EFFECTS OF DH31, DROSOKININ, AND ALLATOSTATIN A ON EPITHELIAL K^+ TRANSPORT AND TISSUE CONTRACTIONS IN THE GUT OF LARVAL *DROSOPHILA MELANOGASTER*

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Abstract

DH31 and drosokinin are known promoters of fluid secretion in *Drosophila* Malpighian tubules, while the effect of allatostatin A on Malpighian tubule fluid secretion is unknown. The expression of these peptides and their receptors is widespread in the larval gut and central nervous system. The scanning ion-selective electrode technique (SIET) was used to measure changes in epithelial K^+ flux in the gut as a proxy for the flow of osmotically-obliged water between the gut lumen and the haemolymph. The primary goal of this study was to investigate the effects of DH31 and drosokinin on the gut as an indicator of coordination of diuretic activity between this tissue and the Malpighian tubules. Such coordination, whereby Malpighian tubule fluid secretion is stimulated concomitantly with fluid uptake by the gut, would be physiologically essential for the maintenance of haemolymph volume and osmolarity. Secondarily, this study sought to characterize the function of allatostatin A with respect to its effect on gut K^+ transport. DH31 stimulated K^+ absorption across the anterior midgut epithelium and reduced K^+ absorption in the middle midgut copper cell zone. Drosokinin increased K^+ absorption across the anterior midgut epithelium and was also stimulatory in the posterior midgut neutral zone. Allatostatin A stimulated K^+ absorption across the epithelia of the anterior midgut and middle midgut copper cell zone, but was inhibitory in the large flat cell zone. The larger surface area of the anterior midgut relative to the middle midgut means that all three peptides were likely net stimulators of K^+ uptake. The reduction in K^+ absorption in the middle midgut by DH31 and allatostatin A may relate to a redistribution of fluid uptake among the regions of the midgut to preserve lumenal pH. DH31 and drosokinin also independently increased the contraction frequency of the anterior midgut, while the contraction frequency of the pyloric sphincter was increased by combined application of both peptides. Stimulation of gut contractions has previously been attributed to these and other diuretic factors in insects. The findings of this investigation implicate DH31, drosokinin, and allatostatin A in the stimulation of midgut K^+ absorption, which may suggest a role for these peptides in altering fluid transport across this epithelium in larval *Drosophila*.

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Introduction

Drosophila melanogaster as an experimental model

The insect gut comprises the foregut, midgut, and hindgut (Figure 1). The foregut and hindgut are derived from ectoderm and are lined with cuticle, limiting their absorptive capacity (Veenstra et al., 2008). The midgut is thus the primary region of absorption and secretion of nutrients and inorganic ions, and is the only gut region possessing epithelial endocrine cells in *Drosophila* (Veenstra et al., 2008). The larval *Drosophila* midgut is divided into anterior, middle, and posterior regions by visual landmarks in the tissue; the junction separates the anterior from the middle region and the iron cells indicate the border between the middle and posterior regions (Figure 1). The acidic middle midgut is visually subdivided into distinct copper cell (anterior) and large flat cell (posterior) zones. The cells of the former zone secrete acid into the lumen and are analogous in function and morphology to the vertebrate stomach's parietal cells (Veenstra, 2009; Shanbhag and Tripathi, 2005). The posterior midgut is subdivided by a change in lumenal pH into neutral (anterior) and alkaline (posterior) zones (Shanbhag and Tripathi, 2005). The Malpighian (renal) tubules drain into the gut lumen via ureters that mark the boundary between the midgut and hindgut. The pyloric sphincter is located immediately posterior to the ureters and represents the anterior limit of the ileum.

The great diversity of dietary intake among insects makes them useful models for the study of homeostatic control involving the gut and Malpighian tubules. Dietary loading experiments have revealed that insects that consume high levels of dietary ions employ homeostatic mechanisms to excrete the excess and thus avoid toxicity or dehydration. For example, the upper Malpighian tubules of the blood-feeding insect *Rhodnius prolixus* are known to rapidly secrete Cl^-, K^+, and

Na^+ ions into the lumen; KCl is reabsorbed by the lower tubule while NaCl and water are excreted (Maddrell, 1969). Water reabsorption in the insect hindgut is also tightly regulated; the larvae of desert beetles produce powder-dry fecal pellets (Machin, 1976), whereas locusts fed a succulent diet reabsorb less than 10% of water secreted by the Malpighian tubules (Loveridge, 1975).

