 568 tyramide solution 1:100 in amplification buffer; Molecular Probes) and incubated in the dark for 2 h at room temperature with constant mixing. Once incubation was complete, the samples were rinsed 3 times with PBS and then washed at room temperature with constant mixing for 1 h, changing the wash buffer every 10 min, followed by an overnight wash in PBS at 4°C. Tissues were mounted in glycerol and viewed under an Axoplan confocal microscope using a helium–neon laser (543 nm) and LSM 510 software (Zeiss, Jena, Germany).

### Immunohistochemistry

Fifth instars were secured on a piece of dental wax in a dissecting dish with the dorsal cuticle uppermost. Under physiological saline (Lane et al., 1975), the dorsal cuticle was removed, exposing the CNS and visceral tissues, and the tissues were then fixed using 2% paraformaldehyde. The fixation and staining of the tissues was performed as described previously (Te Brugge et al., 1999) with some minor modifications. In brief, the tissues were fixed for approximately 2 h at room temperature, washed in PBS, then transferred into 4% Triton X-100 with 2% BSA and 10% normal sheep serum (NSS) for 1 h. The preparations were transferred to the primary antiserum solution and placed on a flatbed shaker at 12°C for 24–48 h. Anti-*Locusta* DH antisera was used at a concentration of 1:1000 in 0.4% Triton X-100 with 2% BSA and 10% NSS. The preparations were then washed in PBS for 24 h at 12°C. The preparations were placed in Cy3-labelled sheep anti-rabbit immunoglobulin solution at 1:200 in PBS with 10% NSS for 12 h, then washed for 18 h at 12°C in PBS. All preparations were mounted in glycerol and were then viewed under an epifluorescence microscope equipped with a drawing tube and/or a confocal microscope (Zeiss, LSM 510).

### Sample preparation for MALDI-TOF tandem mass spectrometry

The CNS from fifth instars was dissected under saline, pooled in 500 µl of methanol–acetic acid–water (90:9:1), and stored overnight at –20°C. Samples were sonicated and centrifuged at 10,000 ***g*** for 10 min. The supernatant was collected and dried in a Speed Vac concentrator (Savant, Farmingdale, NY, USA). These tissue extracts were brought up in 0.1% trifluoroacetic acid (TFA; BDH, Toronto, ON, Canada) in water and applied to a $C_{18}$ Sep-Pak cartridge (Waters Associates, Mississauga, ON, Canada) which had been previously equilibrated with sequential applications of 8 ml methanol, then 8 ml water, then 8 ml water containing 0.1% TFA, and finally 2 ml of 0.1% TFA in water with 10 µg of protease-free bovine serum albumin (BSA, Sigma-Aldrich). The cartridge was then washed sequentially with 5.0 ml of water with 0.1% TFA then 3 ml each of 30% and 60% methanol (Burdick and Jackson, Muskegon, MI, USA) with 0.1% TFA and the eluents collected. The collected extracts were dried in the Speed Vac and frozen at –20°C until use.

### Reversed-phase liquid chromatography (RPLC) purification

The dried Sep-Pak eluents were further purified by RPLC in batches containing 250 or 500 CNS equivalents. Two RPLC purification steps were used and are described below.

#### System A

The dried eluents were brought into solution in 9% acetonitrile (Burdick and Jackson, Muskegon, MI, USA) with 0.1% TFA and filtered using a 0.22 µm Spin-X® filter (Corning, NY, USA). The filtrate was then applied to a RPLC system which utilized a Brownlee $C_{18}$ column (Mandel/Alltech, Guelph, ON, Canada). The RPLC gradient was from 9% to 60% acetonitrile with 0.1% TFA over 34 min (1.5% acetonitrile $min^{-1}$) at a flow rate of 1 ml $min^{-1}$. Fractions were collected, aliquoted, dried and stored at –20°C until use.

#### System B

Biologically active fractions from system A, as determined by Malpighian tubule secretion assay, were further purified through an RPLC system which utilized a Brownlee Spheri 5 phenyl column (Mandel/Alltech). The fractions were brought into solution in 1 ml of 18% acetonitrile with 0.1% TFA. The RPLC gradient was from 18% to 60% acetonitrile over 60 min (0.7% acetonitrile $min^{-1}$) collected in 1 ml fractions. The fractions were aliquoted, dried and stored at –20°C until use.

### Mass spectrometry

The fractions with biological activity, when tested on the *R. prolixus* Malpighian tubule assay, from three separate batches of CNS extractions were dried by Speed Vac and analysed by the Centre of Advanced Protein Technology (Hospital for Sick Children, Toronto, ON, Canada). Samples were analysed using MALDI-TOF mass spectrometry (QStar, Applied Biosystems Inc. Sciex, Concord, ON, Canada).

### Malpighian tubule secretion assay

The Malpighian tubules of fifth instars (5–6 weeks post-feeding as fourth instars) were dissected under physiological saline, and the tubules were freed from trachea and fat with the aid of fine glass rods. The secretion assays were conducted as described previously (Te Brugge et al., 1999). In brief, the upper portions of the tubule were transferred to a 20 µl drop of physiological saline under water-saturated heavy mineral oil. The open end of the tubule was pulled out and wrapped around a minuten pin. Saline containing the various tissue extracts, synthetic Rhopr DH or serotonin was exchanged for the equilibrating saline. The tubules were allowed to secrete for a further 20–30 min. Droplets of urine from the cut end of the tubule were collected and measured under the mineral oil using an eyepiece micrometer and the volume calculated. In experiments testing tissue extracts, a maximal rate of secretion was determined using $10^{-6}$ mol $l^{-1}$ serotonin, with each tubule acting as its own control. The percentage of the maximal rate of secretion was calculated. Mean ± s.e.m. rate of secretion (µl $min^{-1}$) was calculated and compared using Student's unpaired *t*-test.

### Anterior midgut absorption assay

Anterior midguts were removed from fifth instars (5–6 weeks post-feeding as fourth instars) and washed for 5 min in saline. The posterior end of the anterior midgut was ligated with a silk thread. The anterior portion was cut open and the contents of the tissue flushed out. Saline (20–30 µl) was introduced into the lumen of the anterior midgut and then the anterior end of the anterior midgut was also ligated with a silk thread. The anterior midgut was then gently picked up with forceps using the silk ligature, blotted then weighed on a Mettler (AE 240; Mettler-Toledo Inc., Mississauga, ON, Canada) balance. Once weighed, the tissues were placed in a microfuge tube with 100 µl of saline or saline containing serotonin or peptide solution. The tissues were incubated for 30 min at room temperature (23±0.5°C) then gently removed, blotted dry and weighed. The difference in mass, initial minus final, was calculated. A specific gravity of 1 was assumed. The results are expressed as the mean ± s.e.m. rate of absorption (µl $min^{-1}$).

