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Review

Neuroendocrine control of ionic homeostasis in blood-sucking insects

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Summary

The pioneering work of Simon Maddrell established that the rapid postprandial diuresis of the haematophagous insect *Rhodnius prolixus* is controlled by a diuretic hormone and demonstrated the role of the Malpighian tubules in meeting the volumic, osmotic and ionic challenges posed by an enormous blood meal. A number of diuretic and antidiuretic hormones that control secretion of primary urine by Malpighian tubules have now been identified, but little is known of the interplay between these hormones and those that regulate transport processes in the hindgut. This review therefore focuses on the control of ionic homeostasis in *Rhodnius* and mosquitoes, because primary urine is voided virtually unchanged during the rapid diuresis that follows a blood meal. At such times, the hindgut has a negligible impact on the volume and composition of the final urine, and neurohormones acting on the Malpighian tubules have a dominant role in the control of ionic homeostasis.

Key words: *Rhodnius prolixus*, *Aedes aegypti*, *Anopheles gambiae*, Malpighian tubule, diuresis, diuretic hormone, antidiuretic hormone.

Introduction

A review published in 1963 provides an excellent account of the mechanisms of osmotic and ionic regulation in insects, but makes relatively little mention of their control (Shaw and Stobbart, 1963). Reference is made, however, to a short paper submitted to *Nature* by Simon Maddrell in which he presents evidence for the existence of a diuretic hormone in the blood-sucking triatomid bug *Rhodnius prolixus* that stimulates Malpighian tubule secretion and hence the rapid diuresis that follows a blood meal (Maddrell, 1962). The Malpighian tubules of insects generate a variable load of primary urine that is delivered to the hindgut, where it is modified by reabsorptive and secretory processes in the ileum and rectum before being voided as urine or, more usually, as a dry or semi-dry excreta. Most (>90%) of the ions, water and organic solutes secreted by the Malpighian tubules are generally reabsorbed from the hindgut of terrestrial insects, leaving behind concentrated toxic wastes to be excreted. The hindgut therefore normally has a dominant role in determining the composition of the excreta, but, more than 40 years after the demonstration of a diuretic hormone in *Rhodnius*, only one hormone that acts on the hindgut (ion transport peptide, ITP) has been identified, whereas a number of biogenic amines and neuropeptides have been shown to act on Malpighian tubules (Coast et al., 2002; Schooley et al., 2005).

There is evidence to suggest that Malpighian tubules and the hindgut are independently controlled (Coast et al., 1999), which provides enormous scope for the excretory system to regulate haemolymph volume and composition. Little is known, however, of the interplay between the hormones that control primary urine production by the Malpighian tubules and those that control its subsequent modification in the hindgut. The focus of this review will therefore be on homeostatic mechanisms in blood-sucking insects (notably *Rhodnius* and mosquitoes) because transport processes in the hindgut have negligible impact on the volume and composition of urine excreted during the rapid postprandial diuresis. The Malpighian tubules therefore have a dominant role in haemolymph homeostasis, and a considerable amount is known of their control by neurohormones.

The control of ionic homeostasis in *Rhodnius prolixus*

The postprandial diuresis of *Rhodnius*

Nymphs and adults of *Rhodnius prolixus* are obligate blood feeders. Nymphs consume blood meals that are equivalent in mass to 10–12 times their unfed body mass, which greatly restricts their manoeuvrability, thereby making them prone to predation. A large part of the meal comprises unwanted ions (Na^+ and Cl^-) and water, and this excess fluid is rapidly absorbed into the haemolymph, transferred to the Malpighian tubule lumen and expelled as urine during the postprandial diuresis. The nutritious components of the meal, namely red blood cells and plasma proteins, are retained in the expanded anterior midgut, which is a functional crop, and are subsequently passed to more-posterior regions of the midgut for digestion and assimilation, which might not begin until several days after feeding (Wigglesworth, 1943). Between the infrequent blood meals, *Rhodnius* must conserve water and therefore does not urinate.

The rapid diuresis lasts 3–4 h, during which ~50% of the volume load is excreted. Fig. 1 presents an overview of the osmotic and ionic concentrations of fluids in different compartments of a fifth-instar *Rhodnius* nymph during the postprandial diuresis. Fluid is absorbed from the crop at ~400 nl min^{-1}, and the volume of the crop diminishes visibly during diuresis. Fluid absorption appears to be driven by a ouabain-inhibitable Na^+/K^+-ATPase on the basal side (haemolymph side) of the epithelium, and the absorbate is rich in NaCl, contains little K^+ and is isosmotic to the blood meal, which means it is hypo-osmotic to haemolymph (Farmer et al., 1981). The volumic, osmotic and ionic challenges presented by this uptake of fluid into the haemolymph are countered by the Malpighian tubules, which remove the excess salt and water as hypo-osmotic primary urine at ~400 nl min^{-1}. Urine is expelled from the anus

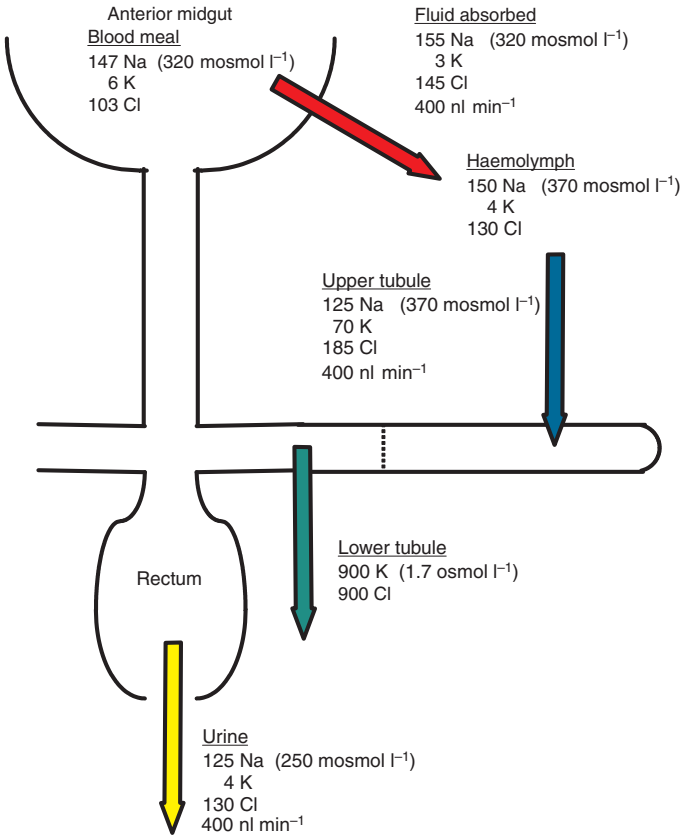


Fig. 1. An overview of osmotic and ionic concentrations and fluid movements during rapid diuresis in a fifth-instar *Rhodnius* nymph. Coloured arrows, used to indicate transport across the anterior midgut and across upper and lower Malpighian tubule segments, correspond to those used for the dose–response curves in Fig. 4. Ion concentrations are given in mmol l^{-1}. Based upon data from Maddrell (Maddrell, 1976).