Drosophila are particularly useful organisms for the study of physiological processes due to their well characterized genome and the amenability of their gut and Malpighian tubules to in vitro studies and electrophysiological techniques. The net K^+ flux by the gut and Malpighian tubules of larval *Drosophila* is generally 3- to 4-fold greater than Na^+ flux, likely because larval *Drosophila* inherently possess a higher capacity for K^+ secretion to cope with the high levels of this ion in rotting fruit (Naikkhwah and O'Donnell, 2012). *Drosophila* exhibit precise osmoregulation of the haemolymph in response to dietary loading with KCl and NaCl. Naikkhwah and O'Donnell (2011) found that chronic exposure to high dietary NaCl or KCl led to only small increases in haemolymph Na^+ concentration and no significant increase in K^+ concentration in adults or larvae after 48 hours of exposure. Increased secretion of Na^+ and K^+ by the Malpighian tubules is one method of eliminating excess salt, but this process is accompanied by reduced K^+ absorption and increased K^+ and Na^+ secretion by the gut (Naikkhwah and O'Donnell, 2012). Reduced K^+ absorption counteracts the chemical gradient promoting K^+ uptake into the haemolymph caused by the high lumenal concentration of this ion (Naikkhwah and O'Donnell, 2011). The rate of secretion of K^+ by the Malpighian tubules exceeds the secretion rate of Na^+ by 4-fold (Naikkhwah and O'Donnell, 2011). In fact, the Malpighian tubules appear to recycle Na^+ because the secretion of this ion in the main segment is

accompanied by its reabsorption in the tubule's lower segment (Naikkhwah and O'Donnell, 2011). Thus, *Drosophila* exhibit considerable phenotypic plasticity in both the gut and Malpighian tubules allowing alterations in Na^+ and K^+ transport in response to dietary salt stress.

Larval Malpighian tubules bathed in haemolymph collected from specimens chronically exposed to an environment rich in K^+ or Na^+ show increased secretion of these ions accompanied by increased fluid secretion (Naikkhwah and O'Donnell, 2011). This response is likely due to the presence of diuretic factors in the haemolymph of salt-stressed larvae (Naikkhwah and O'Donnell, 2011). The effects of insect diuretics are mediated by the second messengers cyclic AMP (cAMP), cyclic GMP (cGMP), and Ca^{2+} (O'Donnell et al., 1996). cAMP and cGMP stimulate cation transport by activating an apical vacuolar-type H^+-ATPase (V-ATPase), while Ca^{2+} increases the permeability of the Malpighian tubule epithelium to Cl^- (O'Donnell et al., 1996). Control of Malpighian tubule fluid secretion by diuretics is therefore dependent on second messengers that are functionally specific for the regulation of cation or anion transport (O'Donnell et al., 1996).

Putative effects of diuretic neuropeptides on gut fluid transport

Several diuretic neuropeptides have been identified based on their stimulation of Malpighian tubule fluid secretion. In various insects, diuretic activity has been ascribed to 5-hydroxytryptamine (5-HT, serotonin), the corticotropin-releasing factor (CRF)-related peptide, cardioacceleratory peptide (CAP) 2b , leucokinin, and the calcitonin-like diuretic hormone 31 (Maddrell, 1969; Audsley et al., 1995; Davies et al., 1995; Terhzaz et al., 1999; Coast et al., 2001). In larval *Drosophila*, the expression of neuropeptides with known or postulated

involvement in water balance regulation in the gut and Malpighian tubules suggests that these factors may exert paracrine control over nearby transporting epithelia or diffuse through the haemolymph as endocrine factors to reach distant targets. True diuretics are expected to produce an overall increase in fecal volume by favouring the excretion of both ions and water. The preservation of haemolymph volume and osmolarity during diuresis requires increased fluid uptake across the gut epithelium. With the midgut being the primary area of nutrient absorption, it is probable that enhanced absorption of water occurs across the epithelia of its constituent regions.