## RESULTS

### Sequence and cloning of Rhopr DH cDNA

Preliminary experiments using degenerate primers designed against conserved regions of the known insect CRF-like peptides and gene sequences did not yield positive results. *In silico* screening of the *R. prolixus* genome trace archives, however, gave rise to several potential hits and primers were designed against these. These primers gave a cDNA product which was a 547 bp sequence encoding an incomplete CRF-like prepropeptide. This partial cDNA was used to design additional gene-specific primers that were used in 5′ and 3′ RACE, yielding a larger sequence of the *R. prolixus* DH gene (Rhopr DH gene), as shown in Fig. 1A (GenBank accession number HM153808). The sequence obtained is 650 nucleotides long and includes a 5′ untranslated region (UTR), an open reading frame (ORF) of 453 nucleotides and a 3′ UTR. The single ORF encodes a prepropeptide of 151 amino acids containing a predicted signal peptide with cleavage probably occurring between residues 17 and 18 (Gly 17 and Ser 18; SignalP3.0, ExPASy Server). The prepropeptide sequence also encodes a cryptic peptide and a CRF-like peptide which is 49 residues long plus a terminal glycine residue (MQRPQGPSLSVANPIEVLRSRLLLEIARRRMKEQDASRVSK-NRQYLQQIG). The putative CRF propeptide is flanked at the N- and C-termini by dibasic (Lys–Arg) residues, dibasic cleavage sites, and a C-terminal glycine residue indicating the sequence is further modified by amidation of the C-terminus. These modifications predict a Rhopr DH peptide with a molecular mass of 5760.7 Da (average) (PeptideMass, ExPASy Server).

### Structure of the *R. prolixus* CRF mRNA

The molecular organization of the Rhopr DH gene based on the BLAST analysis and *in silico* analysis using intron prediction is shown in Fig. 1B. Exon 1 contains the 5′ UTR residues and is at least 116 bp in length. Exon 2 contains the predicted signal peptide and a portion of the cryptic peptide (177 bp). Exon 3 (213 bp) contains the rest of the cryptic peptide and the Rhopr DH peptide sequence, while exon 4 is at least 153 bp long and contains the 3′ UTR. Preliminary studies with northern analysis suggested a larger sequence for the Rhopr DH mRNA of approximately 1.3 kb (not shown). Further *in silico* analysis of the *R. prolixus* genome predicts a sequence of similar value of 1.24 kb. The additional sequence is located in the 3′ UTR, is AT rich (19% GC) and predicts three polyadenylation signals. Primers generated against this region have not yet resulted in any successful sequencing.

### RPLC purification of *R. prolixus* CRF-like material

CRF-like material extracted from 250 and 500 *R. prolixus* CNSs and separated using RPLC over a $C_{18}$ column (system A) was tested for biological activity using Malpighian tubule secretion assays. The CRF-like material eluted in one peak of activity; in fractions 31, 32 and 33 (48–51% acetonitrile) (Fig. 2). The CRF-like material from fraction 31 was dried in a Speed Vac and run again on RPLC using a phenyl column (system B). These fractions were collected, and aliquots were tested in the Malpighian tubule secretion assay. CRF-like material eluted from the phenyl column in fraction 57 (approximately 54% acetonitrile) (Fig. 2). The CRF-like material purified from the batch of 250 CNSs eluted at similar times and percentage acetonitrile to the batch of 500 CNSs in both the $C_{18}$ and phenyl RPLC runs. CRF-like peptide standards were run on system B after the *R. prolixus* samples. The Dippu $DH_{46}$ standard eluted from the column slightly later than the active material at approximately 57% acetonitrile.

### Rhopr DH analysis: MALDI-TOF mass spectrometry

An aliquot of fraction 57 that stimulated Malpighian tubule secretion from the phenyl RPLC run of 500 CNSs was analysed by MALDI-TOF mass spectrometry and shown to contain a mass corresponding to the mass of the predicted amidated Rhopr DH (5760.2, Fig. 3A). This peak was confirmed with two other replicates of RPLC fractions that contained activity in the Malpighian tubule secretion assay. Interestingly, closer observation of the mass spectrometry data

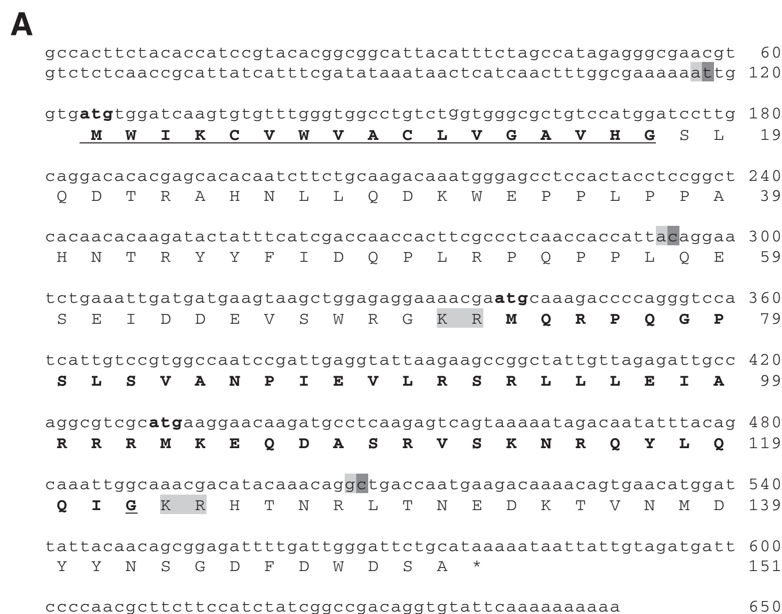

Fig. 1. *Rhodnius prolixus* diuretic hormone (Rhopr DH) sequence. (A) Nucleotide sequence of Rhopr DH cDNA (5′ to 3′) and deduced amino acid sequence of the peptide precursor. The numbering for each sequence is shown on the right. The initial methionine start codon is shown in bold. The predicted signal peptide is indicated in bold and underlined. The amino acid sequence for the mature corticotropin releasing factor (CRF) peptide is shown in bold. Dibasic amino acid pairs are shaded grey and the glycine residue required for amidation is underlined. (B) Molecular organization of the Rhopr DH gene based on BLAST analysis and *in silico* analysis using intron prediction. The boxes represent exons and the dashes represent introns. Exon 1 contains the 5′ untranslated region (UTR), exon 2 contains the predicted signal peptide, exon 3 contains the Rhopr DH peptide sequence and exon 4 contains the 3′ UTR.