every 2–3 min, and transport processes in the hindgut have negligible impact on its volume and composition.

Rhodnius Malpighian tubules comprise distinct upper and lower segments. The upper segment secretes primary urine, and the rate of secretion is increased >1000-fold during the postprandial diuresis. The secreted fluid is isosmotic to haemolymph and, in unstimulated tubules, contains substantially more K^+ than Na^+ (Ramsay, 1952). During diuresis, however, Na^+-rich fluid is secreted, but it nevertheless contains considerable amounts of K^+ (70–80 mmol l^{-1}), which, if it were excreted, would deplete the haemolymph of its total K^+ content within a minute (Maddrell et al., 1993b). Potassium loss is prevented by the selective uptake of KCl from the lower segment of the tubule (Maddrell and Phillips, 1975). The osmotic permeability of this segment is less than that of the upper tubule, and the lower tubule is therefore a diluting segment. The urine expelled from the anus is therefore hypo-osmotic to haemolymph and contains relatively little K^+ (4 mmol l^{-1}).

Coordinating the activities of the anterior midgut and Malpighian tubules

Within 2–3 h of the onset of feeding, a volume of NaCl-rich hypo-osmotic fluid equivalent to 10-times the haemolymph volume is absorbed from the crop of a fifth-instar nymph and expelled as urine. Despite this rapid turnover of ions and water, the volume and composition of the haemolymph change relatively little. Transport processes in the anterior midgut, and the upper and lower segments of the Malpighian tubules, must therefore be precisely coordinated. Maddrell suggested that haemolymph volume could be autonomously regulated by a diuretic hormone that stimulates Malpighian tubule secretion and fluid absorption from the anterior midgut (Maddrell, 1980). The concept is illustrated in Fig. 2. During diuresis, the diuretic hormone concentration in the circulation is somewhat greater (0–50%) than that needed to stimulate maximal tubule secretion (Maddrell, 1964a) but is assumed to be less than that required to maximally stimulate absorption from the crop. Any change in haemolymph volume will alter the diuretic hormone concentration, but will have little effect on tubule secretion, because it is already maximal. It will, however, increase or decrease the rate of absorption of fluid from the crop, thereby restoring haemolymph volume to a set point when the rates of fluid uptake and secretion are equal.

The activities of the upper and lower Malpighian tubule segments must also be closely coordinated during diuresis so as to preserve haemolymph K^+. Importantly, K^+ uptake from the lower tubule segment must be activated in advance of the stimulation of secretion by the upper tubule so as to prevent depletion of haemolymph K^+ (Maddrell et al., 1993b). To achieve this, the lower tubule appears more sensitive to diuretic hormone than the upper tubule, which means it will be stimulated earlier, and its response is more rapid. During diuresis, both tubule segments are maximally stimulated, and haemolymph K^+ is then autonomously regulated by their differing responses to a change in K^+ concentration. Thus, if the haemolymph K^+ concentration falls, K^+ transport by the upper tubule decreases, whereas K^+ uptake from the lower tubule increases, and *vice versa* (Maddrell et al., 1993b).

Rhodnius diuretic hormones

Within a minute of the onset of feeding, diuretic hormone is released into the circulation, and fluid secretion by the upper tubule is stimulated >1000-fold (Maddrell, 1963). The diuretic hormone originates from the mesothoracic ganglion mass (MTGM) and is released from neurohaemal sites along abdominal nerves 1–5 in response to distension of the abdomen by the blood meal (Maddrell, 1964b; Maddrell, 1966). Diuretic activity is largely concentrated in a group of posterior lateral neurosecretory cells, and the hormone content of single cells isolated from the MTGM has been assessed both during and after diuresis (Berlind and Maddrell, 1979). Interestingly, these cells contain a diuretic hormone that is a potent stimulant of the upper tubule but which has no effect on K^+ uptake from the lower tubule, whereas that latter is stimulated by a factor releasable from the MTGM and its associated nerves, which suggests that there might be more than one diuretic hormone present in *Rhodnius* (Maddrell, 1976). Indeed, the MTGM is now known to contain several neurohormones that act on Malpighian tubules (Table 1). Their distribution in the MTGM is shown schematically in Fig. 3. The focus here is on the MTGM because this is the identified source of *Rhodnius* diuretic hormone (Maddrell, 1963), but these neurohormones are also found in other regions of the central nervous system, most notably the brain, and they might be released from the corpora cardiaca.

Serotonin is a diuretic hormone in *Rhodnius*

Of the neurohormones listed in Table 1, only serotonin (5-hydroxytryptamine, 5-HT) has been shown conclusively to function as a diuretic hormone. Serotonin-like immunoreactive

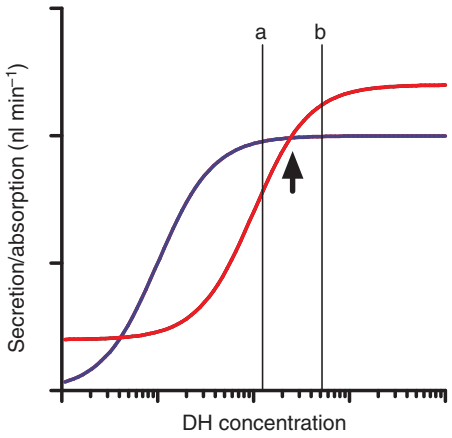


Fig. 2. Hypothetical dose–response curves for a diuretic hormone that stimulates fluid absorption from the anterior midgut (red curve) and fluid secretion by upper Malpighian tubules (blue curve). The vertical lines show how the diuretic hormone concentration will respond to an increase (a) or decrease (b) in haemolymph volume. Changes in diuretic hormone concentration have no effect on fluid secretion, which is already maximal, but will decrease (a) or increase (b) fluid absorption so as to restore haemolymph volume until the two rates are equal (arrow). Redrawn from Maddrell (Maddrell, 1980).

