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A COMPARISON OF THE EFFECTS OF TWO PUTATIVE DIURETIC HORMONES FROM *LOCUSTA MIGRATORIA* ON ISOLATED LOCUST MALPIGHIAN TUBULES

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Summary

Previous work has shown that a peptide related to arginine vasopressin is present in the suboesophageal ganglion of the locust, *Locusta migratoria*. This peptide was determined to be an anti-parallel dimer of the nonapeptide Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2 and was reported to stimulate cyclic AMP production and fluid secretion in a combined Malpighian tubules and midgut preparation from locusts. For these reasons the peptide has been called the arginine-vasopressin-like insect diuretic hormone (AVP-like IDH). Recently, a second diuretic peptide (*Locusta*-DP), which is related to corticotropin releasing factor, has been identified: this is a potent stimulant of fluid secretion and cyclic AMP production by isolated locust tubules. Because water balance in insects is likely to be controlled by a cocktail of hormones acting on both Malpighian tubules and hindgut, this study directly compares the activity of these two peptides in fluid secretion and cyclic AMP production bioassays on one target organ, the isolated Malpighian tubule of *Locusta migratoria*. *Locusta*-DP was synthesised directly, whereas the dimeric AVP-like IDH was obtained by oxidation of a synthetic nonapeptide monomer. Products were separated by RP-HPLC and their structures unequivocally confirmed by enzymatic digestion, sequence analysis and electrospray mass spectrometry. We show that *Locusta*-DP causes strong stimulation of fluid secretion and cyclic AMP production, whereas the AVP-like IDH has no effect in either assay. These findings are discussed in the light of recent work on the anatomy and physiology of the vasopressin-like immunoreactive (VPLI) neurones in the suboesophageal ganglion of *Locusta migratoria*, the proposed source of the AVP-like peptide.

Introduction

It is difficult to define the activity of 'diuretic factors' in insects (Spring, 1990). The

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presence of separate sites of fluid secretion (the Malpighian tubules) and subsequent water reabsorption (the hindgut) allows diuresis to be defined either as primary urine production by Malpighian tubules (before any hindgut reabsorption) or total water loss *via* the rectum (after any hindgut reabsorption). Generally the former definition is used to characterise diuretic factors: they must stimulate primary urine production by Malpighian tubules.

Using a radioimmunoassay with antisera raised against vasopressin, Proux and Rougon-Rapuzzi (1980) have identified arginine vasopressin (AVP)-like immunoreactive factors present in locust central nervous system (CNS) and haemolymph. Because the levels of the factors in haemolymph altered with relative humidity, they suggested that these factors had a role in water balance. Proux *et al.* (1987) subsequently undertook the mammoth task of isolating two AVP-like neuropeptides from extracts of 51000 suboesophageal and thoracic ganglia, which were purified by immunoaffinity and high pressure liquid chromatography. The two neuropeptides, called F1 and F2, were sequenced and synthesized. The amidated nonapeptide F1 (Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2; see Fig. 1B) had no effect on isolated Malpighian tubules, but F2, an antiparallel dimer of F1 (see Fig. 1D), was reported to have a diuretic action (assessed by urine production from a combined Malpighian tubules/midgut preparation) at concentrations around 1×10^{-9} mol l^{-1}. In addition, F2 was reported to stimulate cyclic AMP production by Malpighian tubules (Proux and Hérault, 1988). F2 has been named the arginine-vasopressin-like insect diuretic hormone (AVP-like IDH; Proux *et al.* 1987).

Picquot and Proux (1987) have related haemolymph titre of AVP-like peptides to the excretion of primary urine *in vivo* (assayed by measuring elimination of amaranth dye by Malpighian tubules). They showed three peaks in diuresis over a 24h period, one of which was correlated with a peak in AVP-like peptide titre in the haemolymph. They concluded that AVP-like IDH titre and diuresis are linked, but acknowledged that other factors must also play a role in the control of primary urine production.

Two candidates for these additional diuretic factors are DP-I and DP-II: these partially characterised factors were shown to be present in the brain and corpora cardiaca of *Locusta migratoria* (Morgan and Mordue, 1984). Recently, a diuretic peptide (*Locusta*-DP; see Fig. 1E) extracted from *Locusta migratoria* heads was fully sequenced (Kay *et al.* 1991*b*; Lehmberg *et al.* 1991) and shown to be present in corpora cardiaca (Kay *et al.* 1991*b*). Because synthetic *Locusta*-DP stimulates cyclic AMP production in isolated tubules, it is probably one of the partially characterised factors (DP-I) described by Morgan *et al.* (1987). *Locusta*-DP has no structural similarity to the AVP-like IDH, but has significant sequence homology to vertebrate corticotropin releasing factor (CRF) and, more importantly, has considerable homology with identified diuretic peptides in *Manduca sexta* (Blackburn *et al.* 1991; Kataoka *et al.* 1989), *Acheta domesticus* (Kay *et al.* 1991*a*) and *Periplaneta americana* (Kay *et al.* 1992). These data provide evidence for a family of CRF-like diuretic peptides in insects.

In this study, we compare directly the biological activities of the AVP-like and CRF-like diuretic peptides of *Locusta migratoria* in two bioassays, namely fluid secretion and cyclic AMP production by isolated Malpighian tubules. We find that the CRF-like *Locusta*-DP stimulates fluid secretion fivefold and dramatically increases cyclic AMP

production at physiologically appropriate doses. In contrast, the AVP-like peptide has no effect in either assay, even at pharmacological doses.