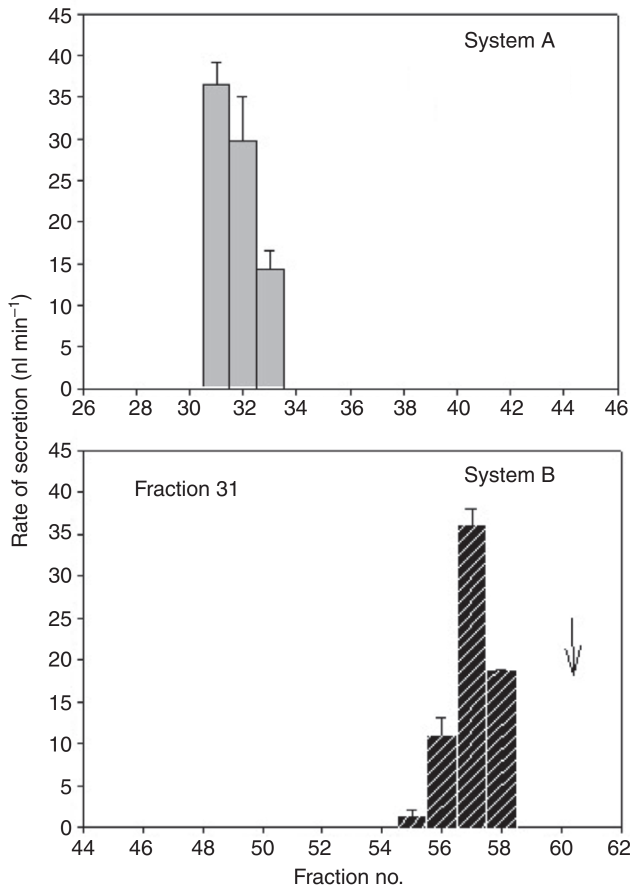


Fig. 2. Purification of Rhopr CRF-like material from 500 *R. prolixus* central nervous systems (CNSs) through RPLC system A ($C_{18}$ column). Subsamples of the fractions collected were tested for bioactivity using the Malpighian tubule secretion assay. Fraction 31 was then applied to system B (phenyl column). The elution time of Dippu $DH_{46}$ is indicted by the arrow. Values are means + s.e.

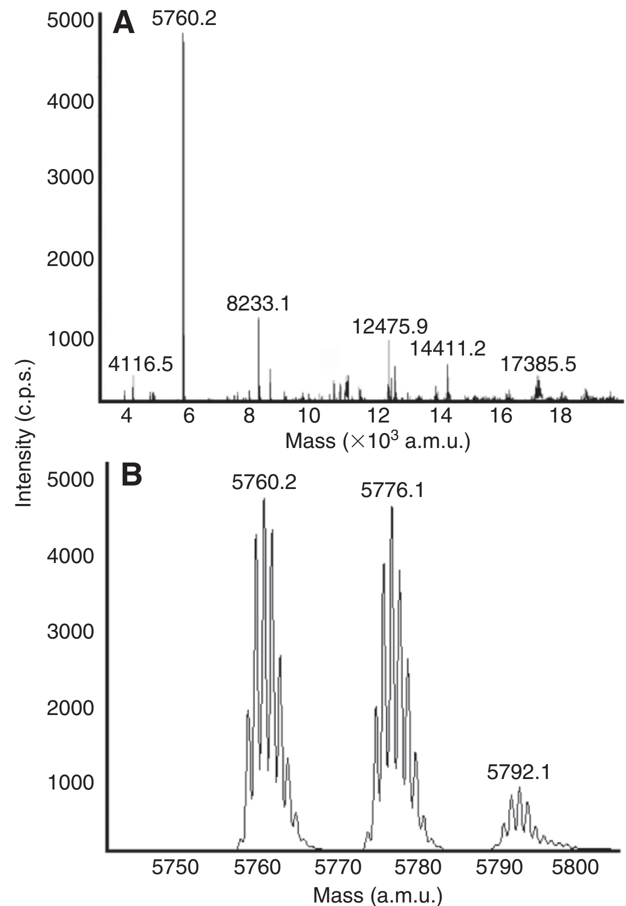


Fig. 3. (A) MALDI-TOF mass spectrometry of CRF-like biologically active material from phenyl column (system B) fraction 57. The peak at 5760.2 Da matches the molecular mass predicted for Rhopr DH. (B) Mass spectrometry over a smaller range of masses from 5700 to 5800 Da showing the three peaks at masses 5760.2, 5776.1 and 5792.1 Da. a.m.u., atomic mass units; c.p.s., counts per second.

revealed the presence of three peaks at masses 5760.2, 5776.1 and 5792.1 Da. These masses correspond to the mass of the predicted peptide, the peptide plus one oxygen molecule and the peptide with two oxygen molecules, consistent with the oxidation of the methionine residue(s) predicted in positions 1 and 31 (**M**QRPQGPSLSVANPIEVLRSRLLLEIARRR**M**KEQDASRVSK-NRQYLQQI-amide, Fig. 3B). The Rhopr DH standard was applied to RPLC system B where it eluted at 53–54% acetonitrile.

### Alignment of the insect CRF-like peptides

The amidated Rhopr DH shows several sequence similarities to other longer forms (greater than 40 amino acids long) of the insect CRF-like sequences (Table 1). The Rhopr DH sequence has 55% identity to *Periplaneta americana* (Peram) DH, 53% identity to Zoone DH and Dippu DH and 51% to Locmi DH (Table 2). The Rhopr DH sequence contains a region – PSLS**VA**NP**IE**VLR**S**RLLLEIARRR (residues 7–29) – that has a high degree of identity with other insect CRF-like sequences. Interestingly, there are several differences; the I (residue 15) and E (residue 16) are conservative substitutions while residues V (residue 11), A (residue 12) and S (residue 20) vary in this region from other closely aligned insect sequences. Conserved residues in the insect sequences are also observed in the amidated C-terminal portion of the CRF-like sequences, including NR (residues 42 and 43), L (residue 46) and I (residue 49).