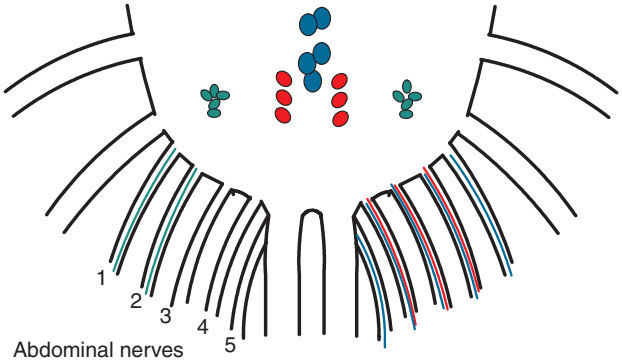


Fig. 3. A schematic diagram of the posterior region of the MTGM showing the localisation of neurosecretory cells containing factors that are known to influence tubule secretion. Serotonin- and Rhopr-DH_{31}-immunoreactive material is present in DUM neurons and axons (blue) that exit *via* abdominal nerves (AN) 1–5. Kinin- and CRF-like DH-immunoreactive material is present in groups of posterior lateral neurosecretory cells and axons (green) exiting *via* AN1 and AN2. Rhopr-CAP_{2b}-immunoreactive material is present in three pairs of ventral medial neurosecretory cells and axons (red) exiting *via* AN2–AN4. Based upon data from Orchard et al. (Orchard et al., 1989), Te Brugge et al. (Te Brugge et al., 2001; Te Brugge et al., 2005) and Paluzzi and Orchard (Paluzzi and Orchard, 2006).

material is present in dorsal unpaired medial (DUM) neurons of the MTGM (Fig. 3) and in axons extending to neurohaemal release sites along abdominal nerves 1–5 (Orchard, 1989). The intensity of staining at these sites is reduced when the insect feeds, and circulating levels of serotonin are elevated within a minute of the onset of feeding (Lange et al., 1989). Moreover, the injection of 5,7-dihydroxytryptamine 24 h before feeding, which depletes nerve terminals of serotonin, either prevents or delays diuresis (Maddrell et al., 1993a).

Serotonin acts through cyclic AMP to maximally stimulate fluid absorption from the crop (Farmer et al., 1981), fluid secretion by the upper tubule (Maddrell et al., 1971) and K^+ uptake from the lower tubule (Maddrell et al., 1993b), making it an excellent candidate for a diuretic hormone that can coordinate all three activities. Normalised dose–response curves for these activities are presented in Fig. 4, along with data for the haemolymph titre of serotonin at various times before and after the onset of feeding. The circulating titre of serotonin increases from ~7 nmol l^{-1} to 115 nmol l^{-1} within 5 min of the onset of feeding (Lange et al., 1989), which is sufficient to maximally stimulate all three target tissues (Fig. 4). Thereafter, the serotonin titre falls, and, 60 min after the onset of feeding, it is ~20 nmol l^{-1}, which has no effect on the crop and upper tubule but will support ~70% of the maximum rate of K^+ uptake from the lower tubule. The postprandial diuresis lasts 3–4 h, however, and this requires the release of a peptide diuretic hormone (Aston, 1979).

Candidates for the peptide diuretic hormone of *Rhodnius*

Possible candidates for the peptide diuretic hormone are listed in Table 1, and their localisation in neurosecretory cells of the MTGM is shown in Fig. 3. CAP_{2b} peptides have antidiuretic activity in *Rhodnius* and are dealt with separately below. Calcitonin (CT)-like diuretic hormone (CT-like DH) immunoreactivity colocalises with serotonin in DUM neurons and their neurohaemal sites. CT-like DH is therefore likely to be released into the circulation along with serotonin shortly after the onset of feeding. A CT-like DH (Rhopr-DH_{31}) has been identified in *Rhodnius* (Te Brugge et al., 2008), but it has little effect on secretion by the upper tubule (Te Brugge et al., 2005) and has no effect on K^+ uptake from the lower tubule (Donini et al., 2008) and fluid absorption from the crop (V. A. Te Brugge and I. Orchard, personal communication).

Kinin and CRF-like peptides have not been identified in *Rhodnius*, but kinin-like and CRF-like immunoreactive material colocalise in 5–6 pairs of posterior lateral neurosecretory cells in

Table 1. Diuretic and antidiuretic hormones present in the MTGM of *Rhodnius* and their actions

Hormone	*Rhodnius* peptide	Sequence	Activity		
			Crop	UMT	LMT
Serotonin			+	+	+
CRF-like DH	(Zoone-DH)	TGAVPSLSIV NPLDVLRQRL LLEIARRRMR QSQDQIQANR EMLQTI-NH_2	+	+	0
CT-like DH	Rhopr-DH_{31}	GLDLGLSRGF SGSQAAKHLM GLAAANYAGG P-NH_2	0	0/+	0
Kinin	(Leuma-KI)	DPAFNSWG-NH_2	0	0	0
CAP_{2b}	Rhopr-CAPA-2	EGGFISFPRV-NH_2	–	–	0

Table shows the effect of hormones on transport across the crop and upper (UMT) and lower (LMT) Malpighian tubule segments. Where the native hormone has not been identified, the sequence given is that of a peptide from the same family that has been tested for activity in *Rhodnius*.
Activity symbols: stimulate, +; inhibit, –; no effect, 0. MTGM, mesothoracic ganglion mass (MTGM).

