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DIURETIC FACTORS CONTROLLING BEETLE MALPIGHIAN TUBULES: FLUID SECRETION AND IMMUNOHISTOCHEMISTRY

by

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DECLARATION

I, Wendy Dianne Holtzhausen, hereby declare that this thesis, submitted for the degree Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this, or any other tertiary institution.

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SUMMARY

Water balance in insects is under neuroendocrine control, and both diuretic and antidiuretic factors are thought to be involved. Despite being the largest and most diverse order of insects, the Coleoptera have been largely neglected in studies of hormonal control of excretion. However, the only insect from which both diuretic and antidiuretic peptides, acting on Malpighian tubules, have been isolated, is a beetle: the mealworm *Tenebrio molitor*. This study reports the effects of different diuretic factors on fluid secretion rates by mealworm tubules and briefly explores cross-reactivity of factors between beetle species, using the scarabaeid *Onthophagus gazella*. Attempts to localise an antidiuretic factor and a diuretic factor in the mealworm nervous system are also described.

The study showed that calcitonin(CT)-like peptides from silkmoth and mosquito increased fluid secretion in mealworm tubules in a dose-dependent manner, but the cockroach CT-like peptide, Dippu-DH_{31}, had no effect. The pharmacological agent thapsigargin, which mimics the action of kinins, caused a small but significant increase in tubule secretion rates. Since mealworm tubules are affected by more than one diuretic factor, the possibility of synergism between different factors was explored by testing CT-like peptides, thapsigargin and the mealworm corticotropin-releasing factor (CRF)-related diuretic peptide Tenmo-DH_{37}, in various combinations and at different concentrations. No synergistic or additive effects were observed. C-terminal fragments of the locust and cockroach CRF-related diuretic peptides, Locmi-DH_{46} and Dippu-DH_{46} respectively, had no significant effect on fluid secretion. This is consistent with previous studies which have shown that the receptor binding and activation sites have to be linked in the same molecule for biological activity. However, the corresponding whole peptides increased fluid secretion, revealing the high degree of cross-reactivity of CRF-related peptides across insect orders. The mealworm diuretic factor Tenmo-DH_{37} increased fluid secretion in isolated tubules of *O. gazella* in a dose-dependent manner,

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demonstrating cross-reactivity with this distantly related beetle species. However, brain homogenates from *O. gazella* significantly inhibited fluid secretion in mealworm tubules. This is unusual, because extracts of insect nervous tissue have generally been shown to be a source of diuretic activity.

Immunohistochemical techniques were used in an attempt to localise the mealworm antidiuretic factor Tenmo-ADFa in the nervous system of *T. molitor*. The antiserum developed against Tenmo-ADFa was found to be inadequately sensitive, and so these efforts were unsuccessful. The same techniques were employed in an attempt to locate the tissue containing a putative beetle CT-like peptide in the mealworm, using antiserum raised against the cockroach CT-like peptide Dippu-DH_{31}. This too did not lead to a result. Experiments were repeated with various modifications, without success. It remains unclear whether the lack of results is due to degradation of the antiserum or if CT-like peptides are not present in detectable amounts in the mealworm nervous system.

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CHAPTER 1

INTRODUCTION

Due to their small size and hence large surface area to volume ratio, insects face many challenges to water balance and therefore exhibit various morphological, behavioural and physiological adaptations to conserve water. Nevertheless, substantial proportions of their water intake can be lost through excretion. The excretory organs of insects, which are responsible for maintaining osmotic and electrolyte balance, comprise the Malpighian tubules and the hindgut (ileum and rectum). The Malpighian tubules, which number from two to 200 depending on the species, perform functions analogous to vertebrate renal tubules and are capable of secreting fluid faster than any other epithelium on a per cell basis (Dow and Davies, 2006). They arise from the junction between the midgut and hindgut and lie free in the haemolymph, except when a rectal complex is present. They secrete primary urine, which is isosmotic to the haemolymph, usually containing relatively more K^+ ions, but in the case of blood-sucking insects, Na^+ ions. From the tubules, primary urine passes into the hindgut, where water, ions, and essential metabolites are reabsorbed according to the requirements of the insect. The selective reabsorption that takes place in the hindgut results in a final urine that is distinctly different in volume and composition to the Malpighian tubule fluid (see Chown and Nicolson, 2004).

Although both Malpighian tubules and hindgut are involved in this process, the tubules remain the main focus of insect excretory physiology studies. This is probably because of their simple structure and the ease with which they can be manipulated for *in vitro* assays. The method used to investigate Malpighian tubule function was first devised by Ramsay (1954). In this assay, isolated tubules are set up in physiological saline and their ends are drawn out into liquid paraffin. Arranged in this way, the tubules continue to secrete for long periods, and the secreted fluid accumulates as discrete droplets in the paraffin. Rates of secretion

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before and after the addition of a test substance can then be calculated to assess diuretic activity. The Ramsay assay has been used for over 50 years with only minor modifications, and has enabled an enormous amount of data to be generated on the hormonal control of fluid secretion in insects (see review by Coast et al., 2002).

Using this method, Ramsay also measured the excretion of sodium and potassium using tubules of the stick insect (Ramsay, 1955). The difference in ion concentration between the secreted fluid and the bathing saline provides information on transport mechanisms (Beyenbach, 2005). Fluid secretion in Malpighian tubules is driven by the active transport of K^+ and/or Na^+ ions into the lumen accompanied by Cl^-, and water follows by osmosis. The voltages generated by the secretion of Na^+, K^+ and Cl^- into the tubule lumen can be measured in isolated perfused tubules, with the sensing voltage electrode in the tubule lumen (Beyenbach, 2005). Such electrical techniques are very useful in augmenting understanding of ion transport mechanisms in Malpighian tubules and diuretic activity has thus been investigated further by measuring electrophysiological parameters (see Nicolson, 1993; Beyenbach, 1995).

The excretory system in insects is under neuroendocrine control, and water balance is regulated by the interaction of an array of diuretic and antidiuretic factors acting on tubule secretion and/or hindgut reabsorption. These factors are usually synthesised in the neurosecretory cells of the brain and released from the corpora cardiaca, which also serve as storage organs for neuroendocrine factors. Both diuretic and antidiuretic factors are thought to be involved (Coast et al., 2002). Traditionally, diuretic factors are considered to act on Malpighian tubules, while antidiuretic factors have been regarded as those acting on the hindgut to increase fluid reabsorption, thus reducing the amount of water lost from the insect. However, antidiuretic factors may also act on tubules to inhibit secretion (see below).

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It is important to note that the ability of factors to stimulate Malpighian tubule secretion *in vitro* does not confirm their function as hormones *in vivo*. In order to qualify as a hormone, stringent criteria have to be met. The substance must be shown to be present in neurosecretory cells, from where it is released into circulation in response to a stimulus. The increase in circulating hormone, at appropriate concentrations, then triggers a response in the target tissue. This response should be mimicked by injection of synthetic peptide and prevented by the blockading of receptors. To date, these requirements have been met only for the locust corticotropin-releasing factor (CRF)-related diuretic peptide Locmi-DH_{46} (Patel et al., 1995). Whether peptides from all the known diuretic families also function as hormones *in vivo* has yet to be established. Nevertheless, some have felt justified in referring to these substances as hormones rather than peptides or factors (Furuya et al., 2000).

Intriguingly, tubules of the Namib Desert tenebrionid beetle *Onymacris plana* were found to respond dramatically to a diuretic factor present in its nervous tissue (Nicolson and Hanrahan, 1986). The need for a potent diuretic in a species that inhabits such a dry environment was further investigated, and *in vivo* experiments showed that most of the fluid secreted by the tubules flows forward into the midgut and is recycled to the haemolymph (Nicolson, 1991). Consequently, the diuretic hormone does not stimulate water loss from the whole insect, but its release would instead result in the rapid clearance of metabolic wastes from the haemolymph. Hence the term “clearance hormone” may be more descriptive (Nicolson, 1991).

Maddrell (1966) demonstrated the presence of diuretic factors in the blood-sucking bug *Rhodnius prolixus* when he tested crude extracts of its nervous tissue on Malpighian tubules. For many years, only tissue homogenates were available to investigate the presence and effects of diuretic factors, but with the development of sophisticated techniques in biochemistry, the isolation and characterisation of these factors have been made possible. The first definitive diuretic factor was isolated and characterised from head extracts of the tobacco hornworm *Manduca sexta* on

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the basis of its ability to induce a post-eclosion diuresis in the butterfly *Pieris rapae* (Kataoka et al., 1989). The *Manduca* diuretic factor was later shown to stimulate fluid secretion in *M. sexta* Malpighian tubules (Audsley et al., 1993). The peptide, now called Manse-DH_{41}, was found to share 29-35% sequence homology with the CRF-related family of vertebrate peptides. Subsequently, many such peptides have been identified, and they now appear to be ubiquitous in insects (Coast et al., 2002).

The three main families of diuretic peptides that have been identified are the CRF-related peptides, the insect kinins and calcitonin (CT)-like peptides (Coast et al., 2002). The biogenic amine serotonin has also been shown to be an effective stimulant of fluid secretion, and cardioacceleratory peptide (CAP_{2b}) has diuretic effects in the tubules of some insects, like *Drosophila* (Davies et al., 1995), but acts as an antidiuretic in others, like *Rhodnius* (Quinlan et al., 1997) and *Tenebrio* (Wiehart et al., 2002a). It is increasingly evident that any single species of insect possesses diuretic factors from two or more peptide families, and the occurrence of more than one diuretic factor from the same peptide family is not uncommon. There may be many reasons for this multiplicity, including synergistic effects between different factors, the control of different physiological processes, and/or multiple actions in different target organs (Coast and Garside, 2005). It is unlikely that a number of diuretic factors present in the same insect would perform similar roles in fluid secretion, and different factors that stimulate secretion in a similar manner *in vitro* may have very different effects on, for instance, ion transport.

The availability of synthetic peptides has also enabled antisera to be raised against them. Antigens (peptides) within a certain tissue can be detected and localised by making use of specific antibody-antigen reactions. The “indirect method” is most often employed and involves an unlabelled primary antibody that reacts with the antigen in the tissues. In turn, a secondary antibody, which is directed against the immunoglobulins of the animal species in which the primary antibody was raised, reacts with the primary antibody. The secondary antibody is labelled with a

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fluorescent dye such as fluorescein isothiocyanate (FITC) which is viewed with a fluorescent microscope. The secondary antibody reacts with numerous different antigenic sites on the primary antibody, and in this way the signal is amplified, making the immunofluorescence method a very sensitive one (see Fig. 1). For more detail see the review by Nässel (1996).

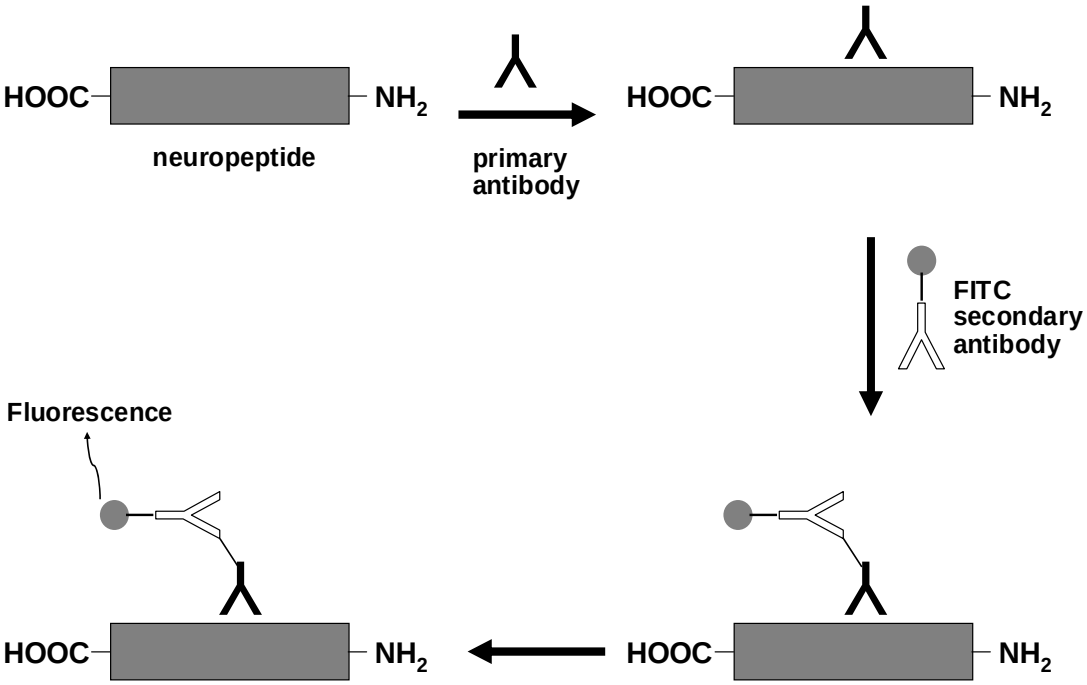

Fig. 1. General principle of immunohistochemistry (whole mount method). The peptide in question is recognised by the primary antibody (raised in rabbit). In turn, the primary antibodies react with the secondary antibody (swine anti-rabbit), to which the fluorescent agent, FITC, is coupled. Fluorescence is visible under blue light (494 nm), thus revealing the location of the peptide in the tissues.

A number of diuretic factors from different peptide families have been mapped in the neuroendocrine systems of insects from the orders Orthoptera, Blattodea, Hemiptera, Lepidoptera and Diptera (Coast et al., 2002). They are expressed throughout the insect central nervous system and in parts of the gut. In some cases, they are co-expressed in the same cells (e.g. Thompson et al., 1995). Immunohisto-chemistry played an important role in demonstrating that Locmi-DH_{46} functions as

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a hormone in *Locusta migratoria* (Patel et al., 1995). In this way, it can provide supporting evidence for the physiological role of novel peptides, and has therefore become an important tool for insect physiologists. Diuretic factors have received more attention than antidiuretic factors and to date, the only insect antidiuretic peptide to be localised is Tenmo-ADFb from the mealworm (Eigenheer at al., 2003).

Despite technological advances such as these, excretory physiology has been investigated in only a very limited number of insects, and beetles, which comprise the largest order of living animals, have been rather neglected in this regard. Yet it is a beetle, the mealworm, *Tenebrio molitor* (Tenebrionidae) that is the only insect for which both diuretic and antidiuretic synthetic endogenous peptides, acting directly on tubules, are available (Eigenheer et al., 2002, 2003; Furuya et al., 1995, 1998).

The two diuretic peptides isolated from *T. molitor*, Tenmo-DH_{37} and Tenmo-$DH_{47,}$ are both CRF-related peptides and, like all members of this peptide family, use cyclic AMP as second messenger (Furuya et al., 1995; 1998). They are of particular interest because they are the only members of the CRF-related family of peptides to have their C-termini in the non-amidated form. Both increase fluid secretion in *Tenebrio* Malpighian tubules, but Tenmo-DH_{37} is the more potent of the two and has been immunohistochemically localised in mealworm brain and abdominal ganglia (Wiehart et al. 2002b). The two antidiuretic factors, Tenmo-ADFa and Tenmo-ADFb, do not belong to any known family of insect peptides and are not structurally related to each other, but both elicit their response via cyclic GMP and act on mealworm tubules to decrease fluid secretion (Eigenheer et al., 2002; 2003). Tenmo-ADFa also inhibits fluid secretion and increases intracellular cyclic GMP in tubules of the mosquito *Aedes aegypti* (Massaro et al., 2004). Tenmo-ADFb was the first insect antidiuretic peptide to be localised using immunohistochemistry. Its presence in the brain of *Tenebrio* gave credence to its role as a neuropeptide in this species (Eigenheer et al., 2003). In the first study to investigate the interactions in

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tubules between endogenous diuretic and antidiuretic factors from the same insect, Wiehart et al. (2002a) demonstrated antagonistic effects between Tenmo-DH_{37} and Tenmo-ADFb and between their second messengers. The sequences of these four mealworm peptides are shown in Table 1.

Table 1. Sequences of *Tenebrio molitor* diuretic and antidiuretic factors

Peptide	Sequence	Monoisotopic mass
Tenmo-DH_{37}	SPTISITAPIDVLRKTWEQERARKQMVKNREFLNSLN	4368.3 Da
Tenmo-DH_{47}	AGALGESGASLSIVNSLDVLRNRLLLEIARKKAKEG ANRNRQILLSL	5026.9 Da
Tenmo-ADFa	VVNTPGHAVSYHVY	1541.8 Da
Tenmo-ADFb	YDDGSYKPHIYGF	1560.7 Da

Electrophysiological effects have been investigated previously in the Namib Desert tenebrionid *O. plana*, where transepithelial and basolateral potentials were strongly affected by the K^+ concentration of the bathing medium (Nicolson and Isaacson, 1987). Stimulation of tubules with cyclic AMP reduced the basal permeability to Cl^- but, even though Na^+ secretion was accelerated, there was no discernible basal permeability to Na^+, suggesting electroneutral entry of Na^+ ions. Electrophysiological studies on *Tenebrio* tubule cells conducted by Wiehart et al. (2003a) showed that fluid secretion is dependent on the presence of K^+ in the bathing solution, and identified the transport mechanisms involved in the uptake of K^+ in *Tenebrio* tubules: K^+ ions are transported across the basolateral membrane via barium sensitive K^+ channels and via the electroneutral $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/K^+-ATPase. Also, the effects on basolateral membrane potentials of glibenclamide, a known blocker of K_{ATP} channels, provide evidence for the presence of K_{ATP} channels in tubule cells (Wiehart et al., 2003b). This study on *Tenebrio* was the first to demonstrate the presence of these channels in insects.