Peptides with diuretic activity in the Malpighian tubules may alternatively act as clearance hormones by promoting the reabsorption of ions and water across the hindgut epithelium, leaving the feces dehydrated but with a high concentration of waste. A similar function has been ascribed to a diuretic hormone in the desert beetle *Onymacris*, a species in which water recycling is of paramount importance. Nicolson (1991) reported a substantial increase in Malpighian tubule fluid secretion rate in this species upon exposure to a diuretic factor contained within extracts of the corpora cardiaca. Concurrently, a greater proportion of the lumenal contents of the gut moved anteriorly into the midgut instead of their usual posteriorly-directed flow. This author proposes that the lumenal contents are directed to the midgut to allow for the reabsorption of water and nutrients. Retroperistaltic movement of gut contents is known to occur in larval *Aedes aegypti*, where some of the fluid released into the pyloric chamber from the Malpighian tubules is directed anteriorly to the midgut (Wigglesworth, 1933). This occurs until a threshold hydrostatic pressure is achieved in the pyloric chamber, at which time the pyloric sphincter dilates and ileal peristalsis moves the midgut contents into the rectum (Stobbart, 1971). In contrast, the rectum is

the site of water recycling in *Schistocerca* and *Manduca*. In both of these species, the rectal epithelium is characterized by extensive apical and basolateral folds that are in close association with mitochondria. This ultrastructural arrangement is suited to the generation of local osmotic gradients that allow for the absorption of fluid that is hypoosmotic to the lumenal contents (Lechleitner et al., 1989; Reynolds and Bellward, 1989). A similar mechanism operates in the rectal papillae of adult dipterans. In adult *Calliphora*, for example, labyrinthine apical membrane folds in papillar cortical cells facilitate local accumulation of ions that create an osmotic gradient favouring the uptake of water from the lumen (Gupta et al., 1980). In adult *Aedes*, apical V-ATPases have been suggested to drive the secondary active transport of ions into the small spaces between papillar membrane folds (Patrick et al., 2006).

DH31

Diuretic hormone 31 (DH31) has been identified as the *Drosophila* orthologue of an insect diuretic hormone that is functionally similar to vertebrate calcitonin, though the sequence similarity between these compounds is limited to six residues (Furuya et al., 2000). DH31 increases *Drosophila* Malpighian tubule fluid secretion and is associated with the activation of an apical V-ATPase involved in electrogenic translocation of protons into the lumen (Coast et al., 2001). This interaction is mediated by increased intracellular cAMP levels and is a common mechanism of tubule regulation among species (Coast et al., 2001; Linton and O'Donnell, 1999). Dames et al. (2006) have confirmed the sufficiency of a rise in intracellular cAMP for the recruitment of the V-ATPase's V_1 subunit to the apical membrane for holoenzyme assembly in the salivary glands of *Calliphora*. Mechanistically, this interaction might depend on the activation of protein kinase A (PKA) by cAMP; Voss et al. (2007) propose that activated PKA

phosphorylates free C subunits of the V_1 complex, initiating holoenzyme assembly via this subunit's interactions with subunits E and G of the V_1 complex and subunit a of the V_0 complex (Inoue and Forgac, 2005). The half-maximal effective concentration (EC_{50}) of DH31 for its stimulation of fluid secretion by *Drosophila* Malpighian tubules was 4.3 nM, a level within the typical range for neurohormones (Coast et al., 2001).

In *Anopheles* mosquitoes, DH31 stimulates natriuresis by the Malpighian tubules following a blood meal (Coast, 2009). The peptide has also been found to play a diuretic role in *Rhodnius*, where it stimulates a 17-fold increase in Malpighian tubule fluid secretion rate at a concentration of 10^{-8} M (Te Brugge et al., 2005). This increase is modest, however, given that it represents less than 2% of the increase elicited by 5-HT. The diuretic potency of DH31 is greater in *Diploptera* and *Locusta*, where Malpighian tubule fluid secretion rate increases to 41% and 50%, respectively, of the maximal rate achieved with the CRF-related peptide DH46 (Furuya et al., 2000). The EC_{50} values of DH31's activity were 9.8 nM and 0.56 nM in *Diploptera* and *Locusta*, respectively. Cations are suggested to be the primary targets for DH31-stimulated transport in the Malpighian tubules, with the activated V-ATPase driving secondary active transport via Na^+/H^+ and K^+/H^+ antiporters (O'Donnell et al., 1996). Research by Ianowski and O'Donnell (2004) suggests that a Na^+-driven $Na^+:K^+:2Cl^-$ cotransporter on the basolateral membrane of principal cells is the primary route of entry for all three ions. A Na^+/K^+-ATPase on the basolateral membrane returns most of the Na^+ to the haemolymph, with the remainder being secreted across the apical membrane (Ianowski and O'Donnell, 2004). The diuretic action of DH31 can thus be explained by the movement of water into the lumen as a passive osmotic consequence of secondary active transport of ions across the apical membrane (O'Donnell et al., 1982). The

resulting secreted fluid is approximately isosmotic with the haemolymph (Linton and O'Donnell, 1999).