Materials and methods

Peptide synthesis and characterisation

The reduced peptide Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2 (Fig. 1A) was synthesized by Alta Bioscience, (Birmingham, UK) using fMOC chemistry on a Biotech BT 7600 (Biotech Instruments, Luton, UK). Material obtained after cleavage of the peptide from the resin was dissolved in 5% acetonitrile/0.1% trifluoroacetic acid (TFA)

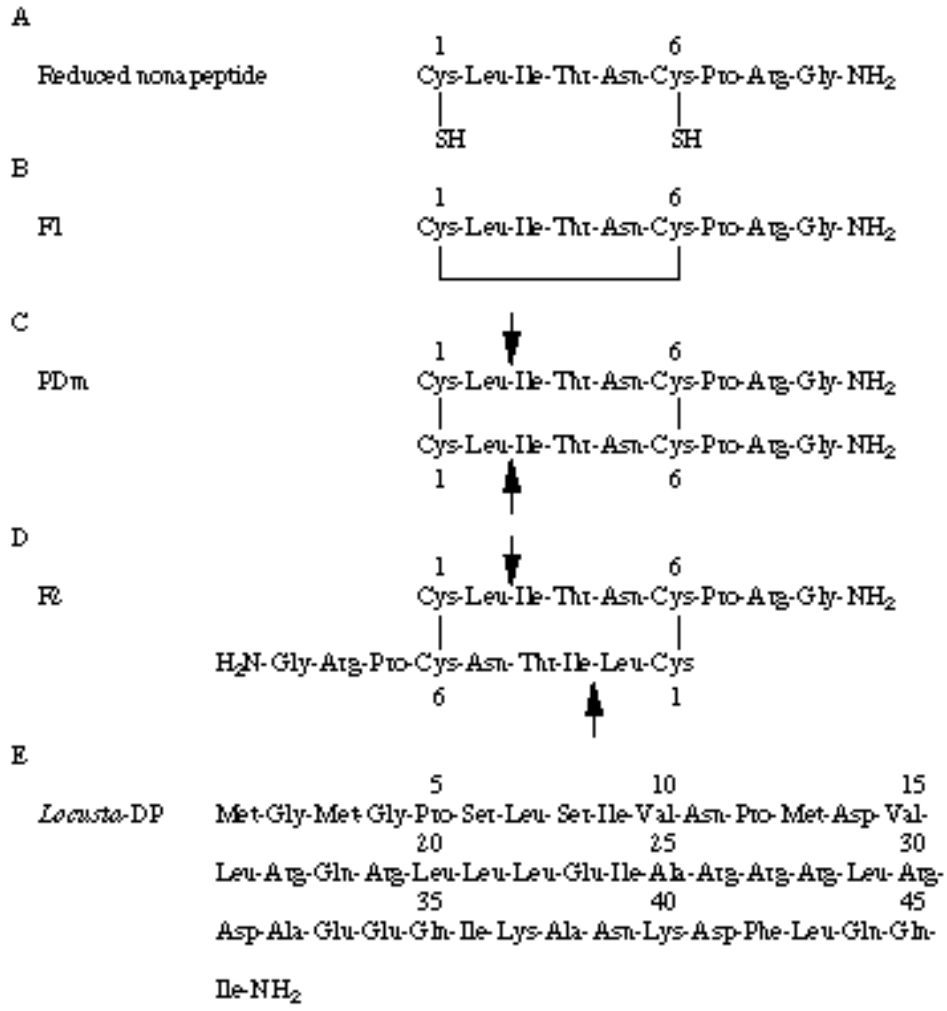

Fig. 1. Primary structures of the peptides investigated in this study. Arrows indicate bonds cleaved by thermolysin.

and loaded onto a Poros R/H reversed-phase perfusion chromatography column (4.6mm×100mm; Progressive Research Systems, Cambridge, UK) equilibrated with the same solvent. The peptide was eluted from the column using a gradient from 0% to 35 % solvent B (solvent A was 5% acetonitrile in 0.1% TFA; solvent B was 60% acetonitrile in 0.07% TFA) over 4min at a flow rate of 4mlmin^{-1}. Peptides were detected by monitoring the absorbance of the eluate at 214nm. The amino acid sequence of the pure peptide was confirmed by automated Edman degradation using a Beckman LF3000 peptide sequencer with an on-line PTH analyser. Mass spectrometry was determined on a TRIO-2 quadrupole mass spectrometer (VG Masslab Ltd, Manchester), fitted with a VG Biotech (Manchester) electrospray device. Peptides were dissolved in acetonitrile:water:acetic acid (50:50:1). Approximately 10pmol was injected. Ionisation was performed with cone voltages of 35V or 55V.

To form the internal 1–6 disulphide-bridged monomer (F1; Fig. 1B), and to generate parallel (PDm; Fig. 1C) and antiparallel (F2; Fig. 1D) dimers from the reduced peptide, we used a glutathione-based oxido-shuffling oxidation system (Jaenicke and Rudolph, 1989). The reduced peptide (250 μg) was dissolved in a minimal volume of water and the solution was brought to 100mmoll^{-1} Tris, pH8.6, containing 1mmoll^{-1} EDTA, 0.3mmoll^{-1} glutathione disulphide and 3.0mmoll^{-1} reduced glutathione (final peptide concentration, 30mgml^{-1}). After incubation at 30˚C for at least 3h, the products were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on an Aquapore C_8 RP300 column (Technicol, Stockport, UK) using an isopropanol solvent system (solvent A was 5% isopropanol in 0.1% TFA; solvent B was 60% isopropanol in 0.07% TFA; see Proux and Beydon, 1992). A gradient of solvent B from 5% to 45 % over 20min at 1.0mlmin^{-1} was used to elute the peptides. In some cases, chromatography of these peptides was performed using a Vydac C_4 column (300Å, 4.6mm×100mm). In these instances, the column was equilibrated at 0% B using the isopropanol solvent system; peptides were eluted using a gradient of 0% to 35% B over 30min at 1mlmin^{-1}.

To distinguish between parallel (PDm) and antiparallel (F2) dimeric peptides produced in the oxidation system, each of the purified dimers (10 μg) was incubated with 0.1 μg of thermolysin in 10 μl of 100mmoll^{-1} Tes, pH7.2, at 30˚C for 2–3h (see Proux *et al.* 1987). The cleavage products were separated by RP-HPLC using the C_8 system. Products were sequenced, and their masses determined, as described above.

Locusta-DP (Fig. 1E) was synthesized using standard fMOC chemistry and purified by RP-HPLC (M. Patel and T. K. Hayes in preparation).

Bioassays

Adult male (14–21 days old) *Locusta migratoria* L. from the colony at Birkbeck College were used as the source of Malpighian tubules in the bioassays.