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The availability of the four peptides from *T. molitor*, all acting directly on the Malpighian tubules, makes it the ideal choice for investigating the complexities of fluid balance in beetle Malpighian tubules.

Chapter 2 reports the effects of various diuretic factors on fluid secretion in mealworm tubules, and the interactions between them. With the exception of a study involving the isolation of an antidiuretic factor from the Colarado potato beetle *Leptinotarsa decemlineata* (Chrysomelidae) (Lavigne et al., 2001), previous fluid secretion studies in beetles have concentrated on tenebrionids (Nicolson and Hanrahan, 1986; Nicolson, 1991; 1992). In this study, we investigate the possibility of the presence of CRF-related peptides in another beetle species, the dung beetle *Onthophagus gazella* (Scarabaeidae) and explore cross-reactivity with this species, which is only distantly related to *T. molitor*.

Chapter 3 describes immunohistochemical studies attempting to localise a calcitonin-like diuretic peptide in *T. molitor* tissues, using antiserum raised against the cockroach CT-like peptide Dippu-DH_{31}. Attempts to localise the antidiuretic factor Tenmo-ADFa in the mealworm nervous system are also reported.

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CHAPTER 2

THE RESPONSE OF MEALWORM (*TENEBRIO MOLITOR*) MALPIGHIAN TUBULES TO SYNTHETIC PEPTIDES, AND CROSS-REACTIVITY STUDIES WITH A DUNG BEETLE (*ONTHOPHAGUS GAZELLA*)

Introduction

Insect water balance is under the control of diuretic and antidiuretic hormones, comprehensively reviewed by Coast et al. (2002). Despite being the largest and most diverse insect order, the Coleoptera have been relatively neglected in studies of neuroendocrine control of Malpighian tubule function. Nicolson and Hanrahan (1986) demonstrated the presence of potent diuretic activity in the Namib Desert tenebrionid beetle, *Onymacris plana,* when they tested homogenates of brain and corpora cardiaca (CC) on isolated tubules. The remarkable stimulation of secretion rates was surprising considering the arid environment that this species inhabits and hence its need to conserve water. *In vivo* experiments demonstrated that the fluid secreted by the tubules is directed to the midgut for recycling to the haemolymph (Nicolson, 1991). In this way, metabolic wastes are rapidly cleared from the haemolymph without associated loss of water, indicating that diuretic hormones may not always lead to diuresis *per se*.

Another tenebrionid, the mealworm *Tenebrio molitor*, is unique in being the only insect from which both diuretic and antidiuretic hormones (acting directly on tubules) have been isolated. The two diuretic peptides, Tenmo-DH_{37} and Tenmo-DH_{47} (named according to the number of amino acid residues in their structure) both belong to the family of corticotropin releasing factor (CRF)-related peptides, which elicit their response via the second messenger cyclic AMP (Furuya et al., 1995, 1998). Tenmo-DH_{37} is the more potent of the two, with EC_{50} values in the nanomolar range (Wiehart et al., 2002a). This peptide has been immunocyto-

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chemically localised in the brain, corpora cardiaca, abdominal ganglia and posterior midgut of *T. molitor* (Wiehart et al., 2002b), further evidence of its physiological function as a diuretic hormone in this insect.

In addition to these two diuretic peptides, the mealworm also possesses two antidiuretic peptides that act directly on Malpighian tubules. Tenmo-ADFa and Tenmo-ADFb both inhibit fluid secretion in mealworm tubules and act via the second messenger cyclic GMP (Eigenheer et al., 2002; 2003). These two peptides are structurally unrelated to each other and to any other family of insect neuropeptides. In the first known example of antagonistic interactions between endogenous neuropeptides acting on Malpighian tubules, Tenmo-ADFa was shown to reverse the stimulatory effect of Tenmo-DH_{37} on mealworm tubules (Wiehart et al., 2002a).

The main families of insect diuretic hormones are the CRF-related peptides, calcitonin (CT)-like peptides and insect kinins (Coast et al., 2002). CRF-related peptides are the best characterised and, because of their similarity to the CRF-related peptides of vertebrates, are grouped with them in the same superfamily (Coast, 1998). In addition to those of *T. molitor*, these peptides have been isolated from Blattodea, Isoptera, Orthoptera, Diptera and Lepidoptera, and are suspected to be universal in insects (Coast et al., 2002). They act by increasing cyclic AMP production in Malpighian tubules and stimulate cation (K^+/Na^+) transport (reviewed by Nicolson, 1993; Beyenbach, 1995; Coast et al., 2002). They range from 30 to 47 amino acid residues in length, and all have a hydrophobic amide at the C-terminus except the CRF-related peptides from *T. molitor*, which are of particular interest because they exist as free acids instead (Furuya et al., 1995, 1998).

Originally known as the myokinins because of their myotropic activity, the kinins were first isolated from the Madeira cockroach, *Leucophaea maderae* (Holman et al., 1986). Hayes et al. (1989) were the first to demonstrate their diuretic activity in Malpighian tubules. However, it is uncertain if this is their function *in vivo*, since

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they are also effective stimulants of hindgut contraction. Kinins are much smaller than the CRF-related peptides: typically 6 - 15 residues long (Coast et al., 2002). They appear to have a non-selective effect on ion secretion by opening a Ca^{2+}-activated anion conductance, thus allowing more Cl^- into the tubule. With the increase in available Cl^-, additional Na^+ and K^+ can be transported into the lumen. Kinins are known from Blattodea, Lepidoptera and Diptera, but have not been isolated from Coleoptera to date (Coast et al., 2002).

More recently, a peptide was isolated from the cockroach *Diploptera punctata* that did not correspond to any known insect peptide but did show some similarity to vertebrate calcitonin (Furuya et al., 2000). The *D. punctata* peptide, subsequently named Dippu-$DH_{31,}$ proved to be the first example of a whole new family of insect diuretic peptides, the CT-like peptides. Dippu-DH_{31} was isolated concurrently with a CRF-related peptide, Dippu-$DH_{46,}$ using the same cyclic AMP assay. It is suspected that CT-like peptides target a different cyclic AMP-dependent effector system or activate a different second messenger pathway to that of CRF-related peptides (Coast et al., 2002). The two peptides differ in their modes of action (Dippu-DH_{31} has no effect on the Na^+/K^+ ratio of secreted fluid) and they act synergistically in *D. punctata* (Furuya et al., 2000). CT-like peptides have since been identified in other orders – Diptera (Coast et al., 2001; Coast et al., 2005), Lepidoptera (D.A. Schooley, unpublished data) and one has been partially sequenced from Hymenoptera (Laenen, 1999; Coast et al., 2002). A CT-like peptide has yet to be isolated from Coleoptera.

This study focuses on beetles in examining the effects and interactions of diuretic peptides on Malpighian tubules. We investigate the following: 1. The effect of thapsigargin, which mimics the action of kinins, and CT-like peptides on fluid secretion in tubules of *T. molitor,* exploring the possibility of synergism between different diuretic factors; 2. Structure/activity relationships, by testing C-terminal fragments of diuretic peptides from different species on mealworm tubules; 3. Cross-reactivity between beetle species, by examining the effects of a *T. molitor*

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diuretic factor on the tubules of a distantly related beetle species, the dung beetle *Onthophagus gazella* (Scarabaeidae).

Materials and methods

Experimental animals

Tenebrio molitor larvae were maintained at room temperature in dry bran. Apple or potato slices were provided on a regular basis as a source of moisture. Care was taken to select mealworms of similar size for experiments. *Onthophagus gazella* were collected near Bronkhorstspruit, east of Pretoria, maintained in buckets containing soil and fed fresh cow dung every 4-6 days. Adult dung beetles were used because larvae and pupae develop in brood balls, making it difficult to determine their stage of development. All experiments were conducted at room temperature (20 – 24°C).

Fluid secretion assays

Mealworms were opened dorsally and their tubules dissected out under Ringer's solution (see below). Tubules were dissected only from feeding mealworms that had bran in the midgut. The length of each tubule in the saline droplet varied, but Nicolson (1992) showed that there is no difference in secretion rates between the different regions of mealworm tubules. The arrangement of tubules of *Onthophagus gazella* adults differs from that of larval and adult mealworms: its four very long transparent tubules seem to be more robust than the shorter, pigmented tubules of the mealworm, and the dung beetle does not possess a rectal complex.

Isolated tubules were transferred to 50 µl droplets of Ringer's solution beneath liquid paraffin in a Sylgard-covered Petri dish. The Ringer used for isolated tubules had the following composition in mM: NaCl, 90; KCl, 50; $MgCl_2$, 5; $CaCl_2$ 2; $NaHCO_3$, 6; NaH_2PO_4, 4; glucose, 50; glycine, 10; proline, 10; serine, 10; histidine,

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10; and glutamine, 10 (Nicolson, 1992). The pH was adjusted to 7.0 with NaOH, and phenol red was used as an indicator. Two tubules were placed in each droplet and both ends were drawn out of the saline into the paraffin and wrapped around minuten pins. Because of the absence of a rectal complex in *O. gazella* the tubules are blind-ending, but the portions of whole tubules used were severed at both ends. The length of each tubule in the Ringer varied, but each tubule served as its own control.

Tubules were allowed approximately 20 min to equilibrate before control measurements were made. They usually secreted at one end only, and the secreted droplets were collected and transferred to the Sylgard-covered base with a fine glass pipette. The diameter of each droplet was measured using a calibrated eyepiece graticule and the volume (nl) and rate of secretion (nl/min) were calculated assuming the droplet to be spherical. After 2-3 control measurements at 10-15 min intervals, the bathing Ringer was replaced with 50 µl of Ringer containing the test substance. Measurements of secreted droplets were again taken at 10-15 min intervals to calculate the rate of secretion in the presence of the test substance. In experiments testing substances in combination, a third step was included, in which the initial test substance was removed and replaced with saline containing 2 test substances. At the end of each experiment, 0.1 mM dibutyryl cyclic AMP (db-cyclic AMP) was added to stimulate maximal rates of secretion and for dose-response curves results were expressed as a percentage of the maximum. Unless otherwise stated, this protocol was used for all experiments. Dibutyryl cyclic AMP was tested on *O. gazella* tubules in the presence of 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cyclic AMP phosphodiesterase activity. IBMX was dissolved in dimethylsulphoxide (DMSO), and stored as a stock solution (250 mM). The desired concentration was obtained by further dilution in Ringer's solution to obtain 0.5 mM IBMX and ≤0.2% DMSO in the final working solution.

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For preparation on tissue homogenates, mealworms and dung beetles were decapitated (dung beetle heads were stored at -20°C until use). For both species, brains (with CC included) were dissected out under Ringer's solution and transferred to a small glass mortar with a drop of Ringer. The tissue was ground up with a glass rod and the homogenate was added to Ringer to give a concentration of 1 brain per 100 µl.

Chemicals

No kinins have yet been isolated from Coleoptera, but thapsigargin can be used as a substitute for kinins as it is a specific inhibitor of Ca^{2+} uptake by intracellular stores, thereby increasing intracellular Ca^{2+} (Thastrup et al., 1990; Yu and Beyenbach, 2002). Thapsigargin was dissolved in DMSO and diluted in Ringer's solution to 1 µM (the final concentration of DMSO was 0.1%). Synthetic peptides (CRF-related peptides Tenmo-DH_{37}, Locmi-$DH_{46,}$ Dippu-$DH_{46,}$ and CT-like peptides Bommo-$DH_{31,}$ Dippu-DH_{31} and Anoga-DH_{31}) were gifts from David Schooley (University of Nevada, Reno, USA). The truncated peptide analogues Locmi-$DH_{32\text{-}46,}$ Dippu-$DH_{32\text{-}46}$ and Dippu-$DH_{17\text{-}31}$ were synthesised by Charoula Kaskani and Constantine Poulos (Department of Chemistry, University of Patras, Patras, Greece). Peptides were dissolved in 60% acetonitrile, in order to minimise adherence to the sides of polypropylene containers. Ringer's solution was used to dilute to the desired concentration, with ≤0.6% acetonitrile in the final working solution (at this concentration, acetonitrile does not affect secretion rates in mealworm tubules; S.W. Nicolson, unpublished data). All other chemicals were purchased from Sigma.

Statistics

Results are presented as the means ± standard error (SE). Each tubule segment served as its own control. Statistical differences were calculated using the paired Student's *t*-test, or 1-way Anova with $P<0.05$ accepted as significant. GraphPad Prism 4.0 was used to fit dose-response curves (non-linear regression).

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Results

The response of T. molitor *tubules to calcitonin-like peptides and thapsigargin*

The CT-like peptides Dippu-$DH_{31,}$ Bommo-DH_{31} and Anoga-DH_{31} (from the cockroach *Periplaneta americana*, the silkmoth *Bombyx mori* and the malaria mosquito *Anopheles gambiae* respectively) were tested for diuretic activity on isolated mealworm tubules. Both Bommo-DH_{31} and Anoga-DH_{31} stimulated fluid secretion in *T. molitor* tubules in a dose-dependent manner (Fig. 1), with EC_{50} values of 0.61 and 14 nM respectively (95% confidence limits: 0.2–1.8 nM for Bommo-DH_{31} and 0.63–320 nM for Anoga-DH_{31}). The highest concentration used was 1 μM and for both peptides the response was approximately 50% of maximum stimulation due to 0.1 mM db-cyclic AMP. However, at a concentration of 1 μM, the cockroach CT-like peptide Dippu-DH_{31} failed to stimulate mealworm tubule secretion (see Fig. 4).

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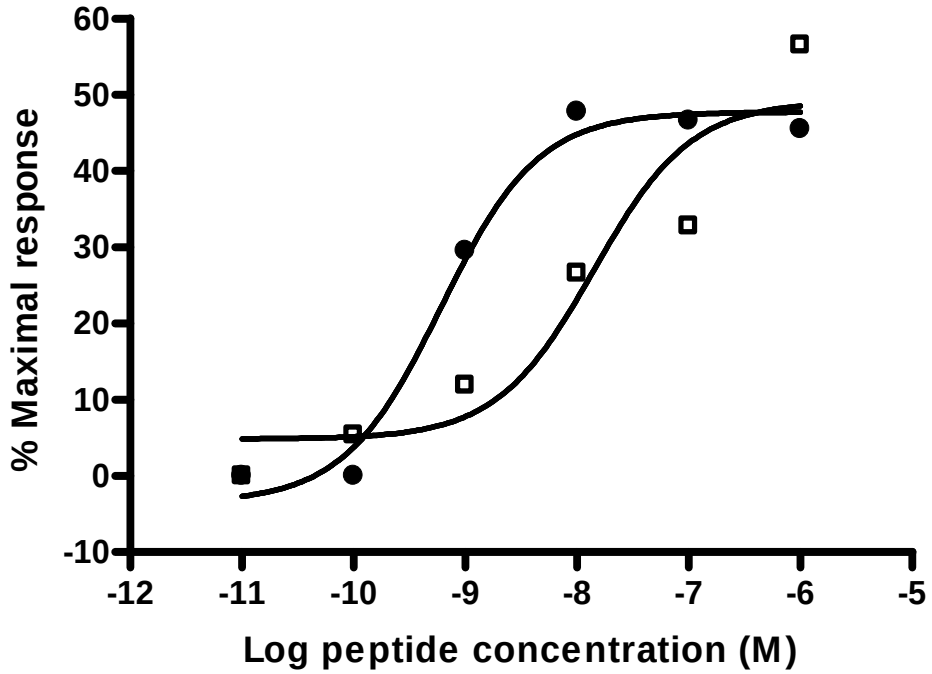

Fig. 1. Dose-response curves for the effect of *Bombyx mori* and *Anopheles gambiae* calcitonin-like peptides on fluid secretion in *Tenebrio* tubules. Data points are the means of 7-10 determinations for Bommo-DH_{31} (filled circles) and 6-8 determinations for Anoga-DH_{31} (open squares), and results are expressed as the percentage of the maximum response obtained in the presence of 0.1 mM dibutyryl cyclic AMP. The EC_{50} values are 0.61 nM for Bommo-DH_{31} and 14 nM for Anoga-DH_{31} (r^2 values for the curve fits are 0.99 and 0.89 respectively). Standard errors of the mean are very small and so are not visible.

The increase in secretion rates in mealworm tubules effected by 1 μM thapsigargin was 22.5% of the maximum response to 0.1 mM db-cyclic AMP (Fig. 2). The increase is significant ($P<0.001$) but very small relative to that caused by cyclic AMP.