Endocrine cells producing DH31 have been found throughout the midgut of larval *Drosophila*, particularly in the anterior midgut and the junction between the anterior and middle midgut (Figure 2; Veenstra, 2009). The peptide is also strongly expressed in the larval central nervous system (CNS; Chintapalli et al., 2007). The DH31-producing cells in the junction have been found by LaJeunesse et al. (2010) to be responsible for the stimulation of peristalsis in this region, a response that required the presence of functional DH31 peptide. Morphologically, the junction acts as a valve regulating the movement of ingested material into the middle midgut (LaJeunesse et al., 2010). Cells in the posterior midgut show only weak immunoreactivity to DH31 antisera, suggesting that this region does not significantly contribute to the production of this peptide (Veenstra, 2009). Interestingly, transition of *Drosophila* larvae into the wandering stage coincides with a dramatic reduction in DH31 production throughout the gut (Veenstra, 2009). The specific locations of DH31 receptor expression have not been mapped, however it is known to be expressed in the crop, midgut, hindgut, and Malpighian tubules of adults (Johnson et al., 2005), and in the latter three structures in larvae (Chintapalli et al., 2007). DH31 receptor expression is weaker in larvae than in adults, however. Since DH31 is produced by midgut endocrine cells and the CNS, the peptide might act as a paracrine signal in the midgut but as an endocrine signal in the Malpighian tubules and hindgut (Veenstra et al., 2008).

Drosokinin

Leucokinins are a class of neuropeptides that are widespread among insects. They were initially characterized for their myotropic activity in the hindgut of the cockroach *Leucophaea maderae*, and were subsequently found to promote diuresis in the Malpighian tubules of *Aedes aegypti* and *Acheta domesticus* (Holman et al., 1987; Hayes et al., 1989; Coast et al., 1990). Leucokinins range in length from six to fifteen amino acids, and a variable C-terminal pentapeptide domain (F*XX*WGamide) similar to that of vertebrate tachykinins endow species-specific leucokinins with myotropic and diuretic activities (Radford et al., 2002; Nachman et al., 1995). The latter activity stimulates Malpighian tubule fluid secretion in several insect species, increasing the conductance of the tubule epithelium to Cl^- ions via intracellular Ca^{2+} signalling (Hayes et al., 1989; O'Donnell et al., 1996, 1998; Pannabecker et al., 1993; Dow and Davies, 2003). Among the various leucokinins, the *Drosophila* leucokinin (drosokinin, DK) most resembles the *Aedes* leucokinin I, a peptide known to stimulate both Malpighian tubule fluid secretion and hindgut contractions in that species (Veenstra et al., 1997). Drosokinin exposure stimulates a rapid (<100 ms) increase in stellate cell intracellular Ca^{2+} levels with an EC_{50} value between 10^{-10} and 10^{-9} M (Radford et al., 2002; Terhzaz et al., 1999). Drosokinin does not affect intracellular Ca^{2+} concentration in principal cells (Terhzaz et al., 1999). Three chloride channels of the CLC family are expressed in *Drosophila* tubules, with some or all of these channels facilitating entry of Cl^- across the basolateral membrane of stellate cells in the Malpighian tubule's main segment (Dow and Davies, 2003). Terhzaz et al. (1999) determined that synthetic drosokinin elicits a three-fold increase in Malpighian tubule fluid secretion with an EC_{50} between 10^{-11} and 10^{-10} M. The secretion rate increases to a maximum after approximately ten minutes of drosokinin exposure and is sustained at an elevated level for at least a further twenty minutes (Terhzaz et al., 1999).