Fluid secretion assay

The fluid secretion assay has been described previously for *Acheta domesticus* tubules (Coast, 1988). In *Locusta*, distal ends of Malpighian tubules are attached to the gut by tracheae; consequently, proximal segments equivalent to approximately 80% of the total

tubule length were employed. The cut distal end of the tubule was occluded by clamping it into a strip of Sylgard (Dow Corning). The proximal end of the tubule was bathed in a 5 μl saline droplet of the following composition in mmol l^{-1}: NaCl, 100; $NaHCO_3$, 4; K_2SO_4, 5; $CaCl_2$, 5; $MgSO_4$, 10; glucose, 10; alanine, 2.9; arginine, 1; asparagine, 1.3; glutamine, 5; glycine, 11.4; histidine, 1.4; lysine, 1.4; proline, 13.1; serine, 1.5; tyrosine, 1.9; valine, 1.8; Hepes, 25; adjusted to pH7 with 1mol l^{-1} NaOH). Secreted fluid escaped from the tubule through a small cut made close to the Sylgard (Fig. 2). At intervals, the drops of secreted fluid were displaced from the tubules and their diameters measured using an eyepiece micrometer. Following a 20min equilibration period, the basal rate of secretion was measured over 20min. The saline drop was replaced with saline containing assay material and the rate of secretion determined over a subsequent 20min test period. Results are expressed as both absolute secretion rates in picolitres per minute (see Fig. 5A,B) and as the change in rate of tubule secretion in pl min^{-1} (see Fig. 5A,C).

Cyclic AMP assay

The assay for cyclic AMP production by isolated Malpighian tubules has been described previously (Coast *et al.* 1991; Kay *et al.* 1991*a*). Briefly, single tubules were transferred to 40 μl of a simple saline (Coast, 1988) containing 10^{-4} mol l^{-1} isobutylmethylxanthine (IBMX) in 1.6ml microcentrifuge tubes. Following a 20min equilibration period, 10 μl of assay material was added and the tubes were incubated for 15min at room temperature. Reactions were stopped by the addition of 200 μl of ice-cold methanol, and intracellular cyclic AMP was released by sonication. Cyclic AMP concentration was determined using a competitive protein binding assay (Coast *et al.* 1991) and expressed as pmol per tubule.

Results

Confirming the structures of the synthetic peptides

Oxidation of the reduced peptide Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2 should produce at least three products: a monomer containing an internal 1–6 disulphide bridge (F1), a parallel dimer (PDm) and an antiparallel dimer (F2). The primary structures of these peptides are depicted in Fig. 1. As shown in Fig. 3, incubation of Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2 in the oxido-shuffling system produced a mixture of products including three major peaks (labelled 1, 2 and 3 in Fig. 3) which could be clearly separated by RP-HPLC. The absence of an absorbance peak in the chromatogram corresponding to the reduced peptide (arrow in Fig. 3) indicated complete conversion of the reduced peptide to oxidized species. To identify the peaks, we used automated Edman degradation sequencing. Peaks 1, 2 and 3 contained peptides which exhibited the expected amino acid sequence. In addition, treatment of the individual peaks with the reducing agent dithiothreitol (DTT) produced in each case a single species which co-eluted on RP-HPLC with the reduced peptide (not illustrated).

Mass spectrometry gave a relative molecular mass of 975.3 for the reduced peptide Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2, whereas the material in peak 1 had a mass

of 972.8. This is consistent with formation of an internal, 1–6 disulphide bridge in the peptide. This peptide is therefore F1. Because the later-eluting peaks 2 and 3 have the same primary sequence as F1 and on reduction with DTT co-elute on RP-HPLC with the