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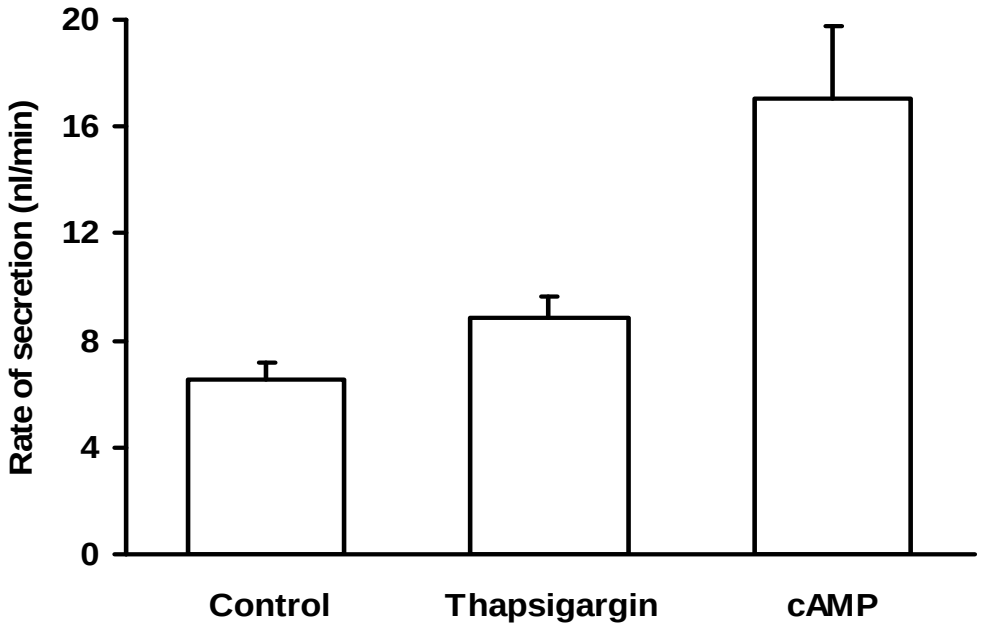

Fig. 2. The effect of thapsigargin, a pharmacological agent that mimics the effects of kinins, on *Tenebrio* tubules. At a concentration of 1 μM, thapsigargin increases fluid secretion to 22.5% of the maximal response to 0.1 mM db-cyclic AMP. The increase is not significant (P>0.05). Bars represent the mean for 7 tubules and vertical lines are SEs.

Interaction between Tenmo-DH$_{37}$, thapsigargin and CT-like peptides

We first measured the response of isolated tubules to 1 nM Tenmo-DH$_{37}$, approximately ten times the EC$_{50}$ for this peptide (Wiehart et al., 2002a), followed by the response to 1 nM Tenmo-DH$_{37}$ together with 1 μM thapsigargin. The results are shown in Fig. 3a. Secretion rates increased in the presence of Tenmo-DH$_{37}$ alone, but when tested in combination with thapsigargin, no further significant increase was observed (P>0.05).

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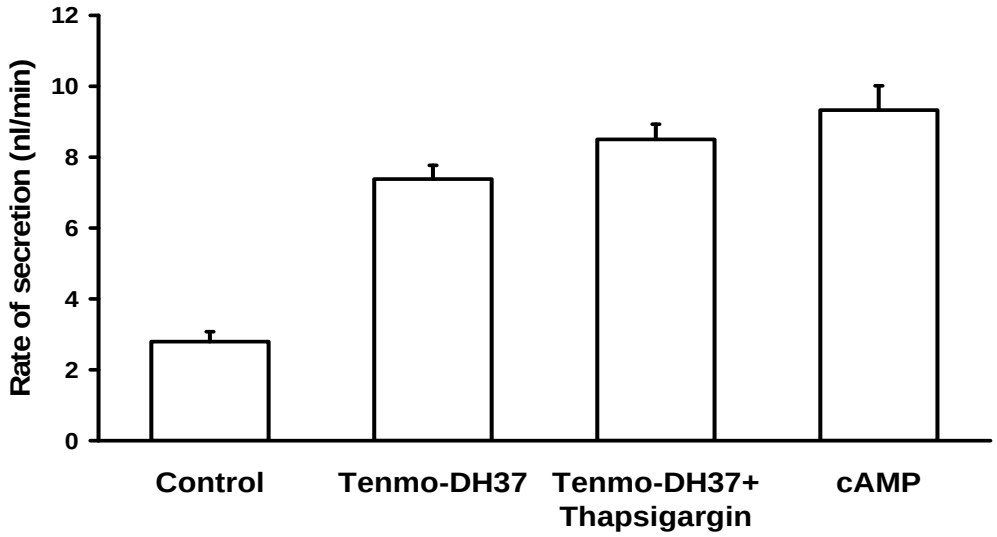

Fig. 3a. No synergistic or additive effects were observed between a CRF-related peptide, Tenmo-DH_{37} and thapsigargin in *Tenebrio* tubules. A small increase occurs with the addition of thapsigargin (1 μM) to Tenmo-DH_{37} (1 nM), but this was not significant (P>0.05; n = 11). Bars represent the means and vertical lines the SEs.

Next, we tested the effects on beetle tubules of combining a CRF-related peptide (Tenmo- DH_{37}) with a calcitonin-like peptide (Bommo-DH_{31}). Tubules were first exposed to 1 nM Tenmo-DH_{37} and then to 1 nM Tenmo-DH_{37} together with 1nM Bommo-DH_{31} (approximately 1.5 times the EC_{50} value determined for this peptide in the mealworm), with no further increase in secretion (results not shown). Another experiment was conducted with 10 nM Tenmo-DH_{37} in combination with 1 μM Bommo-DH_{31} but no significant increase occurred (shown in Fig 3b). The experiment was repeated at other concentrations (10 nM each) and the order of the peptides was reversed, Bommo-DH_{31} being tested first, followed by Bommo-DH_{31} in combination with Tenmo-DH_{37}, but no synergistic or additive effects were observed.

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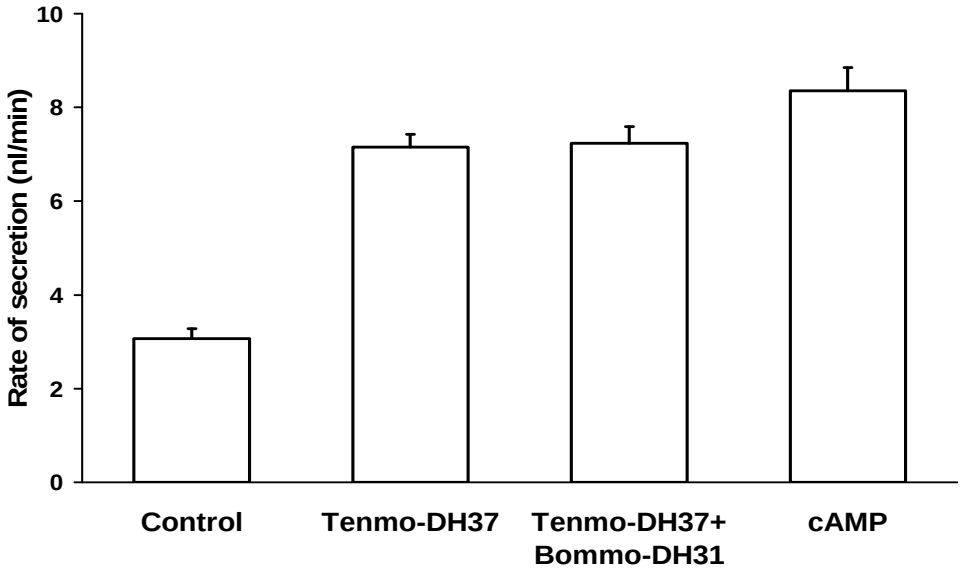

Fig. 3b. At 10 nM, the CRF-related Tenmo-DH_{37} alone increases fluid secretion in mealworm tubules to approximately 80% of maximum, but no further increase occurs with the addition of the CT-like peptide Bommo-DH_{31} at a concentration of 1 µM (P>0.05; n = 7). Bars represent the means and vertical lines the SEs.

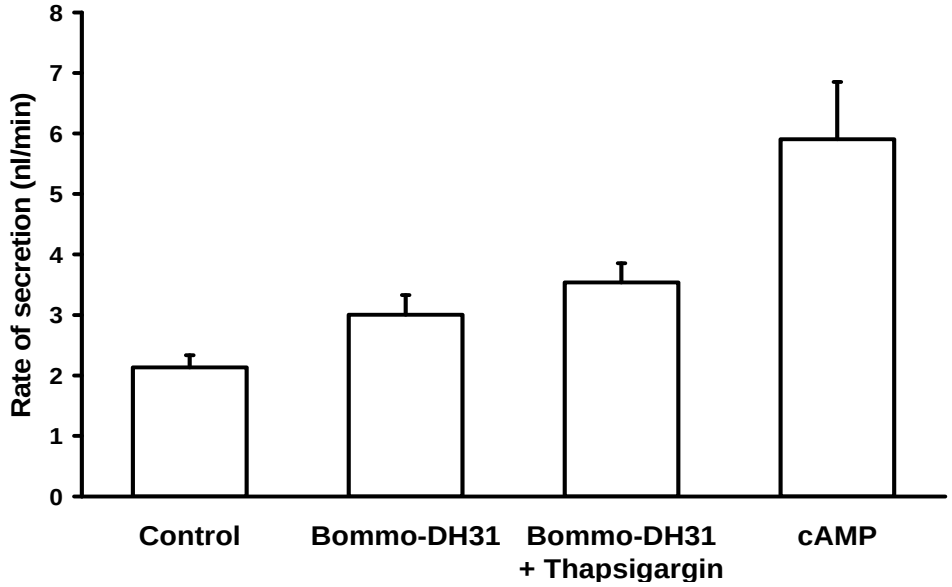

Fig. 3c. No significant increase in fluid secretion rates takes place in the presence of Bommo-DH_{31} (1 nM) and thapsigargin (1 µM) combined (P>0.3; n = 6). Bars represent the means and vertical lines the SEs.

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Effects on fluid secretion rates of C-terminal fragments of CRF-related and CT-like peptides and corresponding whole peptides

C-terminal fragments of diuretic peptides were used to examine the structural requirements for stimulation of fluid secretion in Malpighian tubules of *Tenebrio.*

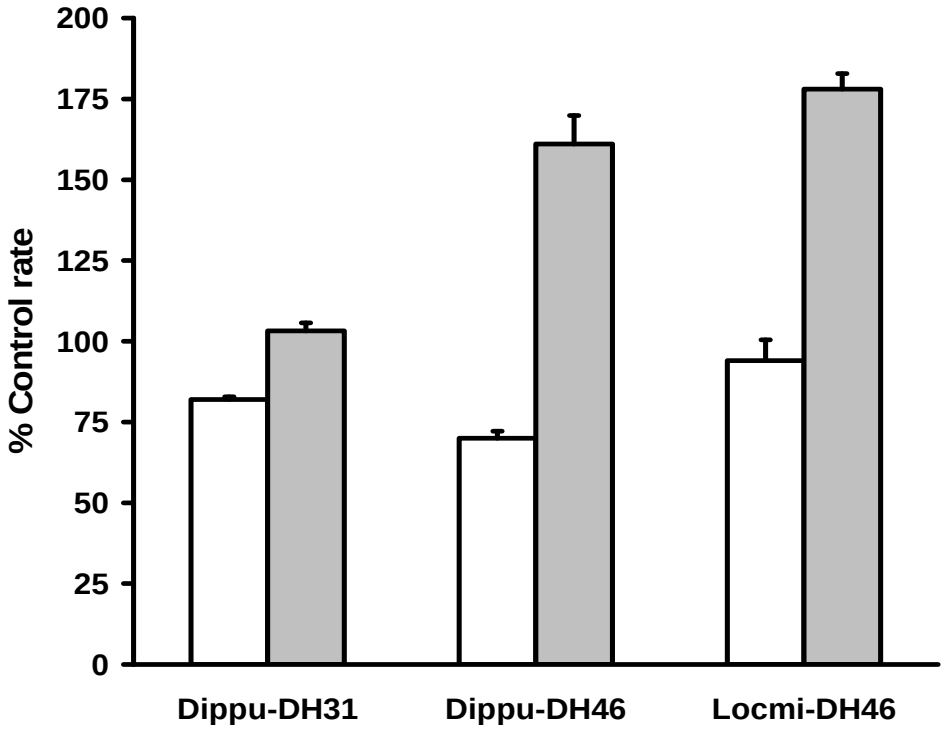

Fig. 4. Comparison of the effects of the C-terminal fragments and corresponding whole peptides of the CT-like peptide Dippu-DH_{31} and the CRF-related peptides Dippu-DH_{46} and Locmi-DH_{46} on fluid secretion in *Tenebrio* Malpighian tubules. Truncated analogues (open bars) were tested at 0.1 mM and whole peptides (filled bars) at 1 µM. A value equal to or less than 100% indicates no increase over control rates (n = 5-6). A decrease can be explained by the normal decline in basal secretion rates over time. The CT-like peptide Dippu-DH_{31} did not stimulate secretion (P>0.05; n = 9). The CRF-related peptides Dippu-DH_{46} and Locmi-DH both significantly increased fluid secretion rates (P<0.001 for both; n = 7-10). Vertical bars are SEs.

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Truncated analogues of the CRF-related peptides Locmi-DH_{46} and Dippu-DH_{46} (Locmi-DH_{32-46} and Dippu-DH_{32-46} respectively) and the CT-like peptide Dippu-DH_{31} (Dippu-DH_{17-31}) were tested for their effects on fluid secretion in mealworm tubules. They were initially tested at 1 μM (results not shown), but even at a concentration of 0.1 mM, there was no stimulation (Fig. 4). Because these peptides had not previously been tested as whole peptides in *T. molitor,* complete sequences were tested as well. The two CRF-related peptides Locmi-DH_{46} and Dippu-DH_{46} both significantly increased fluid secretion in mealworm tubules at a concentration of 1 μM (Fig. 4). Surprisingly, Dippu-DH_{31} did not ($P>0.05$), in contrast to the other CT-like peptides Bommo-DH_{31} and Anoga-DH_{31} (Fig. 1).

The response of O. gazella *tubules to cyclic AMP and the mealworm peptide Tenmo-DH37*

Cyclic AMP appears to be universally involved in fluid secretion by Malpighian tubules. Therefore, before testing other peptides on the tubules of the dung beetle *Onthophagus gazella,* we first explored the effects of exogenous cyclic AMP in this previously unstudied insect. Preliminary studies used the sodium salt of cyclic AMP and the apparent lack of response (results not shown) prompted the use of the more membrane permeant analogue db-cyclic AMP. Dibutyryl cyclic AMP was dissolved in DMSO to obtain a working solution of 0.2%, which has previously been shown to have no effect on tubule secretion (Coast, 1995). Similarly, in this study, there was no change in secretion rates when saline was replaced with saline containing 0.2% DMSO (results not shown). A concentration of 0.1 mM db-cyclic AMP resulted in a significant increase in the fluid secretion rate ($P<0.001$), shown in Fig. 5.

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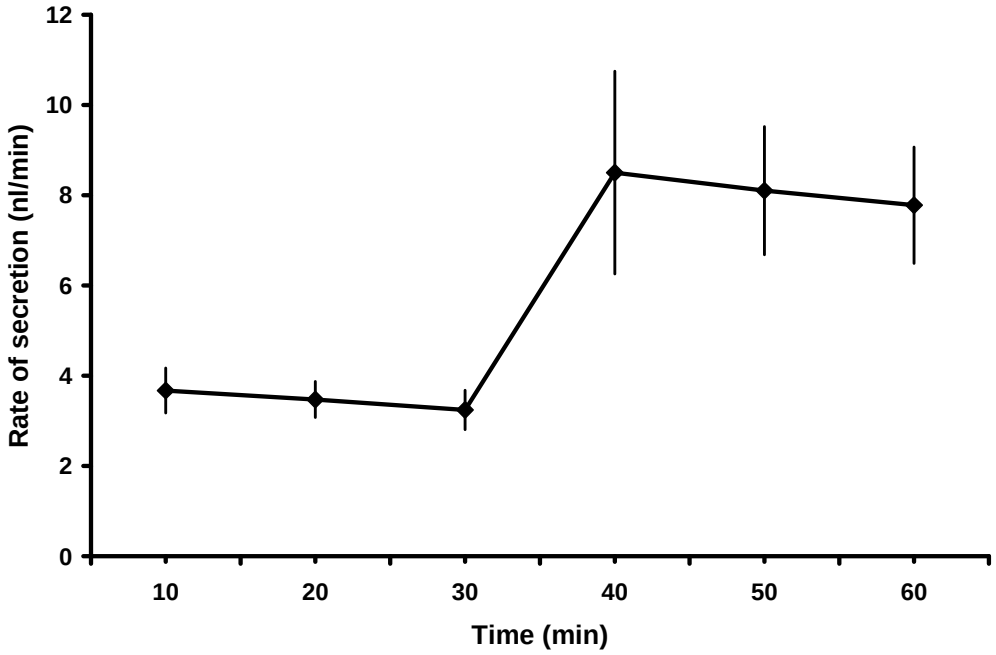

Fig. 5. Dibutyryl cyclic AMP increases fluid secretion in Malpighian tubules of the scarabaeid *Onthophagus gazella* ($P<0.001$; n = 11). cAMP (0.1 mM) was added at Time = 30 min.

Tenmo-DH_{37} stimulated fluid secretion in *O. gazella* tubules in a dose-dependent manner with an EC_{50} value of 0.38 nM (Fig. 6). Maximal stimulation by Tenmo-DH_{37} was obtained at a concentration of 1 µM (68.5% of the maximal response obtained in the presence of 0.1 mM db-cyclic AMP).