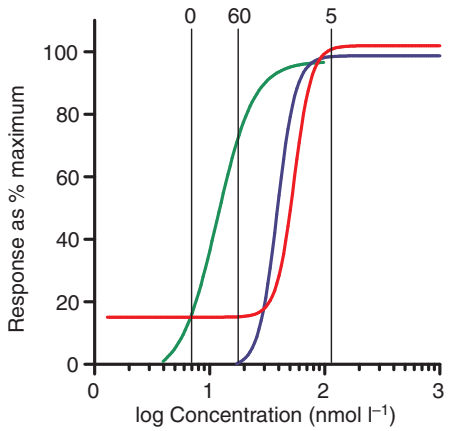


Fig. 4. Normalised dose–response curves for the effects of serotonin in stimulating fluid absorption from the anterior midgut (red curve), fluid secretion by upper Malpighian tubules (blue curve) and K^+ uptake from lower Malpighian tubules (green curve). Vertical lines indicate serotonin concentrations in haemolymph of unfed fifth-instar *Rhodnius* nymphs (0), and at 5 and 60 min after the onset of feeding. Based upon data from Maddrell et al. (Maddrell et al., 1971; Maddrell et al., 1993), Farmer et al. (Farmer et al., 1981) and Lange et al. (Lange et al., 1989).

the MTGM (Te Brugge et al., 2001). These are almost certainly the cells that were shown to contain a diuretic hormone that is released during diuresis (Berlind and Maddrell, 1979). Their axons extend to neurohaemal sites on abdominal nerves 1 and 2, and there is evidence to suggest that both peptides are released into the circulation in response to feeding (Te Brugge and Orchard, 2002). In cross-species assays, kinins have no effect on secretion by the upper segment of *Rhodnius* tubules, and this has been confirmed with HPLC fractions from the MTGM that contain kinin-like immunoreactive material (Te Brugge et al., 2002). Kinins also have no effect on K^+ uptake from the lower tubule (Donini et al., 2008) and fluid absorption from the crop (V. A. Te Brugge and I. Orchard, personal communication).

Cross-species assays with CRF-like DH from *Locusta migratoria* (Locmi-DH) and *Zootermopsis nevadensis* (Zoone-DH) show that both stimulate maximal secretion by the upper segment of *Rhodnius* tubules (Coast, 1996; Te Brugge et al., 2002). In addition, Zoone-DH stimulates fluid absorption from the crop (Te Brugge and I. Orchard, personal communication), but it has no effect on K^+ uptake from the lower tubule (Donini et al., 2008). The latter finding explains why the contents of isolated posterior lateral neurosecretory cells have potent diuretic activity on the upper tubule but no effect on the lower tubule (Maddrell, 1976). Zoone-DH uses cyclic AMP as a second messenger, which is consistent with what is known of the unidentified peptide diuretic hormone (Maddrell et al., 1993a), and it is likely that this is a CRF-like DH. The actions of serotonin and Zoone-DH on the upper tubule appear identical and result in a characteristic triphasic change in transepithelial potential (TEP) (Donini et al., 2008; Ianowski and O'Donnell, 2001; O'Donnell and Maddrell, 1984), which has been attributed to the sequential activation of apical membrane Cl^- channels, the apical membrane V-type H^+-ATPase, and a basal membrane $Na^+/K^+/2Cl^-$ cotransporter. The net result is a massive increase in NaCl and KCl transport into the lumen along with osmotically obliged water.

Serotonin is released rapidly into the haemolymph immediately after the onset of feeding to initiate diuresis, but the circulating titre peaks at 5 min and thereafter declines to levels below those needed to stimulate the fluid absorption from the crop and secretion by upper tubule, which would then require the release of a CRF-like DH. In support of this, the serotonin receptor antagonist ketanserin reduces the diuretic activity of haemolymph sampled 5 min after the onset of feeding by 70%, but by only 30% in samples taken after 1.5 h (Te Brugge and I. Orchard, 2002). The latter effect is considerably greater than would be anticipated, because serotonin levels have then fallen to ~20 nmol l^{-1}, which have little effect on tubule secretion (see Fig. 4). The synergism demonstrated between the peptide diuretic hormone and threshold concentrations of serotonin (Maddrell et al., 1993a) could account for the marked effect of ketanserin on haemolymph diuretic activity at 1.5 h, but surprisingly neither Locmi-DH nor Zoone-DH acts synergistically with serotonin (Coast, 1996; Te Brugge et al., 2002). A different result might be obtained with native *Rhodnius* CRF-like DH, but synergism could not be demonstrated between serotonin and an HPLC fraction from the MTGM that contained CRF-like immunoreactive material (Te Brugge et al., 2002). As there is also no evidence of synergism between serotonin and either kinins or Rhopr-DH_{31}, it is possible that an additional peptide diuretic hormone(s) is present in the MTGM.

Although Zoone-DH mimics serotonin in stimulating fluid secretion by the upper tubule and fluid absorption from the crop, it has no effect on K^+ uptake from the lower tubule (Donini et al., 2008), yet this must continue throughout diuresis to conserve haemolymph K^+. This is probably achieved by the greater potency of serotonin on the lower tubule (see Fig. 4), which would allow ~70% maximal K^+ uptake even when the circulating titre falls to about 20 nmol l^{-1}.

Terminating diuresis

Diuresis ceases 3-4 h after the onset of feeding – by when ~50% of the imbibed salt and water have been voided. The cessation of diuresis was generally assumed to result from the removal of the stimulus for diuretic hormone release (abdominal distension) and the degradation and/or removal of diuretic hormone present in the circulation. Switching off high rates of ion and water movement across the anterior midgut and Malpighian tubules of *Rhodnius* by such a mechanism would be difficult, however, because at all times they must remain precisely coordinated for haemolymph volume and composition to be held constant. This might explain why *Rhodnius* uses an antidiuretic hormone to terminate diuresis, as first demonstrated using a CAP_{2b} (Manse-CAP_{2b}) from the tobacco hornworm, *Manduca sexta*, which acts through cyclic GMP to reduce secretion by upper tubules partially stimulated with serotonin (Quinlan et al., 1997). The native peptide, Rhopr-CAP_{2b} (also known as Rhopr CAPA-2 because it is encoded by the *Rhodnius capability* gene) has subsequently been identified and shown to have potent (IC_{50}=4 nmol l^{-1}) antidiuretic activity on tubules partially stimulated by 50 nmol l^{-1} serotonin (Paluzzi et al., 2008). CAP_{2b}-like immunoreactive material is present in three pairs of ventral medial neurosecretory cells in the MTGM (see Fig. 3), which express the gene encoding CAPA, and axons from these cells extend to neurohaemal areas on abdominal nerves 2–4 (Paluzzi and Orchard, 2006; Paluzzi et al., 2008). The intensity of immunoreactive staining decreases 3–4 h after the onset of feeding (Paluzzi and Orchard, 2006), which is coincident with an increase in the cyclic GMP content of Malpighian tubules *in vivo* (Quinlan et al., 1997) and consistent with release of Rhopr-CAP_{2b} at the time diuresis ceases.