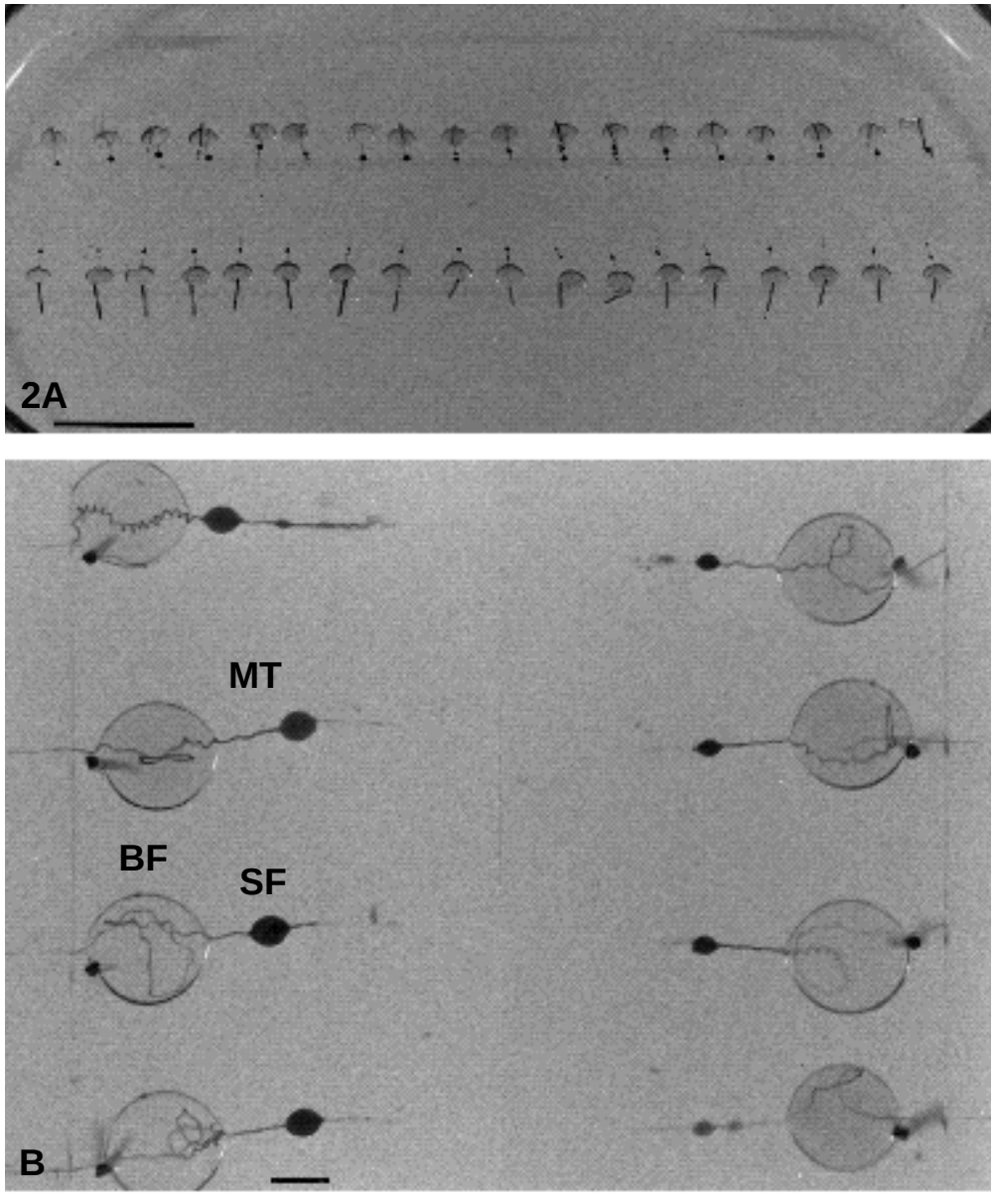

Fig. 2. The isolated Malpighian tubule fluid secretion assay system. (A) Up to 36 tubules from a single locust were run simultaneously. (B) Close up showing tubules treated with *Locusta*-DP (left) and tubules treated with F2 (right). *Locusta*-DP is clearly more efficacious. MT, Malpighian tubule; BF, bathing fluid; SF, secreted fluid (which is dark owing to selective secretion of the phenol red indicator originally present in the bathing fluid). Scale: A, 10mm; B, 1mm.

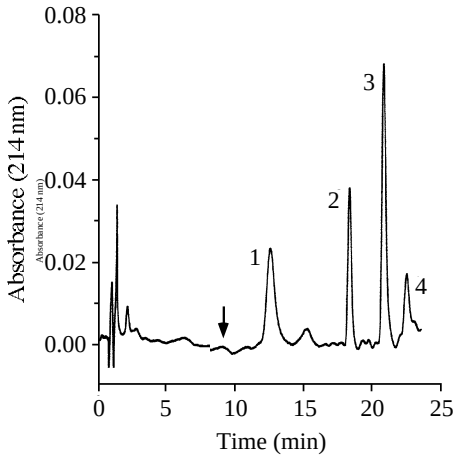

Fig. 3. RP-HPLC separation of oxidation products of the peptide shown in Fig. 1A. The peaks labelled 1, 2 and 3 are identified products subsequently confirmed to be F1, F2 and PDm respectively. Peak 4 was found, by mass spectrometry, to be a trimeric oxidation species. Other peaks in the chromatogram represent exogenous material in the oxidation reaction mixture. The position at which the reduced peptide would elute is indicated by an arrow. The absence of this peak indicates complete conversion of the reduced peptide to oxidized species.

reduced peptide, they were presumed to be the parallel (PDm) and antiparallel (F2) dimers. Mass spectrometry gave relative molecular masses of 1945.2 and 1945.5, respectively, for these products, confirming their dimeric structure. Under conditions of high cone voltage, the difference in the two fragmentation patterns obtained indicates that each dimer has a different structure (Fig. 4). These data alone do not allow us to resolve which of the dimers is parallel and which is antiparallel. To confirm the configuration of the dimers, two approaches were used: further analysis of fragmentation patterns obtained from the peaks, and thermolysin digestion (Proux *et al.* 1987). Species present in the fragmentation pattern of peak 3 exhibited relative molecular masses of 701.9 and 758.5, respectively, which could only have been produced by fragmentation of the parallel dimer (Fig. 4, peak 3). Thermolysin treatment of peak 3 generated two products on HPLC (see Fig. 1C for expected sites of thermolysin cleavage), one of which exhibited a relative molecular mass of 1515.9. This fragment could only have been produced by digestion of PDm. These data show that peak 3 must be PDm.

By default, peak 2 must be the antiparallel dimer, F2. There are no fragments in the high-voltage spectrum which unequivocally confirm this (Fig. 4). However, the configuration of the antiparallel dimer is resolved by thermolysin digestion. Thermolysin treatment of peak 2 generated a single product on HPLC, as expected (Fig. 1D). The measured mass of this product (991.2) indicated that it had been produced by cleavage of the Leu–Ile bond in the antiparallel dimer, confirming that peak 2 is F2.

Synthetic *Locusta*-DP produced a single peak on RP-HPLC (not shown). Sequence and mass analysis of this peak confirmed it to be *Locusta*-DP (M. Patel and T. K. Hayes, in preparation).