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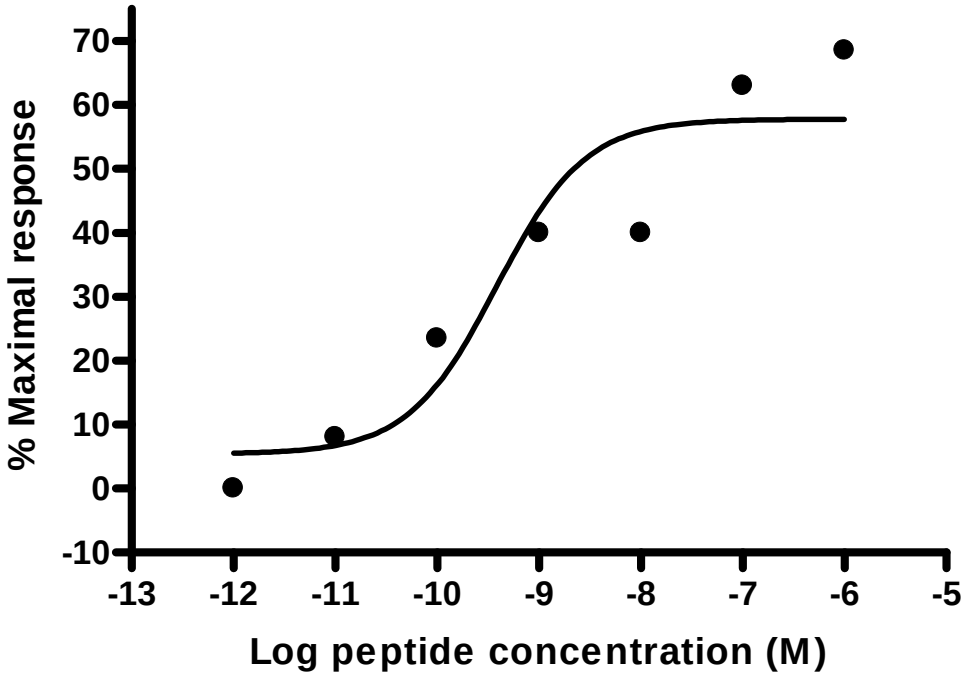

Fig. 6. Dose-response curve for the effect of the mealworm CRF-related diuretic factor Tenmo-DH_{37} on the tubules of *Onthophagus gazella*. Data points are means of 5-9 determinations, SEs are too small to see. Results are expressed as a percentage of the maximal response to 0.1 mM dibutyryl cAMP. EC_{50} = 0.38 nM (r^2 value for the curve fit = 0.89).

Effect of O. gazella *brain homogenate on mealworm tubules*

At a concentration of 1 brain per 100 µl, the brain homogenate of the dung beetle has an antidiuretic effect in mealworm tubules (Fig. 7). Secretion rates decreased by 66% (P<0.005), but increased rapidly when the Ringer containing the homogenate was replaced with Ringer containing 0.1 mM db-cyclic AMP. In contrast, homogenates of mealworm brain had a marked stimulatory effect on mealworm tubules (P<0.001) (Fig. 7).

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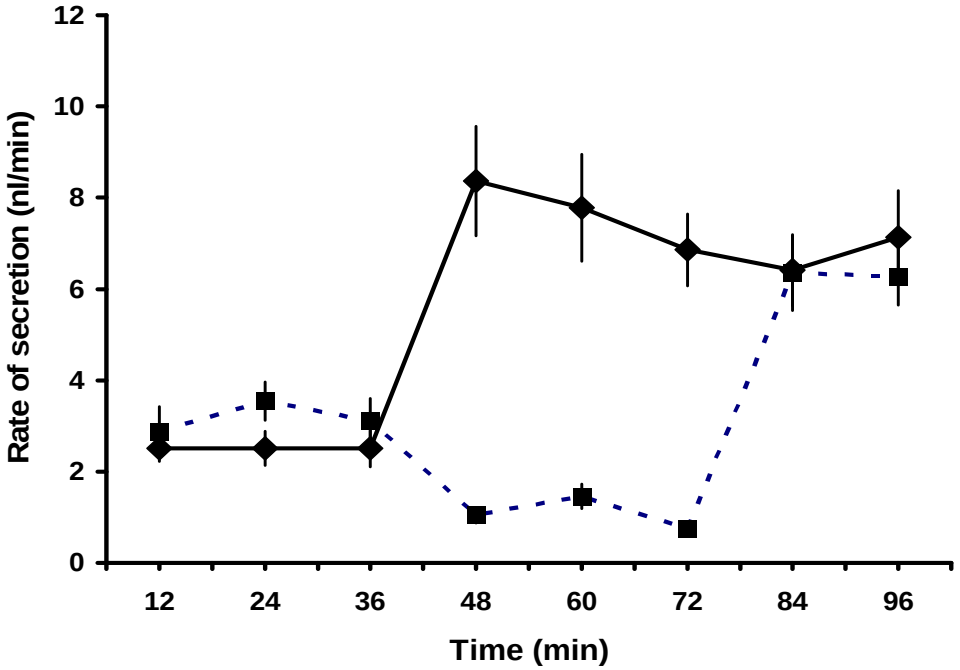

Fig. 7. Secretory response of isolated mealworm tubules to *O. gazella* brain extract (dashed line) compared to the response to *Tenebrio* extract (solid line). Arrow shows time of addition of the brain homogenates (1 brain per 100 µl) and horizontal solid bar indicates time of exposure to 0.1 mM cAMP. Secretion rates declined significantly in tubules exposed to *O. gazella* brain extracts ($P<0.005$; n = 5) but increased in the presence of mealworm brain extracts ($P<0.001$; n = 6). SEs shown by vertical bars.

To further test the inhibitory effect of *O. gazella* brain extracts on mealworm tubules, and to ensure that the decrease was not due to the normal decline of basal secretion rates over time, tubules were first stimulated with 0.1 mM db-cyclic AMP followed by the addition of homogenate (at 1 brain/100 µl) in the presence of db-cyclic AMP. Once more, secretion rates dropped significantly (results not shown).

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Discussion

The availability of a wide range of synthetic diuretic factors has enabled us to examine the interacting effects of these different factors on Malpighian tubule secretion. Because both diuretic and antidiuretic peptides acting on Malpighian tubules have been isolated from *T. molitor*, this species is ideal for further investigation of water and ion regulation in insects in general and beetles in particular.

Effects of calcitonin-like peptides and thapsigargin on mealworm tubules and lack of synergism between diuretic factors

The tubules of *Tenebrio* are sensitive to some CT-like peptides, with both Bommo-DH_{31} and Anoga-DH_{31} having EC_{50} values in the nanomolar range. In fact, with an EC_{50} of 14 nM in *Tenebrio*, Anoga-DH_{31} is slightly more potent than in its source *Anopheles gambiae,* where the EC_{50} was 50 nM (Coast et al., 2005). However, in the mealworm, the maximum stimulation in the presence of these CT-like peptides is much less than that for CRF-related diuretic hormones; thus even though CT-like peptides are potent at low concentrations, their effect in *Tenebrio* tubules is not very powerful. The cockroach CT-like peptide Dippu-DH_{31} failed to stimulate mealworm tubules and attempts to localise a CT-like peptide in *Tenebrio* nervous tissue using the antiserum to Dippu-DH_{31} have not been successful (W.D. Holtzhausen, 2006). A CT-like peptide has been immunocytochemically localised in the blood-feeding bug, *Rhodnius prolixus* using antiserum to Dippu-DH_{31} (Te Brugge et al., 2005). Interestingly, although a CT-like peptide has not yet been isolated from a beetle, one was identified in another tenebrionid during a partial genome sequence of *Tribolium castaneum* (D.A. Schooley, personal communication). The failure to localise a CT-like peptide in *T. molitor* could be because CT-like peptides are not present in detectable amounts in the mealworm nervous system.

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However, data on CT-like peptides are limited and we still do not know much about their mode of action. The different effects of these peptides across and even within orders should be noted. For example, the cockroach CT-like peptide Dippu-DH_{31} has a non-selective effect on the Na^+ and K^+ ratio in the cockroach (Furuya et al., 2000) while Anoga-DH_{31}, recently shown by Coast et al. (2005) to be the previously uncharacterised natriuretic peptide of mosquitoes (Petzel et al., 1985), markedly increases [Na^+] relative to [K^+] in the mosquito (Coast et al., 2005). While the increase in secretion rates induced by thapsigargin was significant, when compared to the effect of CRF-like peptides on mealworm tubules and the effect of kinins on tubules from other insects (flies in particular), the effect was not striking. In the housefly *Musca domestica*, thapsigargin increases secretion rates 4 to 5 fold. In the dipteran suborder Cyclorrhapha, kinins stimulate maximum secretion (as with thapsigargin, 4 to 5 times control rates) (Iaboni et al., 1998) and CRF-related and CT-like peptides are less effective, while in the Nematocera, CT-like peptides stimulate maximal secretion rates and CRF-related peptides are less effective, as are kinins (Coast et al., 2005). This difference in relative importance of the various factors within the Diptera corresponds to phylogeny and may depend on whether anion transport is rate-limiting (Iaboni et al., 1998). Interestingly, in preliminary studies, kinins from other insect orders (leucokinin, achetakinin and muscakinin) failed to induce a response in isolated tubules of *T. molitor* (Wiehart et al., 2002a). This raises the possibility that kinins do not play a diuretic role in *T. molitor*, which would be supported by the failure of thapsigargin to further increase secretion rates in the presence of Tenmo-DH_{37}. It remains to be seen whether kinins do in fact exist in *T. molitor*.

The lack of synergism in mealworm tubules between Tenmo-DH_{37} and the CT-like peptide Bommo-DH_{31} was unexpected, considering the different mechanisms by which these diuretic factors are thought to operate (Coast et al., 2002). In the relatively few studies that have investigated synergistic effects between different factors (Table 1), varied responses have been encountered. For instance, kinins and CRF-related peptides are synergistic in the locust (Locmi-K and Locmi-DH_{46})

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(Coast, 1995), but not in the cricket (achetakinin-I and the CRF-related Achdo-DH_{46}) (Coast and Kay, 1994). Also, CRF-related and CT-like peptides from *D. punctata* (Dippu-DH_{46} and Dippu-DH_{31}) are synergistic in the cockroach, but have only an additive effect in *L. migratoria* (Furuya et al., 2000). In the same way, it is possible that the action of peptides from other insects in mealworm tubules may differ from that of the native peptides.

Table 1. Studies examining potential synergistic or additive interactions between different diuretic factors acting via different second messenger pathways.

Factor 1	Factor 2	Species	Combined effect	Reference
CRF-related + CT-like				
Dippu-DH_{46}	Dippu-DH_{31}	*D. punctata*	Synergistic	Furuya et al. 2000
Dippu-DH_{46}	Dippu-DH_{31}	*L. migratoria*	Additive	Furuya et al. 2000
Locmi-DH	Dippu-DH_{31}	*L. migratoria*	Synergistic	Furuya et al. 2000
CRF-related + kinin				
Locmi-DH	Locmi-K	*L. migratoria*	Synergistic	Coast 1995
Musdo-DP	Musdo-K	*M. domestica*	Synergistic	Holman et al. 1999
Achdo-DP	Achdo-K	*A. domesticus*	Not synergistic or additive	Coast & Kay 1994
Kinin + CT-like				
Musdo-K	Anoga-DH_{31}	*An. gambiae*	Synergistic	Coast et al. 2005
Locmi-K	Dippu-DH_{31}	*L. migratoria*	Synergistic	Furuya et al. 2000
Thapsigargin	Dippu-DH_{31}	*L. migratoria*	Additive	Furuya et al. 2000

Considering the anomaly of a xeric insect such as the mealworm producing at least two potent diuretic factors, it might be presumed that diuresis needs to be controlled precisely in this species. Low concentrations of diuretic factors circulating in the haemolymph would be easier to control, so synergism, which would steepen the dose-response curves and result in a lower concentration necessary for a response (Coast, 1995), would be advantageous. However, while synergism is a plausible explanation for the presence of more than one diuretic

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factor in the same insect, diuretic peptides may have multiple actions or control other physiological processes (Coast et al., 2002). Importantly, different factors that similarly stimulate fluid secretion may have very different effects on ion transport.

Lack of response of T. molitor *tubules to C-terminal fragments of diuretic factors*

Studies using deletion analogues of insect neuropeptides have been carried out to investigate the region of the peptide that binds to and/or activates the relevant receptors. Structure/activity studies in other insects have shown that, for CRF-related peptides, the region towards the N-terminus is important for receptor activation while the C-terminal end is required for receptor binding. For instance, C-terminal fragments of Manse-DH_{41} retain good binding affinity for the *Manduca* receptor but do not stimulate cyclic AMP production (Reagan, 1995). It is known that for many other insect neuropeptides (*A*-type allatostatins, kinins, FRMFamides) the active core resides in the C-terminus of the molecule (Matthew et al., 2006).

Deletion of the first 31 residues of the blattodean and orthopteran CRF-related analogues used in this study completely inhibited the peptides' ability to increase fluid secretion in mealworm tubules. In the locust, Locmi-DH_{32-46} has very low fluid secretion activity, but remarkably, this same fragment retains biological activity in regulating satiety in locusts (Goldsworthy et al., 2003). Hence, it appears that the receptors for controlling satiety have different structure-activity requirements to the receptors in tubules (Goldsworthy et al., 2003). In the latter tissue it appears that the binding and activation regions have to be linked in the same sequence in order for the peptide to be biologically active (Coast et al., 2002). The mealworm diuretic peptides are the only known members of the CRF-related family to have their C-termini in the non-amidated form (Furuya et al., 1995, 1998). There may be important structural requirements of the C-terminus for bioactivity, since Tenmo-DH_{37} had >10 000 times lower activity than Manse-DH_{41} in elevating cyclic AMP in *Manduca* tubules. In the reverse assay, however,

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Manse-DH_{41} is only 17 times less potent than Tenmo-DH_{37} in *Tenebrio* tubules (Furuya et al., 1995). Unlike the truncated analogues, the complete sequences of the locust and cockroach CRF-related peptides are only slightly less powerful in elevating secretion rates in mealworm tubules than Tenmo-DH_{37} (see Wiehart et al., 2002a), indicating a high degree of cross-reactivity in spite of the evolutionary distance between these species and the significant difference in C-terminus structure of their peptides.

Cross-reactivity between beetle species: effects of diuretic factors and brain homogenates

Onthophagus gazella is a dung beetle from the family Scarabaeidae. More than 2000 species of *Onthophagus* have been described, making it the largest genus of beetles, and it is found on every continent except Antarctica (Emlen et al., 2005). Each stage of the life cycle is completely dependent on dung: eggs are laid in individual dung balls underground, the larva hatches inside the dung ball and remains there throughout the pupal stage until emergence as an adult beetle. Unlike tenebrionids, scarabaeids do not have a rectal complex. This structure, present in lepidopteran larvae and in some families of Coleoptera (from the superfamilies Bostrichoidia and Cucujiformia) (Saini, 1964; Lawrence and Britton, 1991), comprises the terminal portions of the Malpighian tubules, which are enclosed in a sheath around the rectum. In tenebrionids the rectal complex is capable of withdrawing water from unsaturated air, as well as from the rectal contents (Machin, 1983).

Onthophagus is only distantly related to *Tenebrio* and although sequence homology for CRF-related peptides is fairly low (around 50%), with varying degrees of interspecific reactivity (Audsley et al., 1995; O'Donnell and Spring, 2000), the tubules of the dung beetle *O. gazella* appear to be very sensitive to the mealworm diuretic hormone Tenmo-DH_{37}. For this peptide in *O. gazella* the EC_{50} is 0.38 nM while in the mealworm itself, the EC_{50} is 0.12 nM and maximal stimulation

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(approximately 100% of maximum) is at 0.1 μM (Wiehart et al., 2002a). Since CRF-related peptides appear to be ubiquitous, it is probable that *O. gazella* also makes use of a CRF-like diuretic factor similar to Tenmo-DH_{37}.

The antidiuretic effect of *O. gazella* brain homogenate was surprising, since the brains and CC of other beetles have previously been shown to be a source of diuretic activity. For example, homogenates from the desert tenebrionid, *Onymacris plana,* produced dramatic increases in fluid secretion rates (Nicolson and Hanrahan, 1986). Also, the tubules of *T. molitor* are stimulated by CC extracts from *Onymacris rugatipennis* (Nicolson, 1992) as well as homogenates of its own brain and CC (Nicolson, 1992; Fig.7). However, antidiuretic factors present in the brain/CC of *O. gazella* may exhibit similar effects to the mealworm antidiuretic factor Tenmo-ADFa, which inhibits fluid secretion rates in mealworm tubules and antagonises the effects of Tenmo-DH_{37} (Wiehart et al., 2002a). The *O. gazella* antidiuretic factor would thereby reverse the effects of the diuretic factor in this insect. An antidiuretic factor has been partially purified from the Colorado potato beetle (Lavigne et al., 2001), which may be related to the *Tenebrio* antidiuretic peptides (Coast et al., 2002).

Te Brugge et al. (2005) showed that the amount of diuretic factor (Dippu-DH_{31}) in the nervous tissue of the blood-sucking bug *Rhodnius prolixus* after feeding appeared to be diminished, indicated by reduced immunoreactivity. Following a blood meal, the diuretic factor needs to be released into circulation to precipitate diuresis. *Onthophagus gazella* feeds on a food source of high water content, and the beetles in this study were decapitated immediately after being removed from dung. In fact, when nervous tissue was dissected out, dung was present in the oesophagus of some of the beetles. These beetles could be releasing their diuretic hormones into circulation on a regular basis, thereby reducing the amount of stored factor in the nervous tissue.