### Immunohistochemistry

Immunohistochemistry was performed on fifth instar CNS using the antisera raised against residues 29–46 of Locmi DH. The pattern of staining was consistent with that which has previously been observed (Te Brugge et al., 1999). Staining was found throughout the CNS on both the dorsal and ventral surfaces. Two areas were of particular interest: the medial NSCs and their projections to the corpus cardiacum (CC); and the posterior lateral NSCs in the MTGM, which send projections out to abdominal nerves 1 and 2 and form neurohaemal-like areas on these nerves. Previous control experiments in which the primary antiserum was omitted or preabsorbed with *Locusta* DH resulted in the abolition of staining in the CNS of *R. prolixus* (Te Brugge et al., 1999).

#### Brain and retrocerebral complex

Numerous bilaterally paired cells were observed throughout the brain and of these 40–46 were very intensely stained (Fig. 4A). These cell bodies were found in the protocerebrum of the brain. Large numbers of immunoreactive cells were found at the base of

Table 1. Clustal W2 sequence alignment of insect corticotropin releasing factor (CRF)-like diuretic hormones (DHs)

| Species | Sequence |
|---|---|
| Musdo DP | ----------NKPSLSIVNPLDVLRQRLLLEIARR-QMKENTRQVELNRAILKNV-NH$_2$ |
| Drome DH$_{44}$ | ----------NKPSLSIVNPLDVLRQRLLLEIARR-QMKENSRQVELNRAILKNV-NH$_2$ |
| Culsa DH | ----------TKPSLSIVNPLDVLRQRIILEMARR-QMRENTRQVERNKAILREI-NH$_2$ |
| Anoga DH | ----------TKPSLSIVNPLDVLRQRIILEMARR-QMRENTRQVELNKALLREI-NH$_2$ |
| Tenmo DH$_{47}$ | ----AGALGESGASLSIVNSLDVLRNRLLLEIARK-KAKEG---ANRNRQILLSL-OH |
| Trica DH$_{47}$ | ----AGALGESGASLSIVNSLDVLRNRLLLEIARK-KAKEG---ANRNRQILLSL-NH$_2$ |
| Acypi DH | NGAMQGESPRSRPSLSIVNSLDVLRQKLMYEVARR-HVDENQKVLSQNHQILKNL-NH$_2$ |
| Apime DH | -----------IGSLSIVNSMDVLRQRVLLEIARR-KALQDQAQIDANRRLLETI-NH$_2$ |
| Nasvi DH | -----------IGSLSVVNSVDVLRERVLLELARR-KAMENQQQLGENQYVFKSV-NH$_2$ |
| Peram DP | --------TGSGPSLSIVNPLDVLRQRLLLEIARR-RMRQSQDQIQANREILQTI-NH$_2$ |
| Zoone DH | --------TGAVPSLSIVNPLDVLRQRLLLEIARR-RMRQSQDQIQANREMLQTI-NH$_2$ |
| Dippu DH$_{46}$ | --------TGTGPSLSIVNPLDVLRQRLLLEIARR-RMRQTQNMIQANRDFLESI-NH$_2$ |
| Locmi DH | --------MGMGPSLSIVNPMDVLRQRLLLEIARR-RLRDAEEQIKANKDFLQQI-NH$_2$ |
| Pedhu DH | ----------MKTSLSIDNPLDVLRQQYYRALARR----MKFKSIEKNREHLIKT-NH$_2$ |
| Achdo DP | ---------TGAQSLSIVAPLDVLRQRLMNELNRRRMRELQGSRIQQNRQLLTSI-NH$_2$ |
| Rhopr DH | ------MQRPQGPSLSVANPIEVLRSRLLLEIARRRMKEQDASRVSKNRQYLQQI-NH$_2$ |
| Manse DH$_{41}$ | ----------RMPSLSIDLPMSVLRQKLSLEKE----RKVHALRAAANRNFLNDI-NH$_2$ |
| Hylli DH$_{41}$ | ----------RMPSLSIDLPMSVLRQKLSLEKE----RKVQALRAAANRNFLNDI-NH$_2$ |
| Bommo DH$_{41}$ | ----------RMPSLSIDMPMSVLRQKLSLENE----RKLQSLRAMANRNFLNDI-NH$_2$ |
| Bommo DH$_{45}$ | ----------KMPSLSINNPMEVLRQRLLLEVARKQMREANQRQAVANRLFLQNV-NH$_2$ |
| Tenmo DH$_{37}$ | -----------SPTISITAPIDVLRKTWEQERARK--------QMVKNREFLNSLN-OH |
| Trica DH$_{37}$ | -----------SPTISITAPIDVLRKTWAKENMRK--------QMQINREYLKNLQ-OH |
| Manse DH$_{30}$ | -------------SFSVNPAVDILQH----RYMEK--------VAQNNRNNLNRV-NH$_2$ |
| Hylli DH$_{30}$ | -------------SFSVNPAVEILQH----RYMEK--------VAQNNRNFLNRV-NH$_2$ |
| Bommo DH$_{34}$ | -------------SFSVNPAVDTLQRGAYNHFLEK--------VVQSNRDYLNRI-NH$_2$ |

*Achaeta domesticus*, Achdo DH$_{46}$ (P23834); *Acyrthosiphon pisum*, Acypi DH (EX615509); *Anopheles gambiae*, Anoga DH$_{44}$ (AGAP003269-PA); *Apis mellifera*, Apime DH$_{43}$ (CAI45289.1); *Bombyx mori*, Bommo DH$_{34}$ (AB298935); Bommo DH$_{41}$ (AB298934); *Culex salinarus*, Culsa DH; *Diploptera punctata*, Dippu DH$_{46}$ (P82373); *Drosophila melanogaster*, Drome DH$_{44}$ (NP_649922.2); *Hyles lineata*, Hylli DH$_{30}$ (P82015), Hylli DH$_{41}$ (P82014); *Locusta migratoria*, Locmi DH$_{46}$ (AAB19827.1); *Manduca sexta*, Manse DH$_{30}$ (P24858), Manse DH$_{41}$ (AAB59200.1); *Musca domesticus*, Musdo DH$_{44}$ (P67800); *Nasonia vitripennis*, Nasvi DH predicted; *Pediculus humanus* corporis, Pedhu DH (XM002423579.1); *Periplaneta Americana*, Peram DH$_{46}$ (P41538); *Tenebrio molitor*, Tenmo DH$_{37}$ (P56618), Tenmo DH$_{47}$ (P56619); *Tribolium castanem*, Trica DH$_{37}$ (GLEAN 02243), Trica DH$_{47}$; *Zootermopsis nevadensis*, Zoone DH$_{46}$ (P82707).

the optic lobes. In addition, numerous cells stained along the posterior margin of the protocerebral lobes, including a cluster of five brightly stained cells in each hemisphere. Twelve to fourteen medial NSCs in each lobe of the brain stained intensely (Fig. 4A). The strong staining in the projections from the medial NSCs could be followed into the CC via the nervi corporis cardiaci. The CC stained very intensely, revealing an extensive plexus of immunoreactive varicosities.