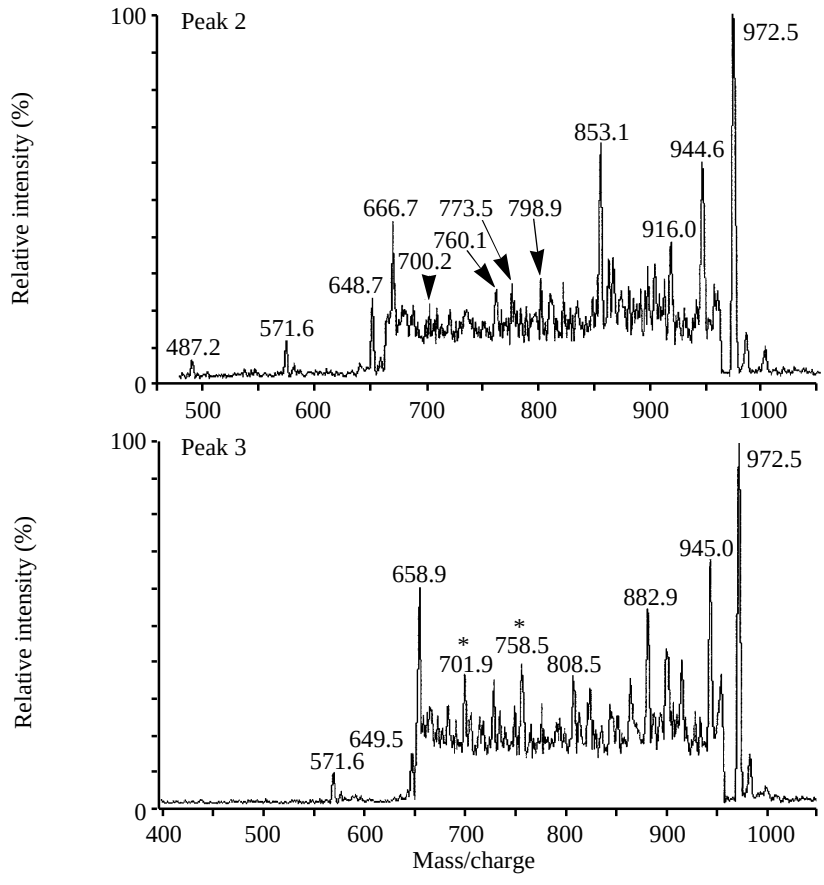

Fig. 4. Electrospray mass spectra of the oxidation products peak 2 and peak 3 from Fig. 3, at elevated cone voltage, confirms that these are both dimers of F1, but are structurally distinct. Only the fragment ion at mass/charge 945 is common to both species and represents a C-terminal fragmentation. The two ions at 701.9 and 758.5 (indicated with asterisks on the spectrum for peak 3) could only have arisen by fragmentation of the parallel dimer, PDm.

Bioassays

Fluid secretion

Previously, the diuretic properties of F2 and *Locusta*-DP had been determined independently, on different assay preparations, by two different groups. Proux *et al.* (1988), using a combined Malpighian tubules and midgut preparation, reported that synthetic F2 optimally stimulated fluid secretion at approximately 10^{-9} mol l^{-1}. No dose–response curve has yet been published for *Locusta*-DP. However, using an isolated tubule bioassay system, Kay *et al.* (1991*b*) reported that the native peptide was active at an estimated concentration of 3.2×10^{-8} mol l^{-1}. We have taken the opportunity to test these two peptides at the same concentration in the same bioassay system: the isolated Malpighian tubule preparation. In this assay system, the basal rate of tubule secretion in control preparations declines by approximately 30% during the second 20min period of

the assay (Fig. 5A). F2 at 10^{-9} mol l^{-1} had no effect on this decline; it neither prevented it, nor exacerbated it. In stark contrast, *Locusta*-DP at the same concentration (10^{-9} mol l^{-1}) elevated the secretion rate fivefold.

These experiments show that *Locusta*-DP is a potent stimulant of fluid secretion and that the effect of F2 is indistinguishable from Ringer substitution. In the face of such a clear negative result, we felt it prudent to test F2 at other concentrations, from an order of magnitude lower to three orders of magnitude higher (10^{-10} mol l^{-1} to 10^{-6} mol l^{-1}). No effect was found at any concentration (Fig. 5B). We also took the opportunity to test F1 and PDm at the same concentrations as F2, and similarly found no stimulatory effect for either of these two peptides. Further, we tested *Locusta*-DP at 5×10^{-8} mol l^{-1} because this is the estimated concentration which produces a response equivalent to that obtained with a supramaximal dose of corpora cardiaca extract (Kay *et al.* 1991*b*). In this instance, a dramatic increase in fluid secretion rate, to nearly 2000pl min^{-1}, was obtained.

The great difference between the efficacy of *Locusta*-DP, as a stimulant of Malpighian tubule primary urine production, and the lack of response to F2, F1 or PDm application, is most clearly illustrated by expressing the results as the change in rate of secretion (test rate minus initial control rate; Fig. 5C).

Cyclic AMP production by Malpighian tubules

Proux and Hérault (1988) found that F2 increases production of cyclic AMP by *Locusta* Malpighian tubules and concluded that the peptide stimulated adenylyl cyclase activity. We therefore compared the abilities of F2 and *Locusta*-DP to stimulate cyclic AMP production in isolated tubules of *Locusta migratoria*. The results parallel those of the fluid secretion assay and demonstrate no stimulatory effect of F2 at doses of 10^{-11} mol l^{-1} to 10^{-7} mol l^{-1} on adenylyl cyclase activity. In contrast, *Locusta*-DP significantly increased cyclic AMP production (to a maximum of approximately 14pmol per tubule) at 5×10^{-8} mol l^{-1}, and even at 10^{-9} mol l^{-1} still gave a significant increase (Student's *t*-test: $P<<0.01$ and $P<0.05$, respectively; Table 1). Neither F1 nor PDm at 10^{-8} mol l^{-1} or 10^{-7} mol l^{-1} had any effect on cyclic AMP production.

Discussion

The results from our bioassays are in marked contrast to the findings of Proux *et al.* (1987, 1988) and Proux and Hérault (1988). These workers used a preparation which included the entire Malpighian tubule mass together with a short section of midgut to assess the effects of AVP-like peptides (F1, F2 and PDm) on fluid secretion. In this preparation, fluid secretion in controls typically declined by approximately 30% over the first hour of the experiment. Addition of F2 after 1h restored secretion rate to the initial level (Proux *et al.* 1987, 1988), but did not significantly increase it above this level. Like the combined Malpighian tubule/midgut preparation, our isolated Malpighian tubule preparation also exhibits a decline of approximately 30% in fluid secretion rate (comparing the initial 20min control period with the subsequent 20min test period). However, in our hands, F2 does not restore fluid secretion to basal levels. Proux and