The drosokinin receptor is located basolaterally on the stellate cells of the Malpighian tubules, in the reproductive organs of both sexes, and in the larval pars intercerebralis, a brain region that is implicated in hormone release (Radford et al., 2002; Zitnan et al., 1993). Cells in the anterior midgut and the posterior midgut neutral zone are strongly immunoreactive to antisera of both short neuropeptide F (sNPF) and the leucokinin receptor (Figure 2; Veenstra, 2009). The immunoreactivity observed by the endocrine cells in these regions is reportedly of greater intensity than that of the stellate cells of the Malpighian tubules (Veenstra, 2009). Immunoreactivity to the leucokinin receptor is notably absent from the midgut of adult *Drosophila* (Veenstra, 2009), and the genes encoding the leucokinin precursor are not highly expressed (Chintapalli et al., 2007). Endocrine cells immunoreactive to the leucokinin receptor are also recognized by allatostatin B and C antisera, particularly in wandering stage *Drosophila* larvae (Veenstra, 2009).

The larval midgut does not display immunoreactivity to leucokinin itself, though this antiserum was recognized within the CNS, where leucokinin is strongly expressed (Veenstra, 2009; Chintapalli et al., 2007). De Haro et al. (2010) localized leucokinergic neurons to four distinct groups within the CNS. Three groups are in the larval brain, corresponding to the lateral protocerebrum, the lateral horn, and the suboesophageal ganglia (de Haro et al., 2010). The neurons of the first group innervate the corpora cardiaca, possibly facilitating drosokinin release from these neurohaemal organs during larval development (de Haro et al., 2010). Innervation of mushroom bodies by the lateral horn leucokinin neurons suggests a role for this neuropeptide in olfaction, while leucokinin neurons of the suboesophageal ganglia relay afferent gustatory signals (de Haro et al., 2010; López-Arias et al., 2011). The fourth group of leucokinergic

neurons consists of seven pairs in the larval abdomen, with one axon from each neuron innervating the body wall muscle 8 (de Haro et al., 2010). Being proximal to both the spiracles and the posterior pair of Malpighian tubules, these authors suggest that this group of neurons is ideally located to exert paracrine control of water balance on these structures. Drosokinin may thus be released from the CNS to bind to receptors in both the gut and the Malpighian tubules, acting as an endocrine factor on the former and a paracrine factor on the latter (Veenstra, 2009).

Leucokinin has been implicated in meal termination signalling in adult *Drosophila*, where mutation of the gene for the peptide or its receptor causes flies to consume larger but fewer meals (Al-Anzi et al., 2010). These authors propose that neurons expressing the leucokinin receptor in the brain and ventral ganglia mediate the brain's sensitivity to signals from stretch receptors in the gut, thereby affecting its response to a satiety stimulus. López-Arias et al. (2011) also showed that inhibition of leucokinin release from abdominal neurons enhanced desiccation-resistance in adult *Drosophila*, likely due to reduced excretory water loss associated with the elimination of basal peptide release.

Allatostatin A

The allatostatins have pleiotropic effects on physiological processes in insects. They were first identified as potent inhibitors of juvenile hormone synthesis by the corpora allata, in opposition to the stimulation of this hormone's synthesis by the allatotropins; juvenile hormone is involved in the developmental progression of larvae to mature adults (Kramer et al., 1991). Allatostatins have been implicated in the decreased synthesis of juvenile hormone from the corpora allata during the final five days of the larval stage of the tobacco hornworm *Manduca sexta* (Janzen et

al., 1991), and have similar effects in other insects such as the cockroach *Diploptera punctata* (Stay et al., 1996), but this effect has not been observed in *Drosophila* (Veenstra et al., 2008). A-, B-, and C-type allatostatins have been identified to date.

Allatostatin A (also known as FGLa-type allatostatin; Coast and Schooley, 2011) was initially characterized for its dose-dependent reduction in both the frequency and amplitude of myogenic hindgut contractions in the cockroach *Diploptera* (Lange et al., 1993). The physiological activity of allatostatin A depends on a conserved C-terminal F/Y*X*FGLamide motif that is shared among its homologues (Audsley and Weaver, 2009). The threshold allatostatin A concentration for its myoinhibitory effect on the *Diploptera* hindgut was at least 10^{-8} M while maximal inhibition was reached between 10^{-5} and 10^{-4} M (Lange et al., 1993). Moreover, allatostatin A at 10^{-6} M was capable of reversing the stimulation of hindgut contractions in this species by proctolin, a peptide that stimulates gut contractions in several insects. Immunostaining revealed allatostatin-like reactivity in the proctodeal nerve, which innervates both the hindgut and rectum with nerve endings between the circular and longitudinal muscle layers (Lange et al., 1993). The *Schistocerca gregaria* homologue of allatostatin A inhibited oviduct contractions in this locust species (Veelaert et al., 1996). Interestingly, however, five isoforms of *Diploptera punctata* allatostatin A did not have myoinhibitory effects in the oviducts of either *Diploptera* or *Locusta* (Lange et al., 1995). Similarly, isoforms of the *Calliphora vomitoria* homologue of this peptide inhibited contractions of the *Leucophaea* foregut but not of the hindgut (Duve et al., 1995). Thus, the tissue-specific actions of allatostatin A are not consistent among species.