### Suboesophageal and prothoracic ganglion

In the suboesophageal ganglion (SOG), numerous positive immunoreactive cells were observed. Strongly immunoreactive cells, however, were only observed at the lateral margins of the SOG and two bilaterally paired cells stained strongly in the midline of the ventral anterior SOG.

In the prothoracic ganglion (PRO), numerous bilaterally paired cells stained at the anterior and posterior regions of the ganglion. Several pairs of axon processes ran through the connectives into each of the ganglia, where they arborized extensively in the neuropile.

Table 2. Percentage identity of amino acid residues to *R. prolixus* DH

| Species | Identity (%) |
|---|---|
| Peram DH | 55.1 |
| Dippu DH$_{46}$ | 53 |
| Zoone DH | 53.1 |
| Locmi DH | 51.1 |
| Drome DH | 50.1 |
| Achdo DH | 44.9 |
| Culsa DH | 42.8 |
| Acypi DH | 36.8 |
| Pedhu DH | 23.5 |
| Bommo DH$_{41}$ | 24.5 |

### Mesothoracic ganglionic mass

The MTGM had numerous bilaterally paired immunoreactive cells (Fig. 4B). There was an extensive immunoreactive neuropile in the anterior, midlateral and central posterior portions of the MTGM. Axon tracts could be followed along the connectives from the prothoracic ganglion into the MTGM. Several cell groups observed were intensely immunoreactive. These included cells in the dorsal central midline of the fused ganglia, where 6–8 paired cells were immunopositive; of these 2–4 stained very strongly. In the same position, but on the ventral surface, a pair of strongly stained cells in the central midline of the MTGM was observed. Anterior, medial and posterior lateral groups of stained cells were also evident. The posterior lateral NSC groups (5–6 bilaterally paired cells) of the MTGM were very intensely stained (Fig. 4B). Processes from these cells were observed to branch centrally and out to abdominal nerves 1 or 2 which resulted in positively stained neurohaemal areas lying on the surface of these nerves (Fig. 4B). Fine axon tracts could also be seen in abdominal nerves 3–5 and in the genital nerves.

## Expression localization using FISH

Rhopr DH mRNA expression was determined using a (DIG)-labelled RNA anti-sense probe. Expression was observed in bilaterally paired cells throughout the CNS on both the dorsal and ventral surfaces of the fifth instar CNS. In the brain, strong expression was observed on the dorsal surface in 12–14 medial NSCs, and two pairs of lateral NSCs (Fig. 4C,E). Five to eight bilaterally paired cells were observed on the margin of the protocerebral lobes of the brain and 10–12

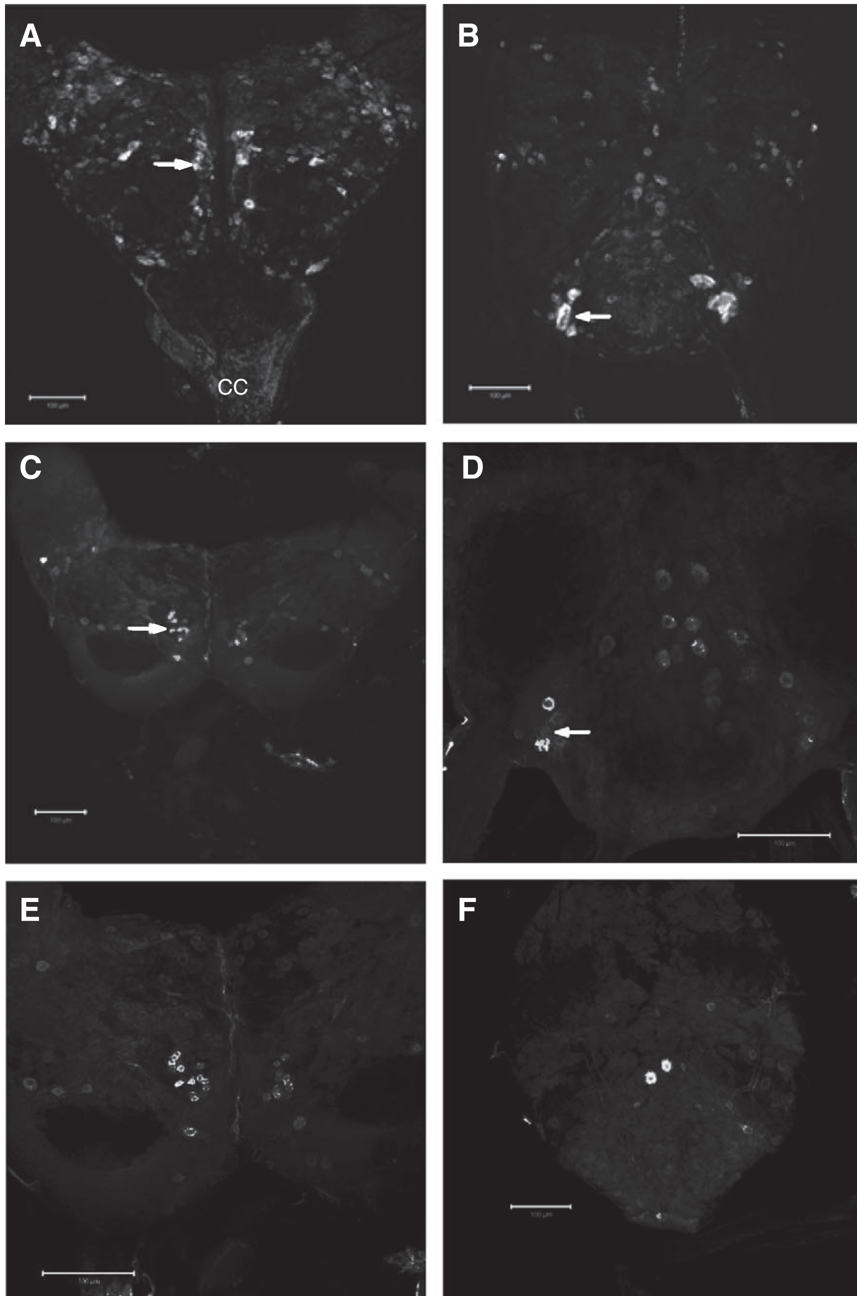


Fig. 4. Whole mount of a fifth instar *R. prolixus* CNS stained using the anti-*Locusta* DH antiserum. (A) Brain and corpus cardiacum (CC). A large number of cells can be seen to be immunoreactive in the optic lobe/brain junction and many cells along the posterior edge of the lobes of the brain. Note the medial neurosecretory cells (arrow). (B) Mesothoracic ganglionic mass (MTGM) showing the strongly stained posterior lateral neurosecretory cells (arrow) and processes. Groups of mid-line and mid-lateral cells are also seen in the MTGM. Expression of Rhopr DH was detected using fluorescent *in situ* hybridization. (C) Dorsal brain cells expressing Rhopr DH are observed at the optic lobe/brain junction, and along the posterior edge of the lobes of the brain. Note the medial neurosecretory cells (arrow). (D) MTGM showing expression of Rhopr DH in posterior lateral neurosecretory cells (arrow) and in paired cells in the mid-line and mid-lateral positions. (E) Higher magnification of the dorsal brain. (F) Rhopr DH expression in ventral MTGM. Note the strong expression in the central pair of cells. Bar in A–E: 100 μm.