It has been suggested that the antidiuretic activity of CAP_{2b} results from the activation of a cyclic-GMP-dependent phosphodiesterase specific for cyclic AMP (Quinlan and O'Donnell, 1998), which is the second messenger used by both serotonin and the CRF-like DH. Although this is an attractive hypothesis, it has yet to be tested, but it is consistent with the observation that high concentrations of cyclic AMP reverse the effects of cyclic GMP (Quinlan and O'Donnell, 1998). Interestingly, with the addition of a high concentration (500 μmol l^{-1}) of exogenous cyclic GMP, upper tubules stimulated with 10 μmol l^{-1} serotonin revert towards their unstimulated state by secreting K^+-rich fluid (Quinlan and O'Donnell, 1998), which could be important for conserving Na^+ once fluid absorption from the crop ceases. The activities of the crop and the upper segment of the Malpighian tubules need to be coordinated, and recently it has been shown that Rhopr-CAP_{2b} also reduces fluid absorption from the anterior midgut stimulated with either serotonin or Zoone-DH (Orchard and Paluzzi, 2009). The termination of diuresis in a coordinated manner would therefore depend on the potency and rate of response of the crop and upper tubule to Rhopr-CAP_{2b}. It is not known whether Rhopr-CAP_{2b} reduces serotonin-stimulated K^+ uptake from the lower tubule, where cyclic AMP is also used as a second messenger, but this might not be necessary. As diuresis ceases, K^+ uptake is likely to be the last process to be turned off because, while the upper tubule remains stimulated, the lower tubule must continue to reabsorb K^+. Serotonin is the only diuretic hormone known to act on the lower tubule, and circulating levels at 3–4 h after the onset of feeding (~20 nmol l^{-1}) lie on the steepest part of the dose–response curve (see Fig. 4), which means that even a small decline in concentration will substantially reduce K^+ uptake.

The control of ionic homeostasis in mosquitoes

Diuresis after a blood meal

Adult female mosquitoes are haematophagous and use proteins from the blood meal for egg development. Males, by contrast, feed only on nectar, and nectar is also used by females as an energy source (Clements, 2000). The blood meals of female mosquitoes are smaller than those imbibed by *Rhodnius* nymphs and are equivalent to 2–3 times the unfed body mass. They differ also in the way they digest and assimilate the meal (Lehane, 2005). *Rhodnius* is 'continuous processor' in that the blood meal is held in the expanded anterior foregut and is slowly passed down to lower regions of the midgut for digestion and assimilation. By contrast, mosquitoes are 'batch processors': digestion and assimilation occur over the entire surface of the food bolus in the midgut. This difference is reflected in the requirements for ionic regulation after the blood meal, because the release of K^+ from blood cells begins not long after feeding in mosquitoes but can be delayed for several days in *Rhodnius*.

Adult female yellow fever mosquitoes, *Aedes aegypti*, typically ingest ~3.5 mg of blood, and gorging is complete within 5 min of the onset of feeding. A few (3–7) drops of urine are voided while feeding, but urine output increases dramatically within minutes of completing the meal (peak phase of diuresis), reaching >40 nl min^{-1} (Williams et al., 1983). Thereafter, urine output gradually declines (post-peak phase of diuresis), until it reaches a lower stable rate (late phase of diuresis) that is ~10% of the peak rate and is maintained for up to 2 h from the onset of feeding. Unfed insects rarely, if ever, void urine. Urine droplets (~17 nl $drop^{-1}$) are voided approximately every 15 s during the peak phase of diuresis (Wheelock et al., 1988), and their composition is similar to that of the primary urine, which is Na^+ rich and isosmotic to haemolymph (Williams et al., 1983). The peak phase is therefore comparable to the rapid diuresis of *Rhodnius* and probably corresponds to a period of rapid absorption of NaCl-rich fluid from the midgut into the haemolymph. Sodium excretion declines during the post-peak phase of diuresis, whereas K^+ excretion increases, and, by the end of this phase, K^+-rich urine is voided. As urine output declines, transport processes in the hindgut have an increasing influence on urine composition. The urine becomes hypo-osmotic to haemolymph, reflecting the uptake of NaCl in the hindgut without an osmotically equivalent volume of water, which is important for preventing dilution of the haemolymph by the blood meal. The osmotic concentration of the urine is more variable during the late phase of diuresis, and K^+ excretion exceeds that of Na^+. In the course of the postprandial diuresis, about 42% of the ingested plasma volume is voided (0.8 μl of urine), with most of this (0.6 μl) occurring during the first 20 min (Williams et al., 1983). At the same time, ~44% of the ingested Na^+ and 144% of the plasma K^+ are expelled, with the additional K^+ most likely coming from the digestion of blood cells in the midgut.

The pattern of diuresis in Anopheline mosquitoes differs from *Aedes* in that a greater volume of urine is voided while the insect is still feeding (Fig. 5A). During this so-called 'pre-diuresis', the