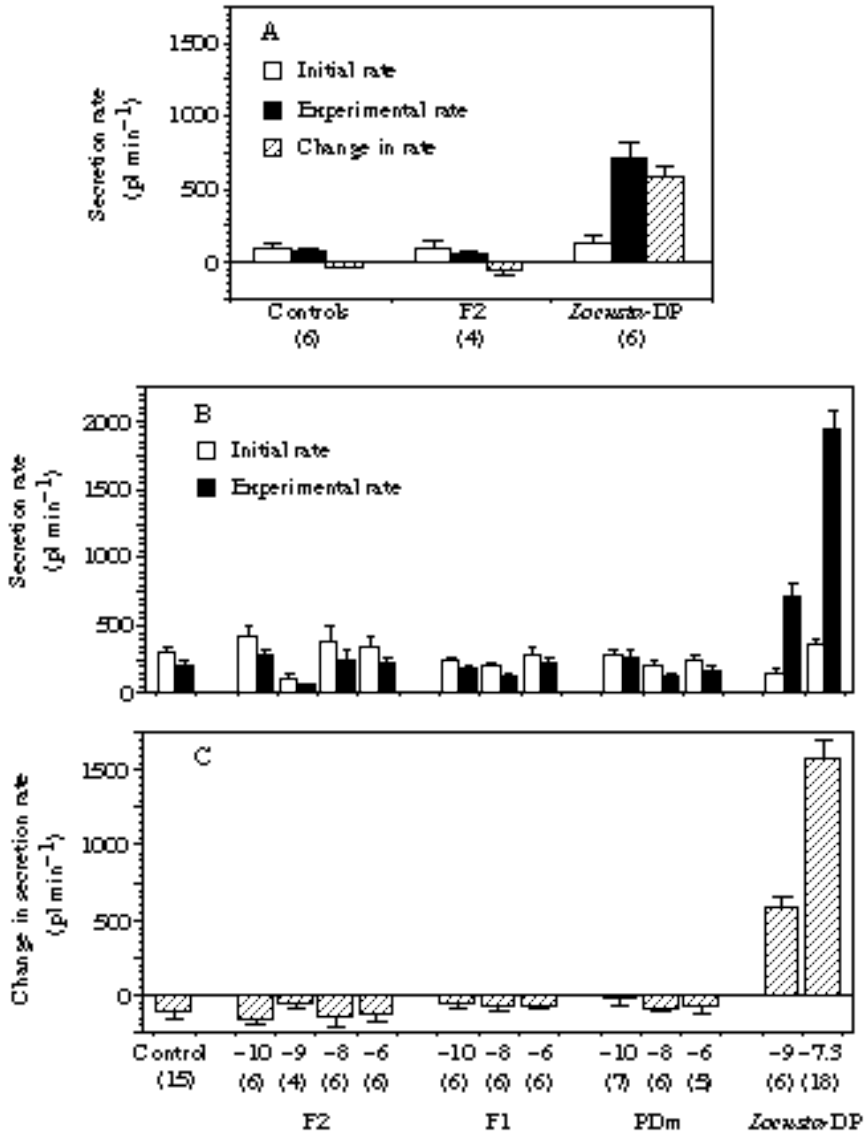

Fig. 5. Peptides F2, F1 and PDm have no significant effect on tubule secretion but *Locusta*-DP is a potent stimulant of fluid secretion. (A) Comparison of the effect of F2 and *Locusta*-DP at 10^{-9} mol l^{-1}. The absolute rate of tubule secretion for the initial control period (open bars) and subsequent test period (filled bars) for controls (Ringer substitution) and the two test compounds. The change in rate of secretion (test minus initial) is indicated by the striped bars. Each bar shows the mean rate of secretion by 4–6 tubules. Numbers of individual tubules tested are given in parentheses and the S.E. is indicated. Only *Locusta*-DP causes a significant increase in secretion (Student's *t*-test, $P<<0.01$). (B) The absolute rates of tubule secretion for tubules treated with F2, F1, PDm and *Locusta*-DP over a range of concentrations. Open and filled bars as in A. Test compound and log[concentration] in mol l^{-1} are indicated below C. Only *Locusta*-DP at 10^{-9} mol l^{-1} or 5×10^{-8} ($10^{-7.3}$) mol l^{-1} causes a significant increase in tubule fluid secretion (Student's *t*-test, $P<<0.01$ for both). (C) The data from B expressed as the change in rate of tubule secretion between control and test periods.

Table 1. *Cyclic AMP production by isolated Malpighian tubules*

Sample	pmol cyclic AMP per tubule	S.E.	(*N*)
Control	0.0	0.04	4
F2 10^{-11} mol l^{-1}	0.0	0.01	4
F2 10^{-10} mol l^{-1}	0.0	0.01	4
F2 10^{-9} mol l^{-1}	−0.2*	0.02	4
F2 10^{-8} mol l^{-1}	0.0	0.01	5
F2 10^{-7} mol l^{-1}	0.0	0.01	5
F1 10^{-8} mol l^{-1}	0.0	0.01	5
F1 10^{-7} mol l^{-1}	0.0	0.01	5
PDm 10^{-8} mol l^{-1}	0.0	0.01	5
PDm 10^{-7} mol l^{-1}	0.0	0.01	5
Locusta-DP 10^{-9} mol l^{-1}	4.9	1.81	4
Locusta-DP 5×10^{-8} mol l^{-1}	13.9	2.01	4

Mean values have been normalised by subtracting the amount of cyclic AMP produced by control tubules.

Peptides F2, F1 and PDm caused no signficant increase in cyclic AMP production. In contrast, *Locusta*-DP at 10^{-9} or 5×10^{-8} mol l^{-1} stimulated production of between 5 and 15pmol of cyclic AMP per tubule.

*In this instance, cyclic AMP concentration was slightly lower than in normalised controls.

Hérault (1988) also found that F2 stimulated cyclic AMP production using their combined Malpighian tubule/midgut preparation. Again, in contrast to this observation, we find no effect of F2 on cyclic AMP production by isolated Malpighian tubules.

It is difficult to reconcile these two very different sets of data. We are confident that we have tested a pure sample of synthetic F2, and our sensitive assays respond unequivocally when stimulated with another peptide, *Locusta*-DP. The difference is therefore striking. One explanation for the disparity may be Proux's (1991) finding that tubules taken from locusts infected with the protozoan *Malameba locustae* showed a dramatic decrease in their response to F2. However, we have examined squash preparations of tubules taken from animals in our colony and find absolutely no evidence of *Malameba* infection under the light microscope. Further, our comparisons of the actions of F2 and *Locusta*-DP were performed in parallel on tubules taken from the same insect, demonstrating that the ability of the tubules to respond to stimulation was not impaired (see Fig. 2). A second explanation is that the tubule and gut preparation used by Proux *et al.* (1988) is open to influence by transport across the gut wall. Thus, if F2 decreased reabsorption of fluid from the short segment of gut present in their preparation by a cyclic-AMP-dependent mechanism (but left Malpighian tubules unaffected), increased rates of fluid secretion and elevated levels of cyclic AMP would result. Our use of single isolated Malpighian tubules, rather than the combined tubule and midgut preparation, has eliminated the possible influence of the gut, and may therefore account for the different results.