cells were observed at the junction of the brain and optic lobes. On the ventral surface, variable expression was observed in a few cells at the border of the brain and the optic lobes. In the SOG, very weak staining was observed, from one pair of cells at the posterior margin of the ganglion. A single pair of weakly staining cells was also observed on the dorsal anterior PRO. On the dorsal surface of the MTGM, weak expression was observed in 1–3 bilaterally paired cells in the anterior and middle lateral positions. Moderate to strong expression was observed in 3–4 paired cells in the posterior central portion of the MTGM, and just posterior and lateral to these central cells were 5–6 pairs of cells with variable expression. Five to six posterior lateral cells were also observed consistently but were not strongly stained (Fig. 4D). On the ventral surface of the MTGM, a single pair of intensely stained cell bodies was observed in the central MTGM as well as a variable number of cells with weak expression (Fig. 4F). Sense controls showed no positive staining in the majority of preparations. In a small number of preparations (4 of 25 preps) 4–6 large cells in the brain showed expression in cells which were not observed in the anti-sense experimental preparations.

### Physiology: Malpighian tubule secretion assay

The Rhopr DH peptide increased the secretion rate of *R. prolixus* Malpighian tubules in a dose-dependent manner. The Rhopr DH had an $EC_{50}$ value of $3.27\times10^{-9}$ mol l$^{-1}$ (Fig. 5), with a threshold between $10^{-10}$ and $10^{-9}$ mol l$^{-1}$, and a maximum at $10^{-7}$ mol l$^{-1}$.

### Physiology: anterior midgut assay

The anterior midgut absorption assay is a sensitive assay to monitor the movement of water across the epithelium of the anterior midgut. In these assays (Fig. 6), Rhopr DH significantly increased the rate of absorption across the anterior midgut epithelium compared with saline controls at a concentration of $10^{-8}$ mol l$^{-1}$.

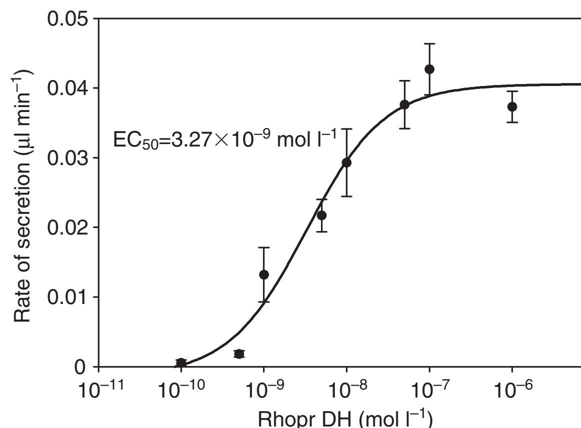


Fig. 5. Dose–response curve for effects of Rhopr DH (plotted on a log scale) on the rate of secretion of *R. prolixus* fifth instar Malpighian tubules. Points are means ± s.e.m., *N*=11–15. Regression curve fitted by Sigmaplot version 11 curve fitting program.

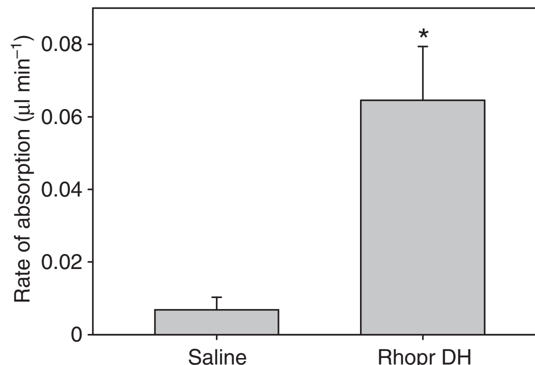


Fig. 6. Absorption assay using the anterior midgut, in the presence of $10^{-8}$ mol l$^{-1}$ Rhopr DH or saline (control). *Significant increase in the rate of absorption (Student's *t*-test, $P<0.05$). Means + s.e.m., *N*=5–10.

### Mass spectrometry

The mass spectrometry confirmed the presence of a peak at mass 5760.2 Da in samples that stimulated *R. prolixus* Malpighian tubules, consistent with the predicted mass of the amidated form of Rhopr DH. The presence of three peaks with masses of 5760.2, 5776.1 and 5792.1 Da correspond to the mass of the predicted peptide, the peptide plus one oxygen molecule and the peptide with two oxygen molecules.

### Comparison of Rhopr DH peptide sequence with other insect sequences

The Rhopr DH sequence is one of the longest insect CRF-like sequences, with only another hemipteran, the pea aphid *Acyrthosiphon pisum* (EX615509), having a longer sequence of 54 residues (Table 1).

In common with the majority of the insect DH/CRF sequences, the Rhopr DH is C-terminally amidated. The insect sequences have only a 6–41% amino acid identity to the vertebrate CRF, urotensin, urocortin and sauvagine peptide family. The insect CRF-like sequences (longer than 40 residues) have the greatest degree of conservation of peptides towards the N-terminus, with the sequence PSLSIVNPLDVLRQRLLLEIARR (consensus sequence for 60% of the peptides). In Rhopr DH, this sequence varies and is PSLS**VA**NP**IE**VLR**S**RLLLEIARR with **VA** in place of IV, **IE** in place of LD and **S** in place of Q. Analysis of the amino acid residues in insect DH sequences with greater than 40 residues shows that there are a high number of arginine (basic) residues (13.2%) and leucine (hydrophobic) residues (15.6%). Basic residues have been implicated in a number of functions including cleavage, protein–protein interaction, and protein–phospholipid and protein–DNA binding (Lovejoy and Barsyte-Lovejoy, 2010). Interestingly, the Rhopr DH contains two methionine residues which may play a role in the biological activity of the peptide as has been suggested for other insect CRF-like peptides (Audsley et al., 1995). A comparison of the insect CRF-like sequences shows a 20–87% amino acid identity (Furuya et al., 1998; Roller et al., 2008) with identical sequences found in *Musca domestica*/*Stomoxys calcitrans* (Clottens et al., 1994). The Rhopr DH sequence has the greatest amino acid identity (50–55%) to the sequences from *D. melanogaster*, *L. migratoria*, *D. punctata*, *Z. nevadensis* and *P. americana*. In common with the C-terminal sequence for Locmi DH, the Rhopr DH ends in QQI-amide. It is interesting that the most successful antisera used to look for CRF-like immunoreactivity in *R. prolixus* (Te Brugge et al., 1999) was that raised against the