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There is speculation that the structure of the C-terminus of Tenmo-DH_{37} and -DH_{47} might be linked to the presence of a rectal complex (Coast et al., 2002), in which case cross-reactivity between the diuretic peptides of *T. molitor* and other coleopterans possessing a rectal complex may be more pronounced. It is interesting that Tenmo-DH_{37}, which has no effect in *Manduca* (possibly other insects too) does stimulate dung beetle tubules. This raises the possibility that the unusual free acid terminus of the mealworm peptides occurs elsewhere in beetles.

It is clear that there is no general pattern for the control of fluid secretion in different species, even within orders. The Malpighian tubules appear to be the target organ, but while various factors may stimulate tubules, their main function may be in other parts of the excretory system, such as the rectal complex. Investigation of diuretic and antidiuretic effects in the rectal complex would prove particularly interesting. It needs to be stressed that while *in vitro* experiments are instructive, the actual physiological role of these peptides remains inconclusive and more *in vivo* studies are necessary to further elucidate our understanding of the function and interaction of different factors involved in beetle excretion.

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CHAPTER 3

ATTEMPTED IMMUNOHISTOCHEMICAL LOCALISATION OF THE MEALWORM ANTIDIURETIC FACTOR TENMO-ADFa AND A CALCITONIN-LIKE PEPTIDE IN THE NERVOUS SYSTEM OF *TENEBRIO MOLITOR*

Introduction

The availability of synthetic insect diuretic and antidiuretic peptides has enabled the production of antisera for immunohistochemical analyses. The use of specific antibodies not only confirms the presence of peptides in certain tissues, but also permits their precise localisation in the insect central nervous system. The detection of the presence and distribution of a number of diuretic factors in insect nervous tissue has further substantiated their role as neurohormones, and enables more detailed studies involving their mode of action (Coast et al., 2002). Immunohisto-chemistry has thus become a valuable tool for insect physiologists (Nässel, 1996).

Until recently, the only beetle to be included in immunohistochemical studies of diuretic factors was the Colorado potato beetle, *Leptinotarsa decemlineata,* in which the distribution of serotonin was shown (Haeften and Schoonveld, 1992). The isolation of diuretic and antidiuretic peptides from the mealworm *Tenebrio molitor* (Tenebrionidae), enables immunohistochemical studies on additional beetle peptides to be carried out using antisera raised against them. Two diuretic and two antidiuretic peptides have been isolated from this species, making it the only insect for which both diuretic and antidiuretic factors acting on Malpighian tubules are known (Furuya *et al*., 1995, 1998; Eigenheer *et al*., 2002, 2003).

Both diuretic peptides isolated from *T. molitor* belong to the family of corticotropin-releasing factor (CRF)-related peptides. One of them, Tenmo-DH_{37}, has been localised in the mealworm nervous system and midgut, revealing

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immunoreactive neurosecretory cells in the brain, corpora cardiaca (CC), abdominal ganglia and posterior midgut (Wiehart *et al*., 2002b). Similarly, one of the two antidiuretic peptides, Tenmo-ADFb, was found in the brain and ventral nerve cord (Eigenheer *et al*., 2003).

The mealworm antidiuretic peptides Tenmo-ADFa and Tenmo-ADFb inhibit fluid secretion in mealworm Malpighian tubules, and both act via the second messenger cyclic GMP. It is not known whether they also promote reabsorption of fluid by the hindgut or cryptonephric complex (Wiehart *et al.* 2002a). Another beetle antidiuretic factor has been isolated and partially sequenced from the Colorado potato beetle *Leptinotarsa decemlineata* (Lavigne et al., 2001). The *L. decemlineata* peptide is similar in size to the *Tenebrio* antidiuretic factors, suggesting that they may be related (Coast et al., 2002). Few antidiuretic peptides have been identified in insects and much ambiguity remains with regard to their exact physiological role (Wiehart *et al.*, 2002b). Tenmo-ADFb is the first insect antidiuretic factor to be localised using immunohistochemistry.

Using immunohistochemical techniques, this study attempted to localise two peptides in the mealworm nervous system: the mealworm antidiuretic factor, Tenmo-ADFa, and a calcitonin-like diuretic peptide, using antiserum raised against Dippu-DH_{31} from the cockroach *Diploptera punctata* (Furuya et al., 2000).

Tenmo-ADFa, a 14 amino-acid peptide with a theoretical mass of 1541.7 Daltons, was isolated from 1500 head equivalents of *T. molitor* (Eigenheer *et al*., 2002). Tenmo-ADFa bears some resemblance to a portion of rabbit big endothelin I (57% identity and an additional 14% similarity) (Eigenheer et al., 2002) and, curiously, is almost identical to the C-terminus of a *Tenebrio* cuticle protein. The other *Tenebrio* antidiuretic factor, Tenmo-ADFb, also shares near identity to another cuticle protein. For this reason, details of its structure and activity were only submitted for publication after immunohistochemistry provided evidence of its distribution in the mealworm nervous system, consistent with a neuropeptide role in this insect

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(Eigenheer et al., 2003). Tenmo-ADFa strongly inhibits fluid secretion in isolated tubules of *T. molitor*: with an EC_{50} of approximately 10 fM, it is 24 000 times more potent than Tenmo-ADFb (Eigenheer *et al*., 2003). It antagonises stimulation of tubules by the diuretic hormone Tenmo-DH_{37} (Wiehart *et al*., 2002a), indicating the possibility of co-ordinated function between the diuretic and antidiuretic hormones in this species. Tenmo-ADFa also has antidiuretic effects in the tubules of the yellow fever mosquito *Aedes aegypti*, acting via the second messenger cyclic GMP (Massaro *et al*., 2004). However, in the mosquito, the concentrations of Na^+, K^+ and Cl^- in secreted fluid remain unchanged in the presence of Tenmo-ADFa, and even though cyclic GMP induces electrophysiological effects in this species, Tenmo-ADFa does not.

Since Tenmo-ADFb is considerably less potent than Tenmo-ADFa, it is possible that the primary function of Tenmo-ADFb may not be antidiuresis. The localisation of Tenmo-ADFa in *Tenebrio* nervous tissue would permit comparison of the distribution patterns of these two antidiuretic peptides in this species. This could substantiate the hypothesis that their action is co-ordinated or indicate whether they have alternate targets. The distribution of the two antidiuretic factors could then also be compared to that of the diuretic hormone Tenmo-DH_{37}.

The calcitonin-like peptide, Dippu-DH_{31}, isolated from the Pacific beetle cockroach *Diploptera punctata*, was the first peptide from the calcitonin-like peptide family to be identified in insects (Furuya et al., 2000). Dippu-DH_{31} increases fluid secretion in tubules of *D. punctata, Locusta migratoria, Schistocerca americana* (Furuya et al., 2000) and *Rhodnius prolixus* (Te Brugge et al., 2005). Using the antiserum to Dippu-DH_{31}, a Dippu-DH_{31}-like peptide has been localised in tissues from the blood-feeding bug *Rhodnius prolixus*, where it was shown to occur over much of the central nervous system, including the brain and CC, as well as in the hindgut and salivary glands (Te Brugge at al., 2005); this strongly suggests an hormonal role for a CT-like peptide in *Rhodnius.* Some CT-like peptides increase fluid secretion in *T. molitor* Malpighian tubules (see Chapter 2), so the localisation of a

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Dippu-DH_{31}-like peptide in this species would provide further evidence for another family of diuretic peptides in beetles. Little is known about CT-like peptides in general, and their role in beetle tubules has not previously been investigated. The localisation of a CT-like peptide in the mealworm would enable comparison with the distribution of the mealworm CRF-related peptide Tenmo-DH_{37}. Knowledge of the distribution patterns of two diuretic factors from different peptide families would provide more clues to their function and possible interaction in *T. molitor*.

The combination of diuretic and antidiuretic hormones in the same species should result in the sensitive regulation of fluid balance, as well as the recycling of fluid by the excretory system (Nicolson, 1991). Hence, the identification of the cells or tissues which might be a source of the factors Tenmo-ADFa and Dippu-DH_{31} would give a clearer indication of their respective physiological functions in beetle excretion and would certainly add to future studies on how each (putative) hormone contributes to water and ion movement in Coleoptera.

Material and Methods

Animals

Tenebrio molitor was maintained under crowded conditions at room temperature. The larvae and adults were kept on oats/bran and regularly given apple or raw potato as a source of moisture. Each life stage (adult, pupa, larva) was maintained separately.

Drugs and peptides

Tenmo-ADFa and Dippu-DH_{31} were provided by D.A. Schooley (Department of Biochemistry, University of Nevada, Reno). All other substances were purchased from Sigma.

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Antiserum development

Initially, an antiserum for Tenmo-ADFa was raised in rabbits using [Cys-0]ADFa-amide, but this conjugate failed to induce an immune response (Schooley, personal communication). A second conjugate, [Cys-15]ADFa-amide, was prepared in order to present the other end of the molecule to the immune system, and this second antiserum was used in the immunohistochemical experiments of this study. Two rabbits, designated A and B, were immunised over a period of 3 months.
The development of the antiserum to Dippu-DH_{31} has been described in detail elsewhere (Te Brugge et al., 2005). The antiserum used in this study was a gift from D.A. Schooley (University of Nevada, Reno, USA).

Dot immunobinding assay (dot blot)

A dot blot is performed to characterise the antisera, that is, to ascertain if antibodies to the peptide concerned were produced in the rabbit, and/or to confirm that the antiserum recognises the pure peptide. The dot blot also gives an indication of the sensitivity and specificity of the antiserum. The standard dot blot protocol used is described below.

The synthetic peptide was dissolved in 10% acetonitrile containing 0.1% trifluoroacetic acid (TFA) to obtain a concentration of 1 µg/µl, which served as the first concentration in a 1:10 dilution series (to 10^{-7} µg/µl). The solution in each Eppendorf® vial was mixed thoroughly after each dilution step using a vortex.

Nitrocellulose membrane paper was cut into strips (approximately 60 x 5mm), taking care not to touch the membranes with bare hands. Sections of about 5 mm were marked off with a pen. One to two µl of each peptide concentration was dropped onto the membranes. The membranes were then wrapped in aluminium foil and baked at 120°C for 30 minutes. In order to reduce background staining, the membranes were placed in a solution of 2% skimmed milk (2 g milk powder in 100

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ml 50 mM Tris-buffered saline (TBS), containing 0.1% Triton-X-100® (see Appendix) (2 h gentle agitation at room temperature).

The experimental strips were placed in a Petri dish to which the primary antiserum was added at a dilution of 1:500 in 50 mM TBS containing 2% skimmed milk. (In the case of Tenmo-ADFa, control strips were placed in a Petri dish containing the pre-immune serum at the same dilution). The Petri dishes were placed on a flatbed shaker to ensure even spread of the antisera and incubated overnight. The nitrocellulose membranes (experimental and control kept separately) were once again rinsed thoroughly with 50 mM TBS (3 rinses of 5 min each), before being incubated with peroxidase-conjugated goat anti-rabbit IgG (GAR). GAR was added at a dilution of 1:500 in 50 mM TBS for 30-40 min. The membranes were rinsed again with TBS (3 x 5 min) and then transferred to Tris stock (diluted 1:10 in distilled water) for 5 min. Two to three drops of hydrogen peroxide (H_2O_2) and 0.025 g 3,3' diaminobenzidine (DAB) were added to 200 ml Tris stock (1/10 dilution). H_2O_2 was used as an enzyme substrate and DAB as chromogenic reagent. The membranes were placed in this solution and the colouring appeared within a few minutes.

Preparation of tissues for PAP-staining

The tissue to be examined was dissected out under Nicolson's (1992) Ringer's solution (Appendix) and transferred to 10% Bouin-Hollande's sublimated fixative (Appendix) for 18-24 h. The tissues were then thoroughly rinsed with distilled water for 12 h. Before fixation in paraffin, the tissues were dehydrated by means of an ethanol series (70%, 95%, 100% ethanol for 2 h each, changing once) followed by 2 h in 50% histosol/50% ethanol and 2 h in 100% histosol.

The tissues were then transferred to 50% histosol / 50% paraplast and kept overnight at 56°C. The following day, they were embedded in a mould of pure paraplast and returned to 56°C for a further 24 hours. Once removed from the heat,

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the moulds were placed in a cold waterbath to cool and harden the paraffin. Cold water ensures that the paraffin hardens rapidly, thus preventing the formation of crystals, which can damage the tissue.

Cutting

The tissue moulds were attached to a wooden block fixed onto an LKB-2218 Historange microtome. The angle of the block was adjusted manually according to how the tissue should be cut. Ovalbumine glycerine solution (Appendix) was used to bind the tissue to the glass slides.

Sections of 4 µm were cut with a microtome using glass knives. The tissue sections were collected from the distilled water in the bath using a small paint brush. The slides were placed on a hot plate at a low heat to dry. Care was taken not to exceed 45°C because this could lead to breakdown of the peptides, resulting in no staining. Thereafter, the slides were placed in a drying cupboard (43°C) for a minimum of 48 h.

Immunohistochemical methods

a) Indirect immunoperoxidase peroxidase method (IIPO)

This protocol was carried out over two consecutive days.

Day 1: The slides were de-paraffinated by placing them in 100% histosol (2 x 5 min) followed by 2 x 5 min in 100% ethanol and 1 min in distilled water. The sublimate was removed by incubating the slides in lugol (2 x 2 min), 5% Na thiosulphate (2 min) and distilled water (1 min). The slides were then placed in TBS for 5 min.

To prevent the antiserum or reagents from streaming off the slides when applied, the back and sides of the slides were first dabbed with paper towels to remove

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excess TBS. The slides were then placed in a moist chamber, to which paper towels moistened with distilled water and TBS had been added. The TBS contains Triton X-100® (prepared as in the Appendix). This detergent is used to perforate the cell membranes and thus improve the penetration of the antiserum.

Firstly, the tissues (on both experiment and control slides) were incubated with pre-immune goat serum (PIG), at a dilution of 1:5, for a period of 45 min. PIG reduces aspecific binding by saturating non-specific binding sites, thereby minimising background staining. The moist chamber was placed on a flatbed shaker to ensure even distribution of PIG on the slides. Between steps, the slides were rinsed thoroughly with TBS for not less than 5 min, replacing the TBS at least 4 times. After each rinse, excess TBS was removed from the slides with paper towels as described above.

The antiserum (primary antibody) was usually used at a dilution of 1:500 in 0.01 M TBS. 0,5 ml of antiserum dilution was placed on each experimental slide and left in the moist chamber for 18-20 h. Incubation with the primary antibody results in the formation of an antigen-antibody$_1$ complex i.e., the primary antibody binds to the corresponding peptide in the tissue.

For Tenmo-ADFa, serum from pre-immune rabbit (PIR) was used as a control. For Dippu-DH$_{31}$, a pre-immune serum was not available, but Tenmo-DH$_{37}$ was used as a positive control to safeguard the protocol. The PIR contains no specific antibodies, since this blood was drawn from the rabbit before the rabbit was immunised with the peptide-conjugate [Cys-15]Tenmo-ADFa-amide. Therefore, there should be no antigen-antibody interaction in the tissue on the control slides and so we expect no colouring to appear. However, if colouring does appear, it is considered to be aspecific. If aspecific staining occurs in the control, then the results would be inconclusive.

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Day 2: Following a thorough rinse with TBS (as described above), the slides were incubated with 0,5 ml goat anti-rabbit (GAR) peroxidase-conjugated secondary antibody (1:30 dilution) for about 30 min. GAR binds to the primary antibody, thereby forming and antibody$_1$-antibody$_2$ complex. The slides were then rinsed with TBS for 5 min. Excess TBS was removed with paper towels before applying the PAP in a 1:300 dilution (0.5 ml on each slide). Incubation with PAP results in an antibody$_2$-PAP complex. In this way the secondary antibody functions as a bridge between the primary antibody and the PAP-complex.

The slides were rinsed thoroughly with TBS and excess TBS was once again wiped away with paper towels. The slides were placed in Tris stock for 5 min. The staining was brought about in 200 ml Tris stock (1/10 dilution) with 25 mg DAB and 20 µl of a 30% H_2O_2 solution. In the presence of peroxidase and H_2O_2, DAB changes into a brownish-coloured insoluble polymer, thus highlighting the location of the antigen (i.e. Tenmo-ADFa) in the tissue. Staining became visible after 2-10 min, after which the slides were immediately taken out of the DAB-H_2O_2 solution and rinsed with distilled water. (If left for too long, the tissues stain too darkly for specific staining to be observed). The tissue on the control and experiment slides was then examined under a light microscope to inspect for staining before dehydration (3 x 5 min in 100% ethanol and 3 x 5 min in 100% histosol). The slides were covered with Depex and a cover slip and then left to dry.

b) Whole mount immunofluorescence

Five days were required for the completion of the whole mount protocol.

Day 1: Nervous tissue and midgut were dissected out under Ringer's solution and transferred directly to clear glass tubes containing approximately 2.5 ml of 2% paraformaldahyde in 10 mM phosphate-buffered saline (PBS) (Appendix). This was made by adding 2 g of paraformaldahyde to 100 ml 10 mM PBS and stirring the solution while heating to 60°C. (The solution must change from milky opaque

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to transparent). Fresh paraformaldehyde solution was made weekly and stored in a fridge at 4°C. While dissecting, the tubes were kept on ice to keep the solution cool and afterwards kept at 4°C overnight.