The use of the isolated tubule preparation in the assessment of diuretic factors is preferable for a number of reasons. The first is that the target is well defined. The second

is that only small quantities of precious test substances are required for the assay. The third, and most important reason, is that those factors found to stimulate fluid secretion in this *in vitro* assay have also been shown to stimulate water elimination *in vivo* (e.g. *Manduca*-DH I in post-eclosion *Pieris rapae*; Kataoka *et al.* 1989; Coast *et al.* 1992) or increase amaranth clearance (e.g. *Locusta*-DP in *Locusta migratoria*; Kay *et al.* 1991*b*; M. Patel, unpublished observations).

The results of our study have failed to confirm a diuretic action for F2 on isolated Malpighian tubules: it neither stimulates fluid secretion nor increases cyclic AMP production. Even if F2 does have a diuretic role, by acting on midgut rather than Malpighian tubules, its effect of only restoring a declining secretion rate to a basal level must be inconsequential in comparison with the fivefold elevation of basal secretion rate obtained by stimulating tubules with *Locusta*-DP. F2 cannot, therefore, be described as a 'potent' diuretic, as we have described *Locusta*-DP. Interestingly, to our knowledge, synthetic F2 has not been shown to promote fluid secretion or amaranth clearance *in vivo*. A correlation has been made between the haemolymph titre of endogenous AVP-like peptides and fluid secretion (amaranth clearance) rate, but these peptides were not unambiguously identified as F1 and F2. Indeed, even this correlation was not strict: starved animals showed a peak in the titre of AVP-like peptides without a concomitant burst of diuresis (Picquot and Proux, 1987). Even the supposition that F2 acts *via* adenylyl cyclase activation (Proux and Hérault, 1988) is questionable: Wenning *et al.* (1991) have shown that F2, albeit at a concentration of 10^{-7} mol l^{-1} (100-fold higher than the effective dose in the locust), stimulates fluid secretion by isolated Malpighian tubules of the centipede *Lithobius forficatus*, but were unable to mimic this effect by application of dibutyryl cyclic AMP. On the basis of these findings, calling F2 the arginine-vasopressin-like insect diuretic hormone (AVP-like IDH; Proux *et al.* 1987) is a misnomer.

What then is the role of F2? An alternative function within the CNS has already been suggested for F1 and F2, based on the anatomy (Thompson *et al.* 1991) and physiology (Thompson and Bacon, 1991) of vasopressin-like immunoreactive (VPLI) neurones, the supposed source of these peptides. These neurones in *Locusta migratoria* have branches which divide repeatedly to form bundles of fibres that extend into proximal regions of most peripheral nerves of the ventral CNS. These fibre bundles are densely beaded and were proposed to be the neurohaemal release site of F1 and F2 (Girardie and Rémy, 1980). However, the morphology of the fibre bundles in peripheral nerves is not consistent with that expected of a neurohaemal organ. Many of the beaded fibres remain deep within the nerve root (Thompson *et al.* 1991), whereas known neurohaemal endings (Maddrell, 1966; Maddrell and Gee, 1974) lie outside the perineurium just under the neural sheath, facilitating release into the haemolymph. Further, the morphology of VPLI neurones in another species of locust, *Schistocerca gregaria*, differs from that in *Locusta migratoria* in that there are no fibre bundles in peripheral nerves (Evans and Cournil, 1990). In a more recent, extensive survey of VPLI cells in 17 species of grasshopper from the superfamily Acridoidea, the presence of peripheral fibre bundles was found to be determined by phylogeny and not habitat, and was restricted to species from the subfamily Oedipodinae (Tyrer *et al.* 1993). This further undermines the suggestion that the peripheral fibre bundles are neurohaemal release sites, but suggests

instead that release occurs centrally, particularly in those species lacking peripheral fibres.

Electrophysiological recording of VPLI cell activity in *Locusta migratoria* has failed to reveal any osmoreceptor input that would be expected from their proposed role in diuresis (Thompson and Bacon, 1991). However, these recordings did reveal that the neurones are more active in the dark than in daylight, this difference in activity being caused by synaptic input from a pair of brain interneurones that are part of an extra-ocular photoreceptor (EOP) system (Thompson and Bacon, 1991). In both invertebrates and lower vertebrates, EOPs are typically implicated in adjusting the clock that regulates circadian rhythms (Saunders, 1982; Cassone, 1990), giving rise to the suggestion that VPLI cells have a role in circadian behaviour. This seems to be a more likely role for these neurones, and their AVP-like peptides, than the control of water balance.

To clarify the situation, a number of key experiments must now be performed. It is imperative that F1 and F2 be shown to exist in VPLI neurones. It is also essential, if a hormonal role is to be supported, that release of F2 from the intact CNS can be demonstrated. Similarly, the release of *Locusta*-DP from the corpora cardiaca into the haemolymph must be demonstrated for it to be assigned any role in water balance *in vivo.* This work is currently in progress. If either of these peptides fails to fulfil this fundamental criterion for a hormone, then attention must switch to the confines of the CNS to determine their function.

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References

BLACKBURN, M. B., KINGAN, T. G., BODNAR, W., SHABANOWITZ, J., HUNT, D. F., KEMPE, T., WAGNER, R. M., RAINA, A. K., SCHNEE, M. E. AND MA, M. C. (1991). Isolation and identification of a new diuretic peptide from the tobacco hornworm, *Manduca sexta*. *Biochem. biophys. Res. Commun.* **181**, 927–932.

CASSONE, V. M. (1990). Effects of melatonin on vertebrate circadian systems. *Trends Neurosci.* **13**, 457–464.

COAST, G. M. (1988). Fluid secretion by single isolated Malpighian tubules of the house cricket, *Acheta domesticus* and their response to diuretic hormone. *Physiol. Ent.* **13**, 381–391.

COAST, G. M., CUSINATO, O., KAY, I. AND GOLDSWORTHY, G. J. (1991). An evaluation of the role of cyclic AMP as an intracellular second messenger in Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect Physiol.* **37**, 563–573.

COAST, G. M., HAYES, T. K., KAY, I. AND CHUNG, J. S.(1992). Effect of *Manduca sexta* diuretic hormone and related peptides on isolated Malpighian tubules of the house cricket, *Acheta domesticus. J. exp. Biol.* **162**, 331–338.

EVANS, P. D. AND COURNIL, I.(1990). Co-localisation of FLRF- and vasopressin-like immunoreactivity in a single pair of sexually dimorphic neurones in the nervous system of the locust. *J. comp. Neurol.* **292**, 331–348.