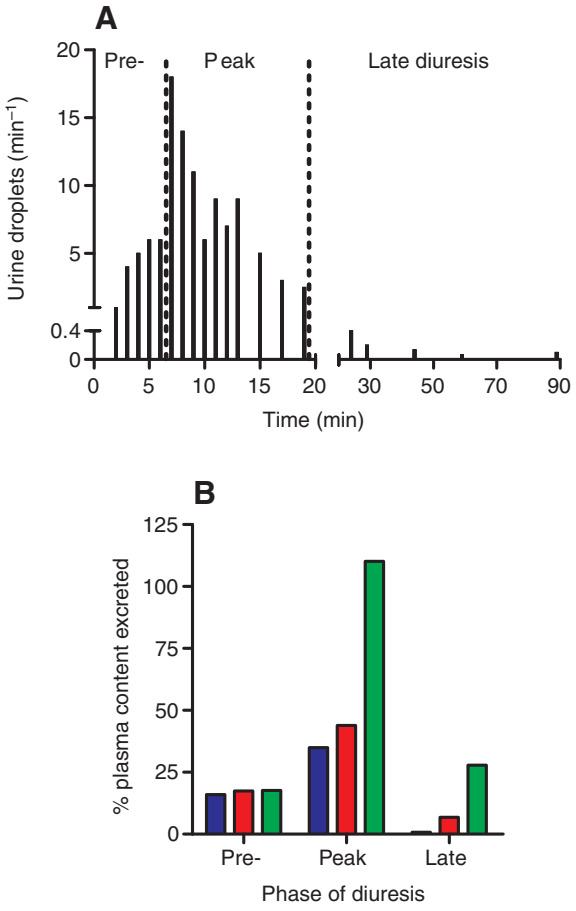


Fig. 5. (A) The rate at which urine droplets are voided during the pre-diuresis and subsequent diuresis of *An. gambiae* fed on a human volunteer. During peak diuresis, urine drops are voided at >1 min^{-1} but are voided less regularly during late diuresis. (B) The percentage of imbibed plasma water (blue bars), Na^+ (red bars) and K^+ (green bars) excreted during pre-diuresis, and the peak and late phases of diuresis by the same mosquito as in (A) (G.M.C., unpublished observations).

fluid voided is frequently pink or reddish in colour and is derived in large part from the blood meal in the midgut rather than the Malpighian tubules (Clements, 2000). Based upon measurements of total body haemoglobin content, we estimate that female malaria mosquitoes (*Anopheles gambiae*), weighing on average 0.88 mg, consume 1.98 µl of blood, but the increase in mass after feeding suggests that 33% of this (0.66 µl) is voided before the meal is completed (G.M.C., unpublished observations). The pre-diuresis allows the insect to imbibe larger meals than would otherwise be possible, and blood cells are concentrated 1.5-fold in the midgut. The fluid voided during the pre-diuresis is similar in composition to plasma and contains 146 mmol l^{-1} Na^+ and 5 mmol l^{-1} K^+, which is consistent with it coming from the blood meal in the midgut where cells are retained (Clements, 2000). The subsequent diuresis is arbitrarily divided into a peak phase, when urine droplets (~5 nl $drop^{-1}$) are voided at >1 min^{-1} and a post-peak phase when urine droplets are voided with much less regularity (Fig. 5A). During the peak phase, about 35% of the imbibed plasma volume is excreted along with 44% of the Na^+ and 110% of the K^+ (Fig. 5B), the additional K^+ probably being released from red blood cells.

The natriuretic hormone of mosquitoes

As in *Rhodnius*, the volumic, osmotic and ionic challenges presented by the blood meal require that excess salt and water absorbed into the haemolymph from the midgut is rapidly eliminated by the excretory system. Transport processes in the midgut and Malpighian tubules must therefore be closely coordinated, but little is known about the former in mosquitoes. At high concentrations (≥10 µmol l^{-1}), serotonin doubles the rate of secretion by *Aedes* tubules (Veenstra, 1988), but this is 100-fold higher than the peak titre measured in *Rhodnius* haemolymph (Lange et al., 1989), and there is no evidence for serotonin functioning as a circulating diuretic hormone. Rather, the peak phase of diuresis and its associated natriuresis are attributed to the release of a peptide diuretic hormone (mosquito natriuretic peptide; MNP) from structures in the head (Beyenbach and Petzel, 1987).

Under control conditions, adult female *Aedes* tubules secrete at ~0.4 nl min^{-1}, but this is accelerated sevenfold by MNP, and the $[Na^+]$:$[K^+]$ ratio of the secreted fluid increases from unity to ~10, as Na^+ transport rises 13-fold, with no change in K^+ transport (Beyenbach, 1995). MNP acts through cyclic AMP, and its diuretic and natriuretic activities are duplicated by the membrane-permeant cyclic AMP analogue dibutyryl-cyclic AMP (db-cyclic AMP) (Petzel et al., 1987; Williams and Beyenbach, 1984). Diuretic activity is detectable in haemolymph from newly fed intact mosquitoes but is not present in either unfed or fed decapitated insects, which suggests that MNP is released into the circulation from structures in the head in response to the blood meal (Wheelock et al., 1988). In support of this, Malpighian tubules removed from mosquitoes 5 min after the onset of a blood meal have significantly higher levels of intracellular cyclic AMP than those from unfed insects (Petzel et al., 1987), consistent with the release of MNP into the circulation. Thereafter, cyclic AMP levels drop back to control levels 10 min after the onset of feeding, but peak again at 25 min, although this was not statistically significant.

MNP has now been shown to be a CT-like DH (Anoga-DH_{31}), which was identified using the Ensembl genome browser with the *Drosophila* CT-like DH_{31} as a query (Coast et al., 2005). The CT-like peptides of *Aedes* and *Anopheles* are identical, and Anoga-DH_{31} duplicates the actions of MNP and db-cyclic AMP on *Aedes* tubules. Representatives of three other families of diuretic hormones have been identified in mosquitoes, namely a CRF-like DH (Anoga-DH_{44}), which was identified using Ensembl with the *Drosophila* orthologue (Drome-DH_{44}) as a query (Coast et al., 2005), three kinins (Veenstra, 1994) and two CAP_{2b} (CAPA) peptides (Pollock et al., 2004). All have diuretic activity, but none has natriuretic activity. The CT-like Anoga-DH_{31} must therefore be released shortly after the onset of feeding to stimulate the peak phase of diuresis and the accompanying natriuresis. Evidence in support of this comes from experiments in which Anoga-DH_{31} was immunoneutralised using an antiserum that was raised against the CT-like DH (Dippu-DH_{31}) of *Diploptera punctata* (Coast, 2007). *Anopheles* mosquitoes did not consistently gorge when presented with a blood meal across an artificial membrane, and we therefore injected females with 1 µl of 0.9% NaCl, which stimulates diuresis, although this is rarely as rapid as after a blood meal (Fig. 6). Insects injected with a 1:50 dilution of pre-immune rabbit serum in saline lost 35% of their weight and 33% of their Na^+ content after 4 h, but these values fell to 23% and 9%, respectively, in females injected with a 1:50 dilution of Dippu-DH_{31} antiserum (Coast, 2007). The greater impact of immunoneutralization on salt excretion is consistent with Anoga-DH_{31} being the only diuretic hormone with natriuretic activity and with evidence for its release in response to the injected salt load. The lesser effect on water loss could be due to release of a different diuretic hormone in response to the volume load.