Allatostatins also modulate feeding behaviour in several insects. For example, feeding is inhibited in larvae of the lepidopteran *Lacanobia oleracea* by allatostatin C and in adult *Blatella germanica* cockroaches by allatostatin A (Audsley et al., 2001; Aguilar et al., 2003). This effect may be associated with the retardation of food movement through the gut lumen due to the peptides' inhibition of gut peristalsis; myoinhibitory activity in the gut has been ascribed to allatostatins C and A in *Lacanobia* and *Blatella*, respectively (Audsley et al., 2001; Aguilar et al., 2003). Hergarden et al. (2012) showed that activation of allatostatin A neurons inhibits feeding in adult *Drosophila*, however this effect was not reproducible by injection with synthetic peptide.

Neurons expressing allatostatin A have been localized to several structures in the adult blowfly *Calliphora vomitoria*, including the medulla of the optic lobes, the suboesophageal and thoracicoabdominal ganglia, and the corpus cardiacum (Duve and Thorpe, 1994). Muscles of the hindgut and heart are innervated by neurons expressing this peptide, which was also found to inhibit ileal peristalsis at concentrations between 10^{-16} and 10^{-13} M (Duve and Thorpe, 1994). Moreover, a group of allatostatin A-producing endocrine cells were localized to the most caudal area of the posterior midgut (Duve and Thorpe, 1994). The widespread distribution of allatostatin A expression in the blowfly led these authors to suggest that this peptide may have multiple functions. Indeed, in contrast to its inhibitory roles in various tissues, allatostatin A has been reported to stimulate the lumenal activity of invertase and amylase in the *Diploptera* midgut (Fusé et al., 1999). The increased activity of these carbohydrate-metabolizing enzymes may reflect enhanced secretion from midgut endocrine cells or activation via phosphorylation (Fusé et al., 1999).

Study of the cellular localization of allatostatin A in the lepidopteran nervous system has revealed patterns of expression that are well suited to the regulation of gut activity. Duve et al. (1999) reported robust allatostatin A expression in the frontal ganglion of several moth species. In *Helicoverpa armigera*, for example, the neuropeptide is expressed in two pairs of neurons descending from the frontal ganglion that terminate at the stomodeal valve after traversing the crop (Duve et al., 1999). Axons also extend from the crop and terminate on longitudinal muscles within the anterior midgut and on the proctodeal valve. As in the cockroach, allatostatin A has been ascribed a myoinhibitory role in this moth species; allatostatin A suppresses peristaltic foregut contractions with half-maximal inhibition between 10^{-10} and 10^{-9} M (Duve et al., 1999). Endocrine cells expressing allatostatin A are present at the extreme ends of the midgut in *Helicoverpa*, but are absent from the foregut and hindgut (Duve et al., 1999). A similar myoinhibitory function has been reported in larval *Manduca*, where allatostatin A is expressed in the brain and abdominal ganglia. In the latter structure, its expression is colocalized with leucokinin and the *Manduca* diuretic hormone in the third lateral neurosecretory cell (L3) of the first through sixth abdominal ganglia (Davis et al., 1997). Allatostatin A is also expressed by the median neurosecretory cell of the ninth neuromere (M_{A9}) and the third lateral neurosecretory cell of the eighth neuromere ($L3_{A8}$) of the terminal abdominal ganglion (Davis et al., 1997). Given that these cells project into the cryptonephridial chamber, and that allatostatin A is colocalized with known diuretic factors, it is plausibly involved in osmoregulation and fluid transport (Davis et al., 1997). Indeed, allatostatin inhibits both peristalsis and epithelial K^+ transport by the locust gut (L. Robertson, A.B. Lange, A. Donini, personal communication).