Day 2: The tissues were rinsed in cold PBS for 4-5 h, changing the PBS every 30 min. This was done by sucking the fluid out with a pipette, taking care not to discard tissues with the solution. The PBS was then replaced with a solution of 4% Triton-X-100®, 2% normal porcine serum (NPS) and 2% bovine serum albumin (BSA) in PBS and left overnight on a flatbed shaker. NPS and BSA reduce aspecific binding.

Day 3: The solution was replaced with 0.4% Triton-X-100®, 2% NPS and 2% BSA in PBS. The primary antibody was added at a dilution of 1:500. The tubes were placed on a shaker bed for 24 - 72 hours.

Day 4: The tissues were rinsed for 4 to 5 hours in cold PBS, which was replaced every half hour. The secondary antiserum, FITC (fluorescein isothiocyanate)-conjugated swine anti-rabbit immunoglobulins diluted 1:20 in PBS, was added in the dark room. The tubes were wrapped in aluminium foil to prevent exposure of the fluorescent agent to light, and placed overnight at 4°C on a shaker bed.

Day 5: From this point on, the protocol was carried out in the dark room, so as to prevent the breakdown of FITC. The tissues were rinsed twice with cold PBS (2 x 10 min) and placed on a clean plastic Petri dish. From here, each tissue was transferred to a glass slide, using forceps. A drop of 90% glycerol containing 0.1% para-phenylenediamine (PPD) - an anti-fading agent, applied to retard the degradation of FITC and thus prolong the life of the fluorescence - was placed on the slide and then covered with a cover slip. Clear nail varnish was used to seal the cover slip onto the slide. Each slide was wrapped in a piece of paper towel and then in aluminium foil and stored at -20°C. The tissues were checked for staining with a conventional fluorescence microscope.

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As a positive control, the whole mount protocol was also carried out using antiserum raised against Tenmo-DH_{37}. The results obtained were compared with those of Wiehart *et al* (2002b).

Mass spectral analysis of Tenmo-ADFa

Mass spectral analysis of mealworm nervous tissue was performed on a matrix-assisted laser desorption ionisation-time-of-flight (MALDI-TOF) mass spectrometer. Brains, corpora cardiaca (CC) and ventral nerve cords (VNC) were analysed separately. The tissues were dissected out under Ringer and transferred to a solution of methanol/H_2O/acetic acid (90:9:1) in Eppendorf® tubes placed on ice. Each tissue type was placed in a separate tube and left in this solution overnight at 4°C. The tissues were kept cool at all times to prevent enzymatic breakdown. The following day, the tubes were placed in a sonication bath to homogenise the tissues (3 min) and then centrifuged (9000 g for 10 min). The supernatant resulting from centrifugation was dried in the SpeedVac. 50 µl 0.1% TFA was added to the sample, which was then placed in a sonication bath for 1 min in order to redissolve the peptides.

A Ziptip C_{18} column was activated with 50% acetonitrile and 0.1% TFA (3 times 10 µl) and washed three times with 10 µl of 0.1% TFA before the sample was loaded onto the column, followed by another wash (3 times 10 µl 0.1% TFA). Peptides were eluted from the column with 4 µl of 70% acetonitrile and 0.1% TFA. 1 µl of the sample was placed on the MALDI multi-sample target plate and mixed with 1 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid in acetone. The mixture was allowed to air-dry before the target was introduced into the instrument, a Reflex IV (Bruker daltonics). The instrument was calibrated using a standard calibration mixture (Bruker daltonics).

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High performance liquid chromatography (HPLC) of Dippu-DH$_{31}$

Using a combination of reversed-phase high performance liquid chromatography (HPLC) and dot blot, peptide extracts of approximately 36 brains and VNC from *T. molitor* larvae were analysed for the presence of a Dippu-DH$_{31}$-like peptide. After dissection, tissues were placed directly into a solution of methanol/water/acetic acid (90:9:1). Pre-purification steps are necessary to remove impurities, such as fats, that would otherwise bind irreversibly to columns.
After homogenisation and centrifugation, the supernatant was dried by vacuum centrifugation (in the SpeedVac). The sample was then redissolved in 0.1% TFA in MilliQ water. Ethylacetate and hexane were added successively to remove lipids and fatty acids. The crude extract was then passed through an activated Sep-Pak C_{18} column (Waters). The column was activated using 10 ml 60% acetonitrile + 0.1% TFA and rinsed with 10 ml 0.1% TFA. The sample was loaded onto the column, and after rinsing again with 10 ml 0.1% TFA, the peptides were eluted with 60% acetonitrile + 0.1% TFA. This sample was then dried in the SpeedVac.

The dried sample was resuspended in 0.1% TFA and applied onto a Waters Symmetry C_{18} column (250 mm by 4.6 mm; 5µm particle size, 10 nm pore size). The linear gradient ran from 2% solvent B, 98% solvent A to 100% B over 60 min (solvent A being 0.1% TFA and solvent B being 60% acetonitrile with 0.1% TFA). The flow rate was 1ml/min and elution was monitored at 214 nm. Fractions of 1ml were collected automatically. Antisera to Dippu-DH$_{31}$ and Tenmo-DH$_{37}$ were used in a dot blot to monitor the HPLC fractions.

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Results

Dot Blots

a) Tenmo-ADFa

The first two dot blots were performed using [Cys-15]ADFa-amide (which was used to immunise the rabbits). The dot blot tests showed that the antisera from Rabbits A and B react with [Cys-15]ADFa-amide at peptide concentrations of 1 μg/μl and 0.1 μg/μl, and that the antiserum from each rabbit appeared to have similar sensitivity (Fig. 2). After obtaining no results in the IHC experiments (see below), another dot blot was carried out with synthetic Tenmo-ADFa. The antiserum reacted with the pure peptide at a concentration of 1 μg/μl only. Aspecific staining was also found at this concentration. Concentrations lower than 1 μg/μl showed no reaction.

The dot blot indicates that the antiserum is not very sensitive to either [Cys-15]Tenmo-ADFa-amide or Tenmo-ADFa. There is also some doubt about its reliability due to the appearance of aspecific staining in the control (using the pre-immune rabbit serum).

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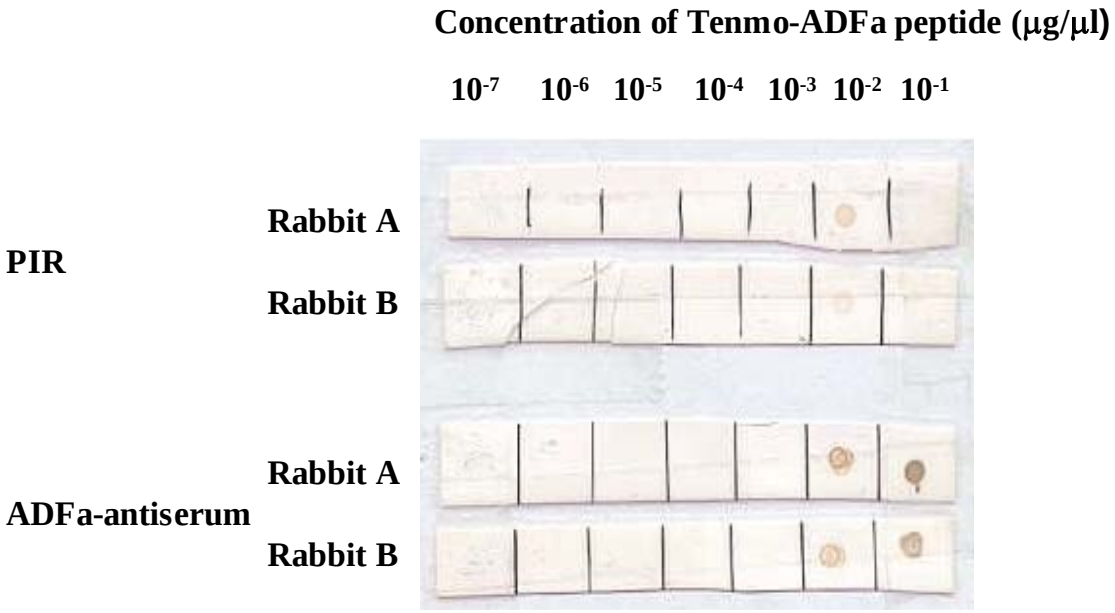

Fig. 2. Dot blot showing the reaction of anti-Tenmo-ADFa (1:500) to [Cys-15]ADFa-amide. Antisera from Rabbit A and B have similar sensitivity but only at the two highest concentrations of peptide, indicating low sensitivity of this antiserum to its antigen. The pre-immune serum also reacts with the peptide, indicating a lack of specificity of the antiserum.

b) Dippu-DH$_{31}$

Initially, an affinity purified antiserum was tested at 1:1000 and 1:500 but did not produce any dots in the dot blot assay. Antisera are usually stable for some time at room temperature (D. Nässel, personal communication) but it is possible that some degradation occurred at room temperature during transportation from the USA.

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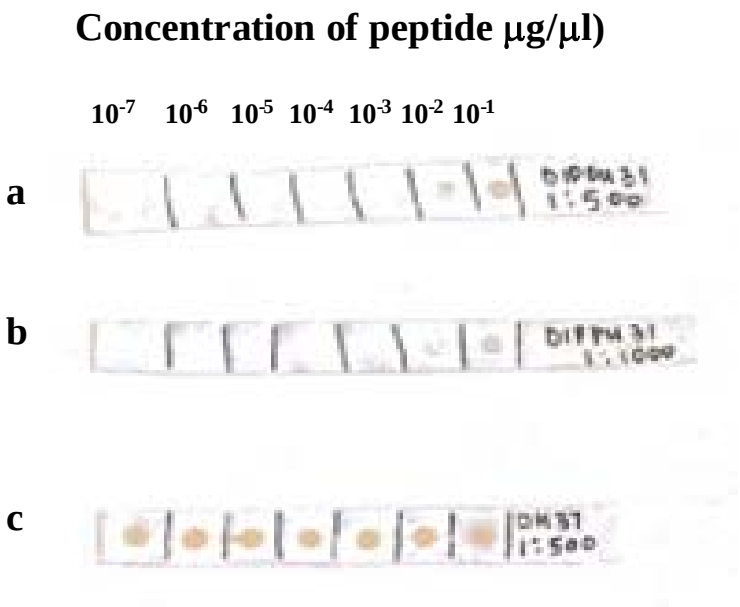

Fig. 3. Dot blot showing the reaction of anti-Dippu-DH_{31} (a) at 1:500 and (b) at 1:1000 to synthetic Dippu-DH_{31}. Dippu-DH_{31} antiserum recognises the peptide at high concentrations only, while the antiserum to Tenmo-DH_{37} (1:500) (c) used as a control recognises pure peptide at concentrations as low as 10^{-7} μg/μl, revealing the very high sensitivity of this antiserum.

Another antiserum, purified and lyophilised, was tested at a dilution of 1:2000. This one was reported to be sensitive at a dilution of 1:4000 (D.A. Schooley, personal communication) but this too did not produce any staining in the dot blot, even after increasing the concentration to 1:500. A third antiserum (affinity purified) in glycerol (1:1) did produce some staining at the two highest concentrations (Fig. 3). Tenmo-DH_{37} (1:500) was used as a control, producing staining from 1μg to 10^{-7}μg.

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Immunohistochemical methods

a) IIPO

For Tenmo-ADFa, brains (with CC intact), VNC, suboesophageal ganglia (SOG), prothoracic ganglia and midguts from larvae, and brains and VNC from adults were used for the IIPO protocol. No staining was found in any part of the nervous tissue or in the midgut. For Dippu-DH_{31}, brains, VNC and midguts from larvae were used, with no staining in any of these tissues.

b) Whole mount

For Tenmo-ADFa, brains, VNC and SOG from adults, pupae and larvae, and midguts from larvae, were used for whole mount staining. No staining using this method was found in any part of the nervous tissue or in the midgut. There was some degree of background staining.

Some modifications to the whole-mount method were attempted. The correct fixation technique is crucial to preserve antigenicity and the duration of fixation depends on the fixative agent used and size of the tissues. Buffered 2% paraformaldehyde is generally used as a fixative and has proven to be successful, but after no staining in this study, paraformaldahyde was replaced with 2% glutaraldehyde.

Furthermore, experiments were conducted in which the concentration of antiserum was increased from 1:500 to 1:200 and also 1:100, and antisera from both rabbits A and B were used. However, even after implementing these changes, no staining was observed. During the course of the study, mealworms were regularly provided with a source of moisture. In a final effort to obtain staining, they were starved of their moisture source for three weeks in order to increase the amount of antidiuretic factor in the nervous system. However this was also unsuccessful.

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For Dippu-DH_{31}, brains, VNC and midguts of mealworm larvae were used. No staining was found in any of the tissues, using any of the antisera (Fig. 4a). The final antiserum in glycerol, despite having a result in the dot blot, did not produce any staining in the tissues. In all cases, the antiserum to Tenmo-DH_{37} was used as a control and results are shown in Fig. 4b.

For experiments testing for the presence of Dippu-DH_{31}-like material, some modifications to the protocol were made in an attempt to obtain staining. The tissues were placed in 4% paraformaldehyde for 5 h after dissection and instead of NPS, goat serum was used. The lyophilised antiserum to Dippu-DH_{31} was used at a concentration of 1:500, and the fluorescent secondary antibody FITC was substituted with Alexa-88 goat anti-rabbit secondary antibody. Antiserum to Tenmo-DH_{37} was used as a control, with the same adjustments to the protocol (results not shown).

In the control experiment using the antiserum to Tenmo-DH_{37}, VNC and brains from larvae were tested. When viewed under the fluorescence microscope, the results obtained matched exactly with those of Wiehart *et al*. (2002b), showing that there was no problem with the protocol or reagents used.

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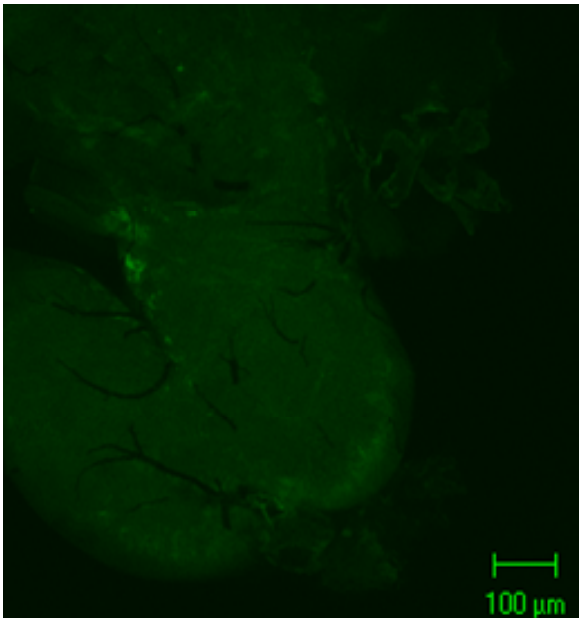
a



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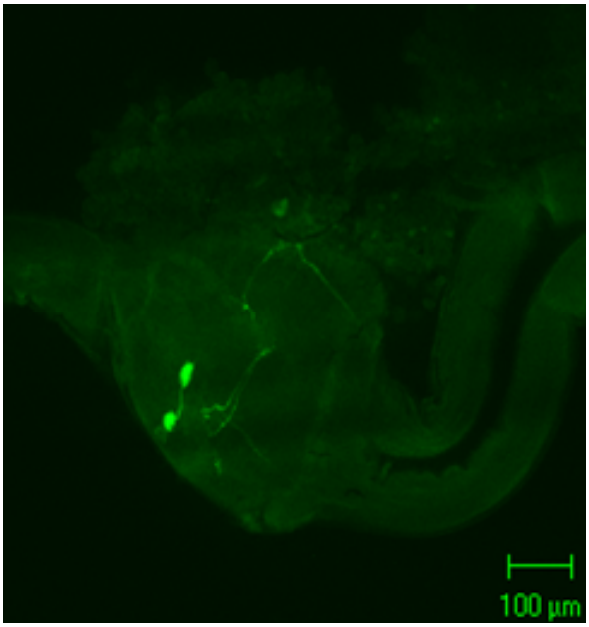

c

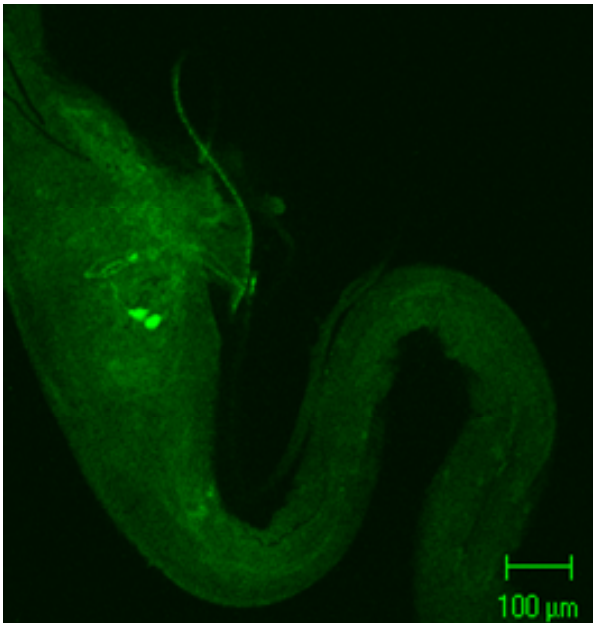

Fig. 4. Whole mount immunohistochemistry with *Tenebrio* tissues viewed by confocal microscopy (Zeiss LSM510 META confocal microscope; excitation with 488 Argon Ion laser. For FITC, excitation = 494 nm and emission = 518 nm.) (a) Section of mealworm VNC treated with anti-Dippu-DH_{31} with no immunoreactivity visible. (b,c) Control using antiserum to Tenmo-DH_{37}, showing immunoreactivity in two bilateral cells in abdominal ganglia of the ventral nerve cord.