Diuresis without natriuresis

The post-peak and late phases of diuresis in *Aedes* are characterised by the excretion of K^+-rich urine (Williams et al., 1983). The urine

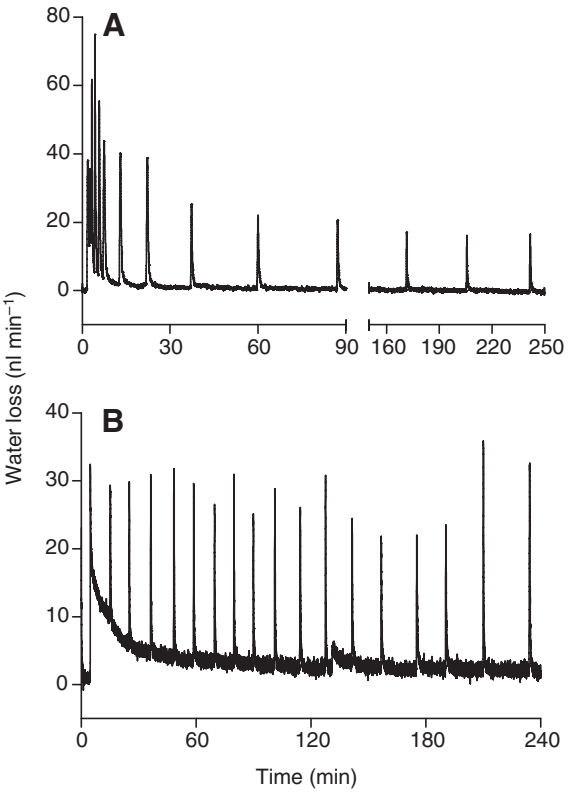


Fig. 6. Water loss from female *An. gambiae* fed on blood (A) and injected with 1 µl of 0.9% NaCl (B). Water loss was recorded using a flow-through humidity meter. The initial peak in the recordings represents water vapour entering the chamber at the start of the experiment, whereas subsequent

is now hypo-osmotic to haemolymph, evidence for its modification in the hindgut, which is likely to be independently controlled. The shift from natriuresis to kaliuresis suggests that primary urine production is no longer stimulated by MNP (i.e. Anoga-DH_{31}). More likely, diuresis is now controlled by a diuretic hormone that has a nonselective effect on active cation transport by Malpighian tubules, which, unlike the CT-like Anoga-DH_{31}, would increase delivery of K^+ to the hindgut and so facilitate the excretion of excess K^+ from digested blood cells. At present, there is no evidence for release of CRF-like, kinin and CAP_{2b} DH after a blood meal, but the nonsignificant peak in tubule cyclic AMP content 25 min after the onset of feeding could be due to the release of the CRF-like Anoga-DH_{44}, which is known to stimulate production of this second messenger (Coast et al., 2005).

Both male and female mosquitoes feed on nectar, which has a low ion content and therefore presents volumic and osmotic challenges coupled with the need to conserve ions. Diuresis commences within seconds of completing a nectar meal, and excess water is voided as hypo-osmotic urine, which requires the stimulation of both tubule secretion and the reabsorption of ions from the hindgut. The hindgut is almost certainly under endocrine control, but this is an unexplored area. As there is no need to void excess Na^+, diuresis is probably not initiated by MNP but is more likely controlled by a diuretic hormone with a nonselective effect on cation transport. If this is correct, the stimulus for diuretic hormone release must differ between blood and nectar meals. Possibly the release of the natriuretic CT-like Anoga-DH_{31} after a blood meal requires abdominal distension to be accompanied by a change in haemolymph Na^+ concentration, which in *Anopheles* rises from 79±4 to 106±3 mmol l^{-1} within 5 min of feeding (G.M.C., unpublished observations). In this context, it is noteworthy that male *Aedes* have a natriuretic peptide, presumably Anoga-DH_{31}, and their tubules have ion-transport mechanisms similar to those of female tubules even though they do not feed on blood (Plawner et al., 1991).

The mode of action of mosquito diuretic hormones

Under control conditions, the fluid secreted by mosquito tubules contains almost-equimolar amounts of Na^+ and K^+, but, when stimulated by the CT-like Anoga-DH_{31} (i.e. MNP), the $[Na^+]$:$[K^+]$ ratio increases to ~10 (Coast et al., 2005). There is considerable evidence to suggest that the natriuretic activity of MNP results from the cyclic-AMP-dependent opening of a Na^+ conductance (Na^+ channel) in the principal cell basal membrane, which makes more Na^+ available to cation/proton antiports in the apical membrane (Beyenbach, 2003b). The K^+ conductance of the basal membrane is normally 3.9 times greater than for Na^+, but the latter almost doubles after stimulation with db-cyclic AMP (Beyenbach and Masia, 2002). This is prevented by the epithelial Na^+ channel (ENaC) blocker amiloride, but, at the concentrations used (0.1 and 1.0 mmol l^{-1}), it could inhibit a number of Na^+-dependent processes (Beyenbach and Masia, 2002). To confirm the role of ENaC proteins in the natriuretic response, they must be shown to be present in the basal membrane and to open in response to cyclic AMP. Natriuresis also requires the activation of a cyclic-AMP-dependent bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter in the basal membrane (Hegarty et al., 1991). The cotransporter will bring additional Cl^- into the cell, which could exit to the lumen through Cl^- channels in the apical membrane (Wright and Beyenbach, 1987) to support a 13-fold increase in transepithelial NaCl transport after stimulation with Anoga-DH_{31} (Coast et al., 2005).

In contrast to the CT-like Anoga-DH_{31}, the CRF-like Anoga-DH_{44} does not have natriuretic activity, even though it stimulates cyclic AMP production (Coast et al., 2005). The action of CRF-like DH in *Aedes* and *Anopheles* tubules is characterised by a triphasic change in TEP, which has been interpreted as resulting from the stimulation of both Ca^{2+}-dependent and cyclic-AMP-dependent transport processes (Clark et al., 1998; Coast et al., 2005). Indeed, the triphasic voltage change can be reproduced by adding a combination of Anoga-DH_{31} and a kinin to *Anopheles* tubules, which suggests that Anoga-DH_{44} activates the basal membrane Na^+ conductance and $Na^+/K^+/2Cl^-$ cotransporter, and opens a Cl^- shunt pathway (see below).