## DISCUSSION

The present work confirms the presence and identity of a CRF-like peptide in *R. prolixus* and its potent biological activity on native tissues. The Rhopr DH cDNA and amino acid sequence have similarities to other insect CRF sequences but contain some interesting differences.

### CRF-like gene organization

The CRF-like gene organization has been studied in vertebrate and invertebrate species. In vertebrates the CRF genes tend to be composed of two exons while in tunicates only a single exon has been demonstrated (Lovejoy and Barsyte-Lovejoy, 2010). In the insects studied to date, including *R. prolixus*, multiple-exon genes have been predicted or observed (present study) (Lovejoy, 2009; Lovejoy and Jahan, 2006). In *Bombyx mori* the CRF-like DH gene is composed of 11 exons (Roller et al., 2008) while in *Tribolium castaneum* (Li et al., 2008) the CRF-like gene is thought to be composed of three exons. In both *B. mori* and *T. castaneum* the CRF-like gene is alternatively spliced, giving rise to short and long forms of CRF-like peptides. While alternative splicing has been suggested in both moths and beetles (Roller et al., 2008; Li et al., 2008) no alternative splicing has been observed in *Drosophila melanogaster* (Cabrero et al., 2002). In all the genes that encode the CRF family members, the exons upstream of the translated portion of the gene encode only a portion of the 5′ UTR.

### Characteristics of CRF-like prepropeptide and mature peptides

The organization of the preprohormone observed in the present work for the Rhopr DH is consistent with that observed in other insects, and indeed conserved in all DH/CRF family members (Lovejoy, 2009; Lovejoy and Jahan, 2006). The Rhopr DH preprohormone is composed of a signal peptide, cryptic peptide and mature CRF-like peptide. In *B. mori* the N-terminal signal peptide and cryptic peptide sequence are found on exons 3 and 4 while the three mature peptides ($DH_{34}$, $DH_{41}$ and $DH_{45}$) are found downstream in exons 5–11 (Roller et al., 2008). Cleavage of the mature Rhopr DH is predicted at the dibasic KR residues, at the N- and C-termini of the peptide sequence, and the presence of the glycine residue predicts a C-terminal amidated mature peptide 49 residues in length.

C-terminal sequence of Locmi DH, residues 29–46, which also ends in LQQI-amide (coupled at the N-terminus to thyroglobulin using glutaraldehyde) (Patel et al., 1994).

### Distribution of the Rhopr DH

Previously we have shown the presence of CRF-like immunoreactivity in *R. prolixus* fifth instar CNS and peripheral tissues (Te Brugge et al., 1999; Te Brugge et al., 2001). The pattern of immunoreactivity in *R. prolixus* CNS in the present work was consistent with that obtained previously. In particular, immunoreactivity was observed in medial NSCs of the brain, which send projections to the CC, and posterior lateral NSCs in the MTGM, which send projections out to abdominal nerves 1 and 2. In all of the other species studied, including *R. prolixus*, strong CRF-like immunoreactivity has been observed in a subset of the medial NSCs of the brain (Te Brugge et al., 1999; Cabrero et al., 2002; Iaboni et al., 1998; Patel et al., 1994; Veenstra and Hagedorn, 1991; Emery et al., 1994). As well, the immunoreactivity observed in the posterior lateral NSCs in the abdominal neuromeres of the MTGM of *R. prolixus* is consistent with the immunoreactivity observed in abdominal ganglia of *L. migratoria* (Patel et al., 1994; Thompson et al., 1995) and *M. sexta* (Chen et al., 1994).

The pattern of gene expression observed in the present study for Rhopr DH mRNA is consistent with the pattern of CRF-like immunoreactivity (present work) (Te Brugge et al., 1999). In both the immunohistochemisty and *in situ* studies strong and consistent expression was observed in medial and lateral NSCs of the brain as well as in neurons along the margin of the protocerebral lobes and at the brain–optic lobe boarder. There was also expression in the MTGM in bilaterally paired cells on the dorsal and ventral surfaces, and, in particular, strong expression was observed in paired central and posterior lateral NSCs. Interestingly, this pattern of expression is consistent with the earlier studies of Maddrell (Maddrell, 1963) who found diuretic activity in all parts of the CNS of *R. prolixus* except for the CC. The majority of the diuretic activity was in the MTGM, and 90% of this activity resided in posterior lateral cell groups.

The expression observed in *R. prolixus* is also consistent with that observed with *in situ* experiments for CRF-like expression in *D. melanogaster* (Cabrero et al., 2002) and *B. mori* (Roller et al., 2008) where $DH_{34}$ and $DH_{41}$ are thought to be co-localized in three pairs of medial NSCs in the protocerebral lobes of the brain. Interestingly, Roller and colleagues (Roller et al., 2008) found that the expression of the third form of CRF found in *B. mori*, $DH_{45}$, was confined to interneurons in the brain.

CRF-like immunoreactivity and expression in posterior lateral NSCs in the MTGM of *R. prolixus* is consistent with the immunoreactivity and expression pattern found in other insects (Patel et al., 1994; Thompson et al., 1995; Chen et al., 1994; Roller et al., 2008). In *B. mori*, $DH_{41}$ and $DH_{34}$ CRF-like expression was observed in dorsolateral cells of the abdominal ganglia (2–7), which are thought to be the lateral NSCs, L3. Expression was also observed in a pair of large dorsolateral neurons in the terminal abdominal ganglion (Roller et al., 2008). Evidence from both immunohistochemistry and *in situ* hybridization therefore suggests that NSCs in both the brain and abdominal ganglia/neuromeres are important sites of insect CRF peptide production.