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Mass spectrometrical analysis for Tenmo-ADFa

MALDI-TOF mass analysis was performed on brains, CC, VNC and SOG from larvae, pupae and adults but did not reveal a mass corresponding to that of Tenmo-ADFa in any of the tissues. Several other masses were observed in all tissues, but these did not correspond to the theoretical mass of any known *Tenebrio* peptides (Fig. 5). [Cys-15]Tenmo-ADFa-amide and Tenmo-ADFa were also subjected to MALDI-TOF MS. This confirmed their mass and therefore presence in Eppendorf® vials. MALDI-TOF MS analysis of the peptide used in the initial dot blots confirmed that it was [Cys-15]ADFa-amide (mass of 1643Da).

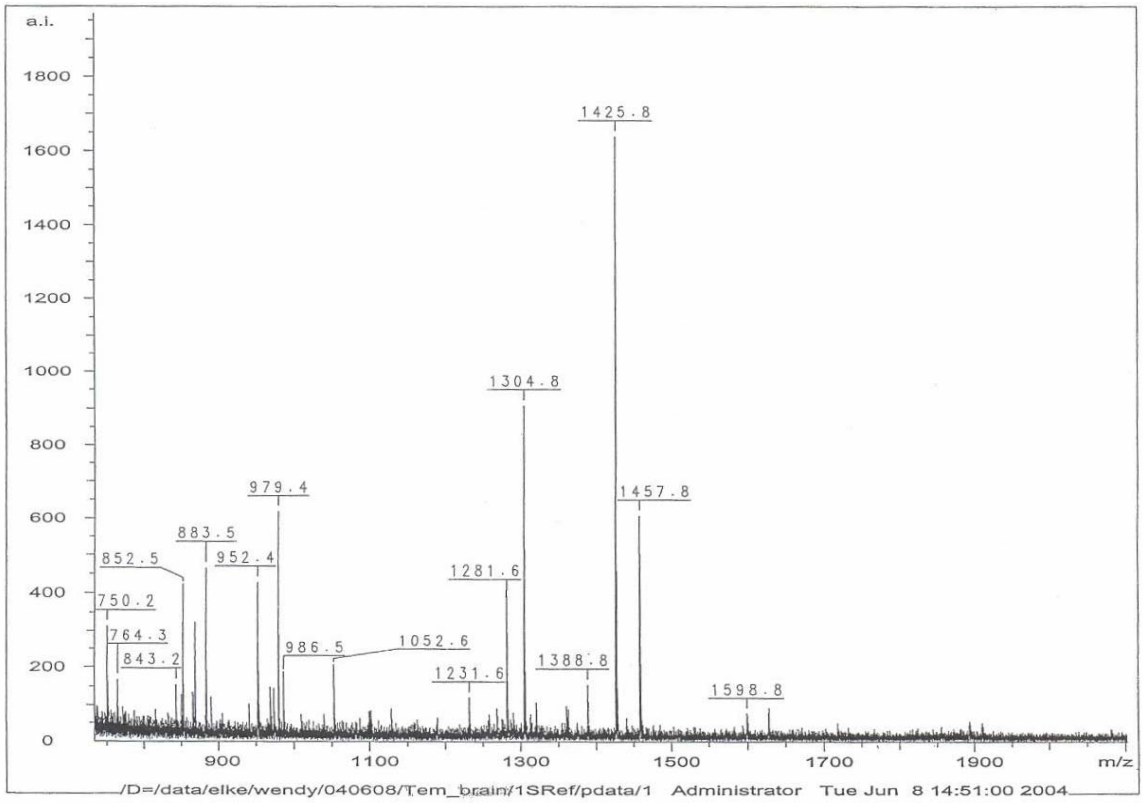

Fig. 5. Example of a mass spectrum obtained by MALDI-TOF MS analysis of *T. molitor* brains. No mass corresponding to that of Tenmo-ADFa (1541.8 Da) was present, but several masses of unknown peptides were revealed.

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HPLC of Dippu-DH$_{31}$

High performance liquid chromatography was performed on nervous tissue from mealworms in an attempt to detect a Dippu-DH$_{31}$-like peptide in crude tissue. Activity was spread over a limited number of fractions (Fig. 6) The HPLC spectrum (214 nm) (Fig. 6) showed no absorbance where Dippu-DH$_{31}$ should elute.

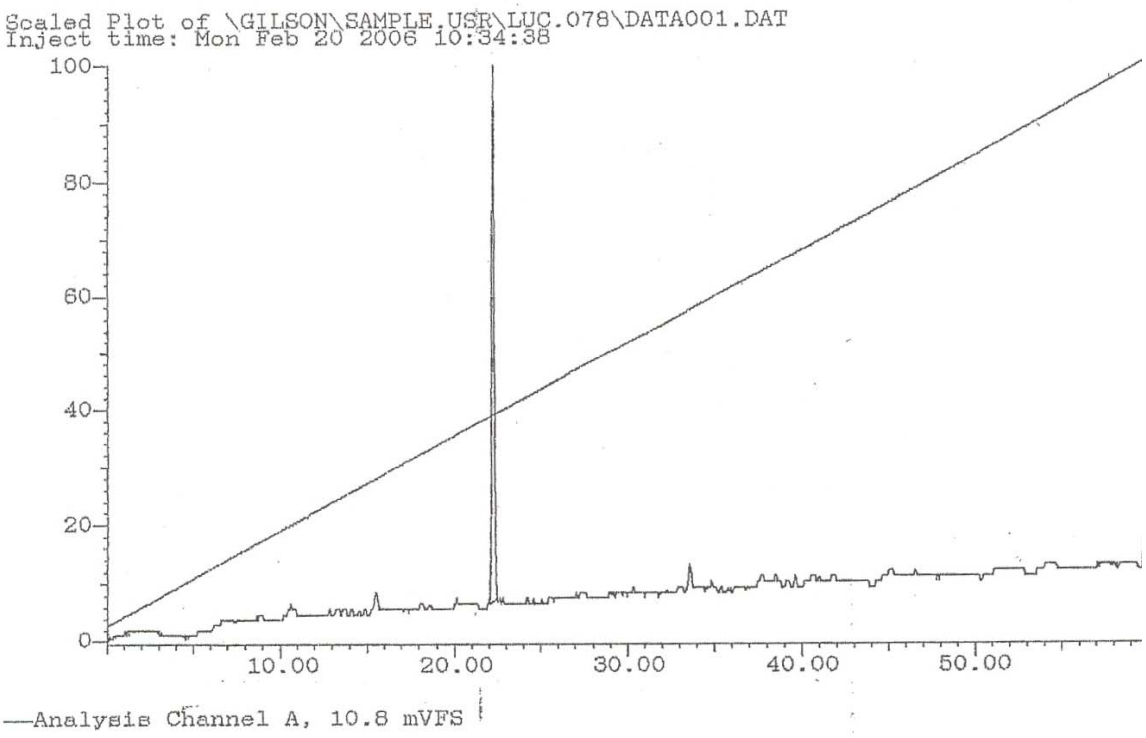

Fig. 6. Chromatogram from the separation of 36 *T. molitor* ventral nerve cords (including brain and CC) by RP-HPLC (Waters Symmetry C$_{18}$). One prominent peak eluted at 22 minutes. 60 fractions of 1 ml each were collected and each was tested in a dot blot for immunoreactivity to anti-Dippu-DH$_{31}$ and anti-Tenmo-DH$_{37}$.

In the dot blot using all the fractions, the antiserum to Dippu-DH$_{31}$ did not recognise anything from the HPLC product, but also did not recognise pure Dippu-DH$_{31}$ (not shown). In a separate dot blot, the antiserum to Tenmo-DH$_{37}$ was also applied to the fractions (Fig. 7). Dots appeared at 7 fractions, indicating the

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presence of peptides that are immunoreactive to Tenmo-DH_{37} antiserum. These fractions corresponded to very small peaks on the chromatogram (Fig. 6). The prominent peak at 22 min did not contain immunoreactivity to Dippu-DH_{31} or Tenmo-DH_{37} antisera. Unlike the Dippu-DH_{31} antiserum, the antiserum to Tenmo-DH_{37} is a crude antiserum and so contains multiple other factors.

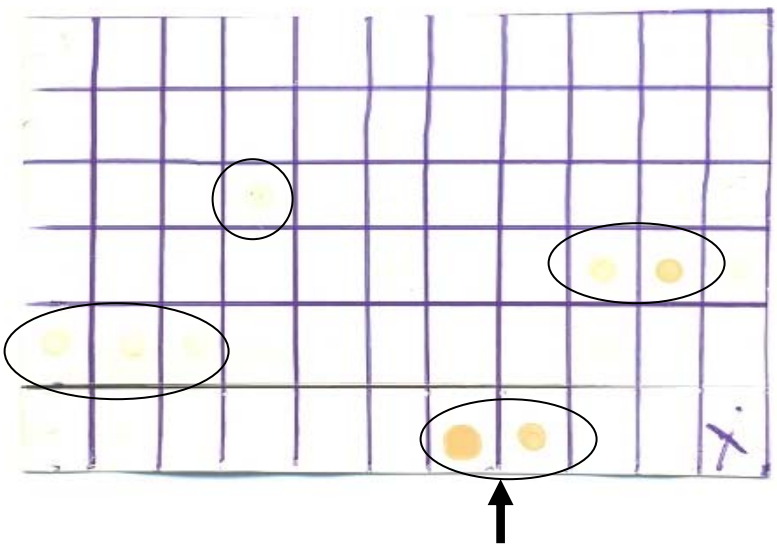

Fig. 7. Dot blot on fractions obtained by RP-HPLC. Several fractions exhibit immunoreactivity to the antiserum to Tenmo-DH_{37} (indicated by circles). Synthetic Tenmo-DH_{37} (10^{-1} to 10^{-2} µg/µl) was applied at blocks indicated by the arrow.

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Discussion

Attempts to localise Tenmo-ADFa in mealworm nervous tissue

Tenmo-ADFa appears to be low in antigenicity, indicated by the failure of the first conjugate to induce an immune response in rabbits (D.A. Schooley, personal communication). The low reactivity between Tenmo-ADFa and its antiserum was evident in the dot blot, where the antiserum reacted with [Cys-15]ADFa-amide and the naturally occurring peptide Tenmo-ADFa only at very high concentrations.

The antigenicity of the molecule (with carrier protein) depends on specific epitopes or antigenic determinants. In the case of Tenmo-ADFa, the only region of the peptide that is antigenic (i.e. able to induce an immune response), is residues 4-7. All other amino-acid residues score negatively in MacVector sequence analysis (D.A. Schooley, personal communication), indicating that the Tenmo-ADFa molecule on the whole has very poor antigenicity.

An antiserum that is produced by regular immunisation is termed a “polyclonal” antiserum, because it will contain a host of antibodies directed against different sections of the antigen (Nässel, 1996). Affinity purification may be an option for the polyclonal antiserum raised against Tenmo-ADFa, as this would extract the antibodies from the crude serum and remove unwanted proteins and immuno-globulins. For a reliable antiserum, it is crucial to use antigens that induce a good immune response (Nässel, 1996), but due to the low antigenicity of Tenmo-ADFa, hence its inability to produce a high titre of specific antigens in the serum, even affinity purification may not be effective.

In further attempts to obtain staining, the concentration of antiserum was increased, but this constitutes only a limited change to the protocol because if the concentration is too high (above 1:100), the formation of bridges between the

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primary and secondary antibodies is incapacitated (Bigbee et al., 1977; Nässel, 1996).

The use of mass spectrometry to detect Tenmo-ADFa in mealworm tissues

Mass spectrometry (MS) is an analytical tool that enables the selective and sensitive detection of peptides and proteins at very low levels, even in single organs of insects (Clynen *et al*., 2001). MS can be used to confirm what is revealed by immunohisto-chemistry, which often suffers from cross-reactivity. This means that the antiserum cannot always differentiate between structurally related peptides. However, mass spectrometry provides an exact mass measurement, making it possible to distinguish between peptides with only one amino acid difference. In this study, MS was used in an attempt to screen for Tenmo-ADFa in tissues of the nervous system of *T. molitor*. In MALDI analysis, the analyte is mixed and co-crystallised with a saturated solution of ultraviolet-absorbing matrix, such as α-cyano-4-hydroxycinnamic acid. Laser radiation of this analyte-matrix mixture results in the vaporisation of the matrix which absorbs the light and causes the analyte to vaporise. This leads to ejection of the analyte peptide's ions into a vacuum flight tube. The m/z value of the ejected ions - which is identical to their mass, as z is typically +1 in MALDI ionisation - is determined as a function of their flight time to the detector. The final output is a mass spectrum, showing peaks which indicate the precise mass of the peptides.

MALDI-TOF MS was used to reveal the presence of Locmi-DH_{46} in the pars intercerebralis and CC of locusts (Clynen *et al*., 2001), but failed to locate Tenmo-ADFa in mealworm tissues. Again, this could be explained by the structure of the peptide. Tenmo-ADFa has no Lys or Arg residues that would enhance positive ionisation and give a high sensitivity response. The only positively charged residues (ionic sites) in the Tenmo-ADFa molecule are two His residues and the alpha-amino group, making Tenmo-ADFa a poor analyte for MALDI-TOF MS (D.A. Schooley, personal communication).

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Attempts to localise a Dippu-DH$_{31}$-like peptide in mealworm nervous tissue

Although the immunohistochemical techniques used in this study failed to localise a Dippu-DH$_{31}$-like peptide in the nervous system of the mealworm, the absence of immunolabelling does not necessarily indicate that a CT-like peptide is not present in *Tenebrio*. CT-like peptides have been found in all insects screened so far. Two CT-like peptides (Anoga-DH$_{31}$ and Bommo-DH$_{31}$) increase secretion rates in *Tenebrio* tubules (Chapter 2), although they cause relatively small increases in secretion rates. This is consistent with findings in other insects (*Diploptera*, *Locusta*, *Rhodnius* and *Anopheles*) where the increase was significant but never maximal (Furuya et al., 2000; Te Brugge et al., 2005; Coast et al., 2005). Surprisingly, Dippu-DH$_{31}$ did not increase fluid secretion in *T. molitor* (Chapter 2). However, the ability of Dippu-DH$_{31}$ to stimulate fluid secretion in Malpighian tubules may not be its main role (Te Brugge et al., 2005), but it may participate in other activities, such as affecting ionic composition.

Interestingly, although a CT-like peptide has not yet been isolated from a beetle, one was identified in another tenebrionid during a partial genome sequence (using bio-informatical tools) of *Tribolium castaneum* (D.A. Schooley, personal communication). Trica-DH$_{31}$ and Dippu-DH$_{31}$ differ from each other at only two residues (Fig. 8). One might expect that a CT-like peptide from *Tenebrio* would strongly resemble that of its close relative, *T. castaneum*, and therefore also Dippu-DH$_{31}$.

It is thus unlikely that the structure of the *T. molitor* CT-like peptide differs so much from that of Dippu-DH$_{31}$ that it cannot be recognised by the Dippu-DH$_{31}$ antiserum. The antiserum to Dippu-DH$_{31}$ was tested for cross-reactivity with other factors, including serotonin, a kinin, *Manduca sexta* cardioacceleratory peptide, as well as Dippu-DH$_{31}$ and calcitonin-like peptides from chicken and *Drosophila* (Drome-DH$_{31}$) (Te Brugge et al., 2005). There was no measurable cross-reactivity

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Fig. 8. Sequence alignment of calcitonin-like peptides with Dippu-DH_{31}. Structures of chicken, Japanese eel, human, pig, rat and salmon CT-like peptides are shown, as well as insect CT-like peptides encoded in the genomes of *Tribolium castaneum, Drosophila melanogaster, Anopheles gambiae, Bombyx mori* and one partial sequence isolated from *Formica polyctena.* Apime-DH_{31} from *Apis mellifera* = Dippu-DH_{31} and Aedae-DH_{31} from *Aedes aegypti* = Anoga-DH_{31}. Red is conserved in the calcitonin family and blue is conserved in the insect CT-like diuretic peptides (and homologues). (Reproduced with permission from D.A. Schooley.)

with anything but the CT-like peptides, indicating that this antiserum is specifically immunoreactive, but also that there is a high degree of cross-reactivity with other CT-like peptides.

The staining found by Te Brugge et al. (2005) of the Dippu-DH_{31}-like peptide in *Rhodnius* was reported to be variable between preparations, with more pronounced immunoreactivity in unfed insects. If this peptide is a circulating neurohormone with a diuretic function, it may be released after feeding, and so be present in lower concentrations in storage organs and throughout the nervous system. In all preparations with mealworm nervous tissue, animals were well-fed and relatively hydrated, possibly reducing the amount of stored CT-like peptide in the nervous tissue.