GIRARDIE, J. AND RÉMY, C. (1980). Particularités histo-cytologiques des prolongements distaux des 2 cellules à 'vasopressine-neurophysine-like' du criquet migrateur. *J. Physiol., Paris* **76**, 265–271.

JAENICKE, R. AND RUDOLF, R. (1989). Folding proteins. In *Protein Structure, a Practical Approach* (ed. T. E. Creighton), pp. 191–223. Oxford: IRL Press.

KATAOKA, H., TREOTSCHLER, R. G., JORGE, P. L., KRAMER, S. J., CARNEY, R. L. AND SCHOOLEY, D. A. (1989). Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta. Proc. natn. Acad. Sci. U.S.A.* **86**, 2976–2980.

KAY, I., COAST, G. M., CUSINATO, O., WHEELER, C. H., TOTTY, N. F. AND GOLDSWORTHY, G. J. (1991*a*). Isolation and characterisation of a diuretic peptide from *Acheta domesticus:* evidence for a family of insect diuretic peptides. *Biol. Chem. Hoppe-Seyler* **372**, 505–512.

KAY, I., PATEL, M., COAST, G. M., TOTTY, N. F., MALLET, A. I. AND GOLDSWORTHY, G. J. (1992). Isolation, characterisation and biological activity of a CRF-related diuretic peptide from *Periplaneta americana* L. *Regulatory Peptides* **42**, 111–122.

KAY, I., WHEELER, C. H., COAST, G. M., TOTTY, N. F., CUSINATO, O., PATEL, M. AND GOLDSWORTHY, G. J.(1991*b*). Characterisation of a diuretic peptide from *Locusta migratoria. Biol. Chem. Hoppe-Seyler* **372**, 929–934.

LEHMBERG, E., OTA, R. B., FURUYA, K., KING, D. S., APPLEBAUM, S. W., FERENZ, H.-J. AND SCHOOLEY, D. A. (1991). Identification of a diuretic hormone of *Locusta migratoria*. *Biochem. biophys. Res. Commun.* **179**, 1036–1041.

MADDRELL, S. H. P.(1966). The site of release of the diuretic hormone in *Rhodnius.* A new neurohaemal system in insects. *J. exp. Biol.* **45**, 499–508.

MADDRELL, S. H. P. AND GEE, J. D. (1974). Potassium induced release of the diuretic hormones of *Rhodnius prolixus* and *Glossina austeni*: Ca dependence, time course and localization of neurohaemal areas. *J. exp. Biol.* **61**, 155–171.

MORGAN, P. J. AND MORDUE, W.(1984). Diuretic hormone: another peptide with widespread distribution within the insect CNS. *Physiol. Ent.* **9**, 197–206.

MORGAN, P. J., SIEGERT, K. J. AND MORDUE, W. (1987). Preliminary characterisation of locust diuretic peptide (DP-1) and another corpus cardiacum peptide (LCCP). *Insect Biochem.* **17**, 383–388.

PICQUOT, M. AND PROUX, J. (1987). Relationship between excretion of primary urine and haemolymph level of diuretic hormone in the migratory locust. *Physiol. Ent.* **12**, 455–460.

PROUX, J. AND ROUGON-RAPUZZI, G. (1980). Evidence for vasopressin-like molecule in migratory locust. Radioimmunological measurements in different tissues: correlation with various states of hydration. *Gen. comp. Endocr.* **42**, 378–383.

PROUX, J. P. (1991). Lack of responsiveness of Malpighian tubules to the AVP-like insect diuretic hormone on migratory locusts infected with the protozoan *Malameba locustae*. *J. Invert. Path.* **58**, 353–361.

PROUX, J. P. AND BEYDON, P. (1992). Rapid chromatographic separation of the AVP-like locust peptides from synthetic and biological mixtures. Quest for these peptides in some insect, batrachian and mammalian nervous systems. *Insect Biochem. molec. Biol.* **22**, 185–192.

PROUX, J. P. AND HÉRAULT, J.-P. (1988). Cyclic AMP: a second messenger of the newly characterized AVP-like insect diuretic hormone, the migratory locust diuretic hormone. *Neuropeptides* **12**, 7–12.

PROUX, J. P., MILLER, C. A., LI, J. P., CARNEY, R. L., GIRARDIE, A., DELAAGE, M. AND SCHOOLEY, D. A. (1987). Identification of an arginine vasopressin-like diuretic hormone from *Locusta migratoria*. *Biochem. biophys. Res. Commun.* **149**, 180–186.

PROUX, J. P., PICQUOT, M., HÉRAULT, J.-P. AND FOURNIER, B. (1988). Diuretic activity of a newly identified neuropeptide – the arginine-vasopressin-like insect diuretic hormone: use of an improved bioassay. *J. Insect Physiol.* **34**, 919–927.

SAUNDERS, D. S. (1982). Photoperiodism in animals and plants. In *Biological Timekeeping* (ed. J. Brady), pp.65–82. Cambridge: Cambridge University Press.

SPRING, J. H.(1990). Endocrine regulation of diuresis in insects. *J. Insect Physiol.* **36**, 13–22.

THOMPSON, K. S. J. AND BACON, J. P. (1991). The vasopressin-like immunoreactive (VPLI) neurons of the locust, *Locusta migratoria.* II. Physiology. *J. comp. Physiol.* **168**, 619–630.

THOMPSON, K. S. J., TYRER, N. M., MAY, S. T. AND BACON, J. P. (1991). The vasopressin-like immunoreactive (VPLI) neurons of the locust, *Locusta migratoria.* I. Anatomy. *J. comp. Physiol.* **168**, 605–617.

TYRER, N. M., DAVIS, N. T., ARBAS, E. A., THOMPSON, K. S. J. AND BACON, J. P. (1993). The morphology of the vasopressin like immunoreactive (VPLI) neurons in many species of grasshopper. *J. comp. Neurol.* (in press).

WENNING, A., GREISINGER, U. AND PROUX, J. P. (1991). Insect-like characteristics of the Malpighian tubules of a non-insect: fluid secretion in the centipede *Lithobius forficatus* (myriapod: chilopoda). *J. exp. Biol.* **158**, 165–180.