Kinins stimulate secretion by mosquito tubules (Beyenbach, 1995; Hayes et al., 1989), but their effect is modest (a twofold increase) compared with that of CT-like Anoga-DH_{31} (sevenfold increase) and CRF-like Anoga-DH_{44} (threefold). Kinins act to open a Ca^{2+}-dependent Cl^--selective shunt pathway that lies outside of the principal cells, which results in a nonselective increase in the active transport of Na^+ and K^+ into the lumen (Pannabecker et al., 1993; Yu and Beyenbach, 2001). A question remains as to whether the Cl^- shunt pathway is paracellular or transcellular, through stellate cells (Beyenbach, 2003a; O'Donnell et al., 1998; Radford et al., 2002; Yu and Beyenbach, 2002), but the latter appears more likely given that a kinin receptor is present on stellate cells in *Anopheles stephensi* (Radford et al., 2004).

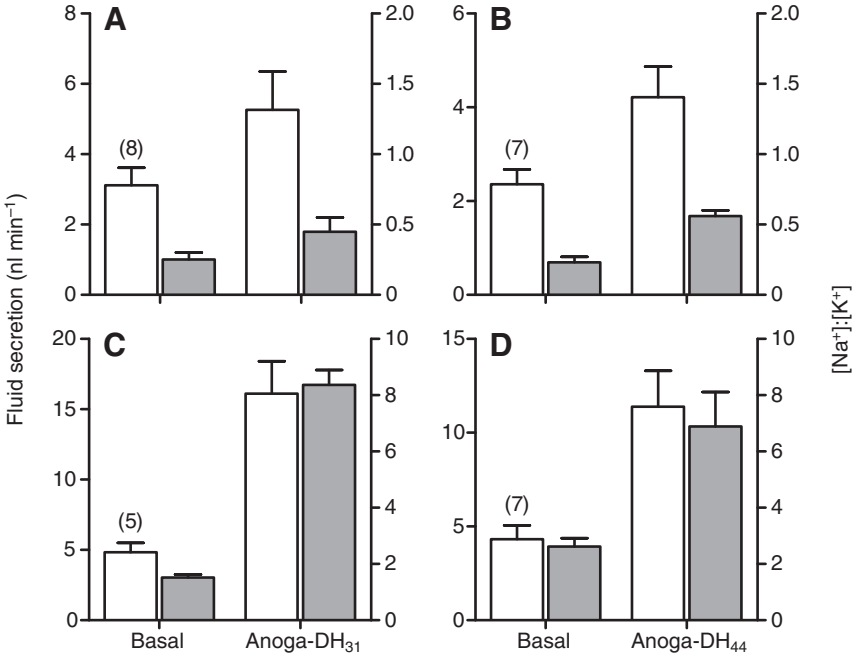
CAP_{2b} peptides from *An. gambiae* and *Drosophila* stimulate cyclic GMP production by *An. stephensi* tubules and cause a modest (approximately twofold) increase in fluid secretion, although the latter response is very variable and not dose–response dependent (Pollock et al., 2004). When tested on *Aedes* tubules, 1 μmol l^{-1} *Drosophila* CAPA-1 peptide produced a small increase in secretion but had no effect on the $[Na^+]$:$[K^+]$ ratio of secreted fluid (G.M.C., unpublished observations).

Comparing the activities of CT-like and CRF-like DH in dipteran insects

The CT-like Anoga-DH_{31} and CRF-like Anoga-DH_{44} have markedly different effects on ion transport by mosquito tubules. The former has natriuretic activity and therefore has an important role in the peak phase of diuresis after a blood meal, which is associated with excretion of excess NaCl (and water), whereas the CRF-like DH has a nonselective effect on Na^+ and K^+ transport and might stimulate diuresis at times when natriuresis would be inappropriate, for example during kaliuresis and after a nectar meal. It is therefore of interest to compare the activity of these peptides in two other species of dipteran flies with different feeding habits, the stable fly *Stomoxys calcitrans*, in which both sexes are obligate blood feeders, and the housefly *Musca domestica*, which does not feed on blood. Under control conditions, *Musca* tubules secrete K^+-rich fluid, and the $[Na^+]$:$[K^+]$ ratio changes little after stimulation by either peptide (Fig. 7A,B). By contrast, when *Stomoxys* tubules are bathed in the same saline, the $[Na^+]$:$[K^+]$ ratio of the secreted fluid is somewhat greater than unity under control conditions and, unlike mosquitoes, both CT-like and CRF-like peptides have natriuretic activity (Fig. 7C,D). The difference between stable fly and mosquito tubules would appear to reflect their diets: *Stomoxys* never consumes nectar and is therefore not confronted with a water load in the absence of a salt load.

Concluding remarks

The focus of this review has been on *Rhodnius* and mosquitoes because, during the rapid diuresis that follows a blood meal, the



peaks correspond to the expulsion of drops of urine from the anus (G.M.C., unpublished observations).

excreted urine is virtually identical to fluid secreted by the Malpighian tubules. At such times, the Malpighian tubules have a dominant role in the regulation of haemolymph volume and composition, removing excess salt and water absorbed from the blood meal in the midgut. Normally, however, the composition of the final excreta is determined by the hindgut, but, with the notable exception of work on locusts, little is known of the control of transport processes in the ileum and rectum. Studies of the hormonal control of the locust hindgut involved the use of flat-sheet and everted sac preparations (Phillips et al., 1986), techniques that can be used only with relatively large insects. Self-referencing ion-selective microelectrodes can, however, measure ion gradients in unstirred layers close to the surface of semi-intact and isolated tissues, allowing net transepithelial ion fluxes to be calculated. It should therefore be possible to study both midgut and hindgut function in model organisms, such as *Drosophila*, and to take advantage of the genetic and molecular biological techniques they have to offer. A number of important questions need to be addressed, including the identification of hormones that contribute to the control of hindgut function, and elucidation of the interplay between hormones acting on the midgut, Malpighian tubules and hindgut, which is essential for the control of haemolymph ionic homeostasis.

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