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Furthermore, the failure of the antiserum to produce dots in the dot blot shows that the antiserum was not reliable in detecting the pure peptide Dippu-DH_{31}. If the antiserum does not recognise the pure peptide in this preliminary test, it usually indicates that there is something amiss with either the peptide used or with the antiserum. However, for the experiments conducted in this study, positive controls were set up, rendering perfect results. The Dippu-DH_{31} antiserum was reported to have good stability – after all, antibodies circulate in our own bodies at temperatures of 37°C so should not degrade readily at room temperature.

Most perplexing is that immunostaining has been found using this antiserum in both *Manduca sexta* (D.A. Schooley, personal communication) and *Drosophila melanogaster* (Dick Nässel, personal communication). Dippu-DH_{31}-like material was present in the central nervous system, but only very weak staining was observed in the brains of both insects. It is possible that the antiserum is not very effective in holometabolous insects, where staining in the brain is very faint. It could be that antigens in the tissue are not accessible to the antibodies because they are masked by different proteins. It is however very likely that a Trica-DH_{31} homologue or similar peptide is present in *Tenebrio,* making the failure to produce staining in the nervous tissue of this insect using Dippu-DH_{31} antiserum rather puzzling!

HPLC of Dippu-DH_{31}

HPLC techniques are used to analyse the peptide composition of nervous tissue and has become a vital step in the isolation of neuropeptides. The success of HPLC depends on the abundance of the peptide in the tissues and also the quantity of the tissue source used. In this study the amount of peptide isolated may have been minute. Also, a loss of immunoreactive AVP-like insect diuretic hormone on passage of extracts through Sep-Pak C_{18} cartridges (Schooley et al., 1987) has been reported, and such losses would be higher for larger peptides such as Dippu-DH_{31} (Coast et al., 2002).

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Troubleshooting

Immunohistochemistry is but the first step towards classifying a peptide as a hormone. While major advances in immunohistochemical techniques have taken place over the last few years, when attempting to localise a new peptide in a new insect species for the first time, one may encounter frustrating methodological problems. These may occur at any of a number of steps, including antiserum production, fixation conditions and the detection system employed. Consequently, troubleshooting should include investigating each step of the protocol, since optimisation of the immunohistochemical methods may be necessary for the antiserum in question as well as for different neuroactive compounds in different tissues and different insect species (Nässel, 1996). It is therefore pivotal to have access to original descriptions of the production and characterisation of each antiserum and the first insect applications.

Affinity purification serves to enhance the specificity of the antiserum, but may lower the titre of antibodies present, because antibodies with the highest affinity for the antigen cannot be dissociated from the antigen and remain on the column during purification steps, producing an antiserum with lower antibody titre and therefore lower affinity. This may be the case with the Dippu-DH_{31} antiserum.

When tested in model systems *in vitro* (such as the dot blot), antibodies may seem highly specific to the antigen used at immunisation. Antisera to neuropeptides often behave differently in model systems and fixed tissue so the specificity that is characterised in nitrocellulose membrane may not indicate accurately how the antiserum behaves in the actual tissues. Hence, one has to rely on several independent test methods for the reliable identification of antigens in tissue. In this study, the antiserum to Dippu-DH_{31} did not work in the dot blot but despite this, it was felt that it would be worth trying out in tissue, since the antiserum may behave differently in the actual tissue.

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The fixation protocol is essential to preserve antigenicity and may need to be adjusted for the antigen to be studied. The antiserum dilution should be thoroughly tested: if the antibody titre is too high, it may hamper the formation of bridges between primary and secondary antibodies, but if it is too low, only very weak immunolabelling will be produced. It is also important to optimise the titre of secondary antiserum. However, since the control slides gave a positive result in accordance with previous findings for Tenmo-DH_{37} (Wiehart et al., 2002b), it is unlikely that the problem lay with the reagents or the specimens in this study. The protocols used here have been established for numerous other peptides including Tenmo-ADFb in *Tenebrio* (Eigenheer *et al*., 2003) and Dippu-DH_{31} in *Rhodnius* (Te Brugge et al., 2005).

Since the immunohistochemical localisation of both Tenmo-ADFa and Dippu-DH_{31} failed, the physiological function of these peptides *in vitro* remains to be clarified. The successful immunohistochemical localisation of a second antidiuretic factor and a CT-like peptide in *Tenebrio* in the future would help to distinguish the neurohormones that control excretory processes in beetles and their potential interactions. The co-localisation of diuretic and antidiuretic factors in *T. molitor* would substantiate the hypothesis that their release is co-ordinated (Wiehart *et al*., 2002b).

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APPENDIX: SOLUTIONS FOR IMMUNOHISTOCHEMICAL PROTOCOLS

Ringer's solution (Nicolson, 1992)

In mmol/litre:

90 NaCl, 50 KCl, 5 $MgCl_2$, 2 $CaCl_2$, 6 $NaHCO_3$, 4 NaH_2PO_4, 50 Glucose.

Store at 4-6°C

Bouin Hollande (sublimated fixative)

Solution A

25 g cupric acetate dissolved in 1 l distilled water

Add 40 g picric acid

Filter the solution

Add 100 ml 40% formaldehyde

Add 10 ml acetic acid (minimum 96%)

Solution B

Saturated $HgCl_2$ solution

For use:

Mix 9 parts A with 1 part B

Ovalbumine glycerine

2 g ovalbumine dissolved in 100 ml water

Add 100 ml glycerine

Add a little menthol/camphor

Lugol

15 g iodide (I_2)

30 g potassium iodide (KI)

Add distilled water to 3 l

Preserve in the dark

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Tris stock solution

60.5 g trishydroxymethylaminomethane (Tris) dissolved in 1 l distilled water

pH 7.6

Store at 4°C

DAB/H_2O_2 solution

200 ml Tris stock (diluted 1/10)

25 mg DAB

2 ml H_2O_2 (0.3%) added just before use

Tris-buffered saline (TBS)

30 g Tris

225 g NaCl

0.5 g NaN_3

Add water to 2.5 l

pH 7.6

Store at 4°C

For use:

Dilute 10 times and add 0.1% Triton X-100®

Phosphate-buffered saline (PBS)

0.345 g $NaH_2PO_4.H_2O$

1.33 g $Na_2HPO_4.2H_2O$

8.474 g NaCl

pH 7.2

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CHAPTER 4

CONCLUSION

Water balance of beetles

In diversity, adaptability and distribution, beetles are perhaps the most successful of all living creatures, and are in many ways ecologically and economically important to humans. This eminent group of animals formed the focus of the present study, which investigated different aspects of the role of diuretic factors in insect excretion. Insects can lose substantial proportions of their water content through evaporation and/or excretion and, interestingly, beetles represent the extremes in lethal water-loss values – 17% of body water in *Carabus* beetles and 89% in the semiaquatic *Peltyodytes muticus* (Benoit et al., 2005). Generally, beetle species adapted for life in xeric environments have low rates of water loss and possess physiological and morphological adaptations to maximise water retention. An exception to this is seen in the burying beetle *Nicrophorus marginatus* (Silphidae), which has naturally high rates of water loss, making it highly susceptible to death by desiccation (Bedick et al., 2006). The burying beetle overcomes this problem behaviourally, by limiting activity during the hottest hours of the day. A more typical example can be found in the spider beetles *Mezium affine* and *Gibbium aequinoctiale* (Ptininae), pests of stored products. In these insects, conservation of water is of primary importance, and water loss rates are so low that they are comparable to those of diapausing insects (Benoit et al., 2005). The Namib Desert tenebrionids, *Onymacris* in particular, are noted for their very low rates of evaporative water loss (Nicolson, 1990). The success of this genus in one of the driest environments on earth is attributable to the combination of various well-developed adaptations to minimise loss of cuticular, respiratory and excretory water (Nicolson, 1990). Flightlessness is another adaptation commonly encountered amongst dwellers of arid environments: the fusion of the elytra creates a subelytral cavity, and abdominal spiracles open internally into this air-filled space. In this

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way, water loss in species such as the Namib Desert tenebrionids (Nicolson, 1990) and spider beetles (Benoit et al., 2005) is retarded.

The Tenebrionidae is the most abundantly represented family in arid regions (Cloudsley-Thompson, 2001). Nevertheless, the Malpighian tubules of both *Onymacris plana* and *Tenebrio molitor* respond readily to neuroendocrine factors: fluid secretion in the tubules of *O. plana* is dramatically stimulated by diuretic hormones (Nicolson and Hanrahan, 1986), and the tubules of *T. molitor* are sensitive to diuretic peptides from two different peptide families. Termination of diuresis in these xeric insects is therefore vital to avoid potentially lethal dehydration. One way in which diuresis could be controlled is by rapid inactivation of the hormones or their effects. Nicolson and Hanrahan (1986) showed that the diuretic hormone in *O. plana* is destroyed by prolonged contact with haemolymph, although it is not known if this alone would be sufficient to limit diuresis. Alternatively, precise control of diuresis could be achieved by the coordinated release of antidiuretic hormones. The presence of two potent antidiuretic peptides in *Tenebrio* suggests interaction between the diuretic and antidiuretic factors in this species. However, while antagonistic action between Tenmo-DH_{37} and Tenmo-ADFb and between their second messengers has been demonstrated (Wiehart et al., 2002), nothing is known about the circulating levels of these different factors and what triggers their release.

The dung beetle *Onthophagus gazella* (Scarabaeidae) has very different habitat, dietary and energy requirements to those of *Tenebrio*. Dung beetles are K-strategists – they lay a few eggs at a time, each in an individual brood ball made of dung. The egg hatches and the larva develops and pupates inside the brood ball, from which it emerges as an adult beetle. *Onthophagus gazella* adults are strong fliers, which would be necessary to search for moist dung, a patchy food source. In contrast, mealworms lay many eggs at a time, fly only rarely, spend their entire life cycle in the same food source (dry cereal), and can survive long periods with minimal preformed water. Despite these differences, and the evolutionary distance

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between *Tenebrio* and *Onthophagus*, the mealworm diuretic hormone Tenmo-DH_{37} has a potent effect on dung beetle tubules. This is especially interesting when considering the unusual structure of *Tenebrio*'s CRF-related diuretic peptides. The high level of conservation in the primary structure of CRF-related peptides may be due to the importance of these peptides in overall basic functions, meaning that drastic evolutionary changes in the structure of these peptides across insect species would not be necessary. Water balance in dung beetles has not been investigated before, and this is the first study to explore the effects of diuretic peptides on the tubules of a scarabaeid.

Malpighian tubules and insect physiology

While Malpighian tubules are excellent material for the study of processes involved in fluid secretion, it is increasingly evident that they play a far bigger role in the insect's overall physiology than was previously supposed. Mechanisms of inorganic ion transport have been extensively studied, but tubules have recently been shown to also play an important role in the clearance of organic solutes (Dow and Davies, 2006). Rheault et al. (2006) demonstrated that active transport of organic cations by Malpighian tubules is common among species from different orders. They examined transepithelial transport of nicotine (a plant alkaloid) and tetraethylammonium (TEA - a xenobiotic) in tubules of nine insect species from six orders, and found that transepithelial transport of TEA and nicotine occurs through separate pathways. Furthermore, tubules react to bacterial challenge by secreting antimicrobial peptides, and this has proved an effective killing response. Malpighian tubules therefore constitute an autonomous immune system, entirely independent of the insect fat body (Dow and Davies, 2006).

The removal or metabolism of waste products and the careful regulation of excretory water loss are basic functions in all insects, for which the Malpighian tubules, and therefore the diuretic peptides which act on them, are partly responsible. The adults of all major pest species are terrestrial and so interference

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with water balance would have adverse effects on survival. Hormones control many aspects of physiology, and there is speculation that disruption of the normal synthesis of diuretic hormones could be detrimental to the insect, potentially inducing dehydration and death (Gäde and Goldsworthy, 2003). Diuretic peptides have also been shown to disrupt insect growth and development and increase mortality. Injection of kinins into the haemolymph of *Heliothis virescens* larvae lead to a reduction in weight gain and increased mortality (Seinsche et al., 2000). Similarly, Ma et al. (2000) found that when larvae of the tobacco hornworm *Manduca sexta* were fed Manse-DH_{30} at doses of of 1.5 ng to 15000 ng, mortality increased. However, when injected into the haemolymph, Manse-DH_{30} had no effect, suggesting that the peptide acts as an antifeedant. Tubules and their controlling peptides could therefore be an attractive target for the development of insecticides, although much research into this possibility is still required.

Relevance of this study and future directions

The importance of beetles and their neglect in past excretory physiological studies have already been underscored. The availability of both diuretic and antidiuretic peptides from *Tenebrio* makes it an ideal subject for further investigation of insect excretion. Nevertheless, it is worthwhile to consider the applicability of *Tenebrio* to the study of other insects, since some differences may be important.

Some insect species exhibit regional specialisation along the length of their Malpighian tubules, and others have differentiation in the type of cells that make up the tubule epithelium. The latter is well known in Diptera, where tubules comprise principal and stellate cells (Dow and Davies, 2006). These cells appear to be targeted by different diuretic factors: leukokinins act on the stellate cells of tubules while CT-like peptides appear to act on principal cells (see Johnson et al., 2004, 2005; O'Donnell et al., 1996). Likewise, the tubules of the tenebrionid *Onymacris plana* are composed of two cell types: type II cells are relatively smaller and less common than type I cells, the structure of which appears to be influenced by

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different conditions to which the beetles were exposed (Hanrahan and Nicolson, 1987). Regional specialisation along the length of the tubule is also known. For example, tubules of the cricket *Teleogryllus oceanicus* are differentiated into distal and main segments, and each responds very differently to the same diuretic factor (Xu and Marshall, 2000). Synergism between diuretic factors may thus be due to actions on different cells or regions within the tubules. There are apparently no structural (Meyran, 1982) or functional heterogeneities in *Tenebrio* tubules (Nicolson, 1992), but it is not clear if this would explain the lack of synergism observed.

It is possible that diuretic factors have different effects between life stages, but this is not often investigated (except, perhaps, in mosquitoes). Adult and larval tubules of *Tenebrio* do not show significantly different secretion rates, nor do they respond differently to diuretic factors (Wiehart et al., 2002; Rheault et al. 2006). However, future studies should take into consideration the different phases in the life cycle of the dung beetle, as the morphology and behaviour of the adults differ considerably from those of the larvae. Peptides may also display different effects at different concentrations (Clark et al., 1998) and some studies that demonstrated synergism between factors have used high doses (in the micromolar range) (e.g. Iaboni et al., 1998; Coast et al., 2005). Finally, some diuretic peptides have opposing effects within orders (as in the Diptera in particular – see Chapter 2), but similar effects across orders. Thus, dose-dependent effects and varying effects within and between orders make it difficult to compare results between studies.

Like the CRF-related peptides, insect CT-like peptides are highly conserved within and even across orders. Given the fairly potent response of *Tenebrio* tubules to some CT-like peptides (Chapter 2), it is expected that *Tenebrio* possesses its own CT-like peptide and, possibly, that its structure may be similar to that of Trica-DH_{31} (identified during a BLAST search of the *Tribolium* genome). The unsuccessful attempts to localise a CT-like peptide in the nervous tissue of *Tenebrio*, using antiserum to the cockroach CT-like peptide Dippu-DH_{31}, were thus

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very disappointing. Interestingly, Trica-DH_{31} is identical in structure to Dippu-DH_{31} (see Chapter 3, Fig. 8), but the cockroach CT-like peptide failed to stimulate secretion in *Tenebrio* tubules.

The inhibitory effect of *Onthophagus gazella* brain homogenates on mealworm fluid secretion was unexpected, since brain and corpora cardiaca homogenates have invariably been shown to stimulate secretion. In the present study, this inhibitory effect was initially assumed to be a weakening of the tubules over time, but in repeat experiments the response of tubules to cyclic AMP verified that they were still viable (see Chapter 2). The decline in fluid secretion in otherwise healthy tubules was significant, and it therefore seems plausible to attribute it to an antidiuretic factor present in the dung beetle brain. It is likely that similar observations have previously been dismissed and consequently, published studies report only increases in fluid secretion rates in the presence of homogenates. However, the presence of biogenic amines or multiple peptides in nervous tissue may obscure the effects observed, and so results obtained using crude homogenates should be interpreted with reservation. For example, the addition of tissue homogenates and cyclic AMP to the tubules of *O. plana* caused dramatic but opposing effects on the transepithelial potential (Nicolson and Isaacson, 1987). This example also demonstrates that changes in fluid secretion are not necessarily reflected in the electrophysiology of the tubule and different factors that similarly stimulate fluid secretion may have very different effects on ion fluxes. For instance, the mealworm antidiuretic factor Tenmo-ADFa significantly inhibits fluid secretion in tubules of the mosquito *Aedes aegypti*, without changing the concentrations of Na^+, K^+ and Cl^- in the secreted fluid or affecting membrane potential or resistance (Massaro et al., 2004).

Diuretic and antidiuretic peptides have been identified from a limited number of species (Coast et al., 2002), so the factors isolated to date, as well as their reported effects, are unlikely to represent the enormous diversity of insects. Novel insect neuropeptides are being isolated and characterised at a rapid pace and new

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technologies facilitate the use of a combination of different techniques to identify their physiological functions (e.g. Clynen et al., 2002). The complexity of the system can only be fully appreciated by using an “integrative” approach (Dow and Davies, 2002) and in future, multidisciplinary approaches wiil advance understanding of the physiological context.

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