There is also evidence for the co-localization of CRF-like peptides with other peptides in the cells of the brain and abdominal ganglia/neuromeres. In *R. prolixus* the medial NSCs show immunoreactivity for several neuropeptides, including RF-amide-like (Tsang and Orchard, 1991) and $DH_{31}$-like peptides (Te Brugge et al., 2005). Co-localization of kinin-like and CRF-like immunoreactivity is also observed in *R. prolixus* in the posterior lateral NSCs of the MTGM (Te Brugge et al., 2001). In *M. sexta,* the lateral NSCs produce CRF-like and kinin-like peptides as well as ecdysis-triggering hormone receptor (Chen et al., 1994; Kim et al., 2006).

As was indicated above, the pattern of gene expression observed for Rhopr DH mRNA is consistent with the pattern of CRF-like immunoreactivity; however, more cells were observed in all parts of the CNS using immunohistochemisty. This observation could be due to several factors. (1) The antiserum used is non-native and is cross-reacting with other peptides. The *Locusta* antiserum has been shown to specifically recognize the CRF-like *Locusta* DH in *L. migratoria* using a variety of techniques (Patel et al., 1994; Audsley et al., 1997). Preabsorption of the *Locusta* DH antiserum with *Locusta* DH abolished staining in the CNS, indicating a degree of specificity of the antiserum. However, the blocking of staining does not remove the possibility that the antiserum cross-reacts with another peptide(s) (see Nässel, 1996). (2) It is possible that more than one CRF-related peptide is present in *R. prolixus* and that the CRF antiserum is able to recognize these peptides while the Rhopr DH mRNA expression using the anti-sense probe is more specific, recognizing only one specific mRNA encoding the peptide. Multiple CRF-like peptides have been found in other species and preliminary studies have suggested this may also be the case in *R. prolixus* (V.T.B. and D.A.S., unpublished). If present, these peptides could have differential expression as was observed in *B. mori* (see above) (Roller et al., 2008).

### Activity of Rhopr DH on native tissues

In insects, the CRF-like peptides have been shown to increase cAMP (Coast, 1996), transepithelial potential (O'Donnell et al., 1996; Nicolson, 1993) and the secretion rate of Malpighian tubules (Coast, 1996). The CRF-like peptides increase fluid secretion in a dose-dependent manner with $EC_{50}$ values for most species in the nanomolar range (Coast, 1995; Coast, 1996). Previously, we have demonstrated that the non-native CRF-like peptides Zoone DH and Dippu $DH_{46}$ increase the rate of secretion of *R. prolixus* Malpighian tubules in a dose-dependent manner, with the two CRF-like peptides having a similar dose–response curve (TeBrugge et al., 2002). The $EC_{50}$ values were $6.7\times10^{-7}$ mol l$^{-1}$ and $5.5\times10^{-7}$ mol l$^{-1}$ for Zoone DH and Dippu $DH_{46}$, respectively, with a threshold at $2\times10^{-7}$ mol l$^{-1}$, and maximum at approximately $10^{-6}$ mol l$^{-1}$. The dose–response curve for the native Rhopr DH tested on fifth instar *R. prolixus* Malpighian tubules had an $EC_{50}$ of $3.27\times10^{-9}$ mol l$^{-1}$. This represents a 200-fold difference in the $EC_{50}$ value of the native peptide compared with the non-native CRF-like peptides tested.

Both non-native CRF-like peptides, as well as serotonin, increase the cAMP content of *R. prolixus* Malpighian tubules in the presence or absence of 3-isobutyl-1-methylxanthine (IBMX). Zoone DH, in the presence of IBMX, increases the cAMP content in a dose-dependent manner with an $EC_{50}$ of $7.5\times10^{-7}$ mol l$^{-1}$, a threshold at $5\times10^{-8}$ mol l$^{-1}$ and maximum at approximately $2\times10^{-6}$ mol l$^{-1}$ (Te Brugge et al., 2002). However, in both *A. aegypti* and *A. domesticus*, CRF-like peptides have been shown to activate cAMP as well as alternative pathways. In *A. aegypti*, the *Culex salinarus* CRF-like peptide has a biphasic response; at low concentrations the peptide stimulates $Cl^-$ shunt conductance (*via* $Ca^+$) and depolarizes the transepithelial potential (TEP), which is positive. At higher concentrations, however, the peptide stimulates both the shunt conductance and the cAMP-dependent movement of $Na^+$ through the principal cells (Clark et al., 1998).

In *R. prolixus*, studies comparing the activity of Zoone DH and 5HT on ion transport and transepithelial potential across the upper segments of Malpighian tubules (Donini et al., 2008), coupled with secretion and second messenger studies (Te Brugge et al., 1999; Te Brugge et al., 2002), suggest that CRF-like peptides and 5HT activate the same second messenger pathways and ion-transport mechanisms in the upper tubule cells. Interestingly, however, Donini and colleagues (Donini et al., 2008) have suggested that the lower portion of the Malpighian tubules, where the reabsorption of $K^+$ occurs, is stimulated only by 5HT, not by Zoone DH. Studies are currently underway to look at the activity of the native peptide on the lower tubule.

Previously, both 5HT and Zoone DH have been shown to have activity on the anterior midgut of *R. prolixus*, elevating cAMP content (Te Brugge et al., 2009; Barrett et al., 1993), increasing the frequency of contractions (Te Brugge et al., 2009), and increasing ion and water movement (Te Brugge et al., 2009; Farmer et al., 1981). The present work demonstrates that the native peptide, Rhopr DH, is biologically active, increasing the rate of absorption from the anterior midgut, as well as secretion by the Malpighian tubules, suggesting that the activities of the anterior midgut and upper Malpighian tubules are coordinated by both 5HT and Rhopr DH.

Barrett and Orchard (Barrett and Orchard, 1990) suggested a possible synergistic role for 5HT and the diuretic peptide, and Maddrell and colleagues (Maddrell et al., 1993) showed that, indeed, serotonin acts synergistically with forskolin and the peptide DHs, to increase fluid secretion. Studies are underway to explore the interaction of the native Rhopr DH peptide and 5HT as well as the native $DH_{31}$ and kinin-like peptides.

The use of traditional methods of peptide purification did not prove fruitful in *R. prolixus* because of low quantities of the CRF-like peptide and the possible loss of activity due to the oxidation of the methionine residues. Other attempts with degenerate primers using DNA sequences from other insects also did not prove to be useful. Use of the *R. prolixus* genome has enabled confirmation of the presence and sequencing of the long sought-after native CRF-like peptide, demonstrating the importance and usefulness of the information from the *R. prolixus* genome. The identification of this Rhopr DH and other neuropeptide sequences is key to furthering our understanding of the post-feeding diuresis in *R. prolixus*.

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