In larval *Drosophila*, allatostatin A production is confined to cells in the alkaline zone of the posterior midgut and in the hindgut (Figure 2; Veenstra, 2009; Chintapalli et al., 2007). Allatostatin A4, the only one of the four *Drosophila* isoforms for which expression data is available, is also strongly expressed in the larval CNS (Chintapalli et al., 2007). Immunocytochemical localization of allatostatin A neurons in *Drosophila* larvae revealed expression in the ventral and suboesophageal ganglia, but not innervation of the midgut (Yoon and Stay, 1995). Consistent with the findings of Veenstra (2009), these authors reported approximately 40 endocrine cells in the posterior midgut epithelium that strongly express allatostatin A. Lenz et al. (2001) confirmed the existence of at least two *Drosophila* allatostatin A receptors (DAR). Expression of DAR-1 is confined to the brain and thoracicoabdominal ganglion in adults, but is essentially absent in larval *Drosophila* (Chintapalli et al., 2007). DAR-2 is expressed in the Malpighian tubules, midgut, and hindgut of larval *Drosophila*, suggesting that allatostatin A may play a role in the inhibition of muscle contraction or in the regulation of water balance (Chintapalli et al., 2007; Veenstra et al., 2008). Of the four *Drosophila* isoforms, allatostatin A4 most effectively initiates a second messenger cascade upon binding to DAR-2 with an EC_{50} of 10^{-8} M (Lenz et al., 2001).

Hypothesis

The *Drosophila* neuropeptides DH31 and drosokinin are known to stimulate fluid secretion by the Malpighian tubules in this species. These peptides promote the secretion of K^+ and Na^+ ions across the Malpighian tubule epithelium, creating osmotic gradients that favour the diffusion of water into the tubule lumen (see Coast et al., 2001; Terhzaz et al., 1999). Increased outflow of ions and water through the Malpighian tubules is expected to be complemented by enhanced

fluid uptake across the midgut epithelium. In this way, the diuretic activities of DH31 and drosokinin would be coordinated between the gut and renal systems, maintaining haemolymph volume and osmolarity within homeostatic limits. Since K^+ is the main dietary cation of larval *Drosophila*, changes in K^+ flux across the gut epithelium are expected to contribute to, and thus indicate, changes in the osmotic flow of water between the gut lumen and the haemolymph.

If these peptides function as true diuretics, they are expected to promote cation and water secretion across the ileal epithelium. Given that the Malpighian tubules also play a major role in the elimination of toxins and wastes from the haemolymph, these peptides may alternatively act as haemolymph clearance factors. In this capacity, they are expected to stimulate fluid secretion by the tubules followed by reabsorption of ions and water in the ileum with wastes remaining in the lumen for excretion. Because myostimulatory activities have also been attributed to these peptides (e.g. LaJeunesse et al., 2010; Veenstra et al., 1997), they are additionally expected to increase the frequency of gut contractions in larval *Drosophila*. This effect may enhance the circulation of neuropeptides and ions in the haemolymph to augment diuresis (e.g. Te Brugge et al., 2008). The expression of DH31, drosokinin, and their respective receptors in the CNS, midgut, hindgut, and Malpighian tubules supports their postulated involvement in water balance regulation in the gut (see Veenstra, 2009; Chintapalli et al., 2007).

Allatostatin A is known to inhibit physiological processes in insects, including juvenile hormone synthesis and muscle contractions in various tissues (see Janzen et al., 1991; Lange et al., 1993; Veelaert et al., 1996). Expression of allatostatin A and its receptor in the midgut, hindgut, and Malpighian tubules of larval *Drosophila* (Veenstra, 2009; Chintapalli et al., 2007) suggests that it

may also contribute to the regulation of water balance. Thus, this peptide is expected to alter epithelial K^+ transport in the midgut to drive the flow of osmotically-obliged water between the gut lumen and haemolymph.

Objectives

The goal of this study was to characterize DH31, drosokinin, and allatostatin A in the larval *Drosophila* gut with respect to their effects on epithelial K^+ transport. The effects of the former two peptides on contraction frequency of the anterior midgut and pyloric sphincter were also investigated.

Figure 1. Schematic diagram of a dissected larval *Drosophila melanogaster* gut. J, junction between anterior and middle midgut; CC, copper cells; LFC, large flat cells (adapted from Naikkhwah and O'Donnell, 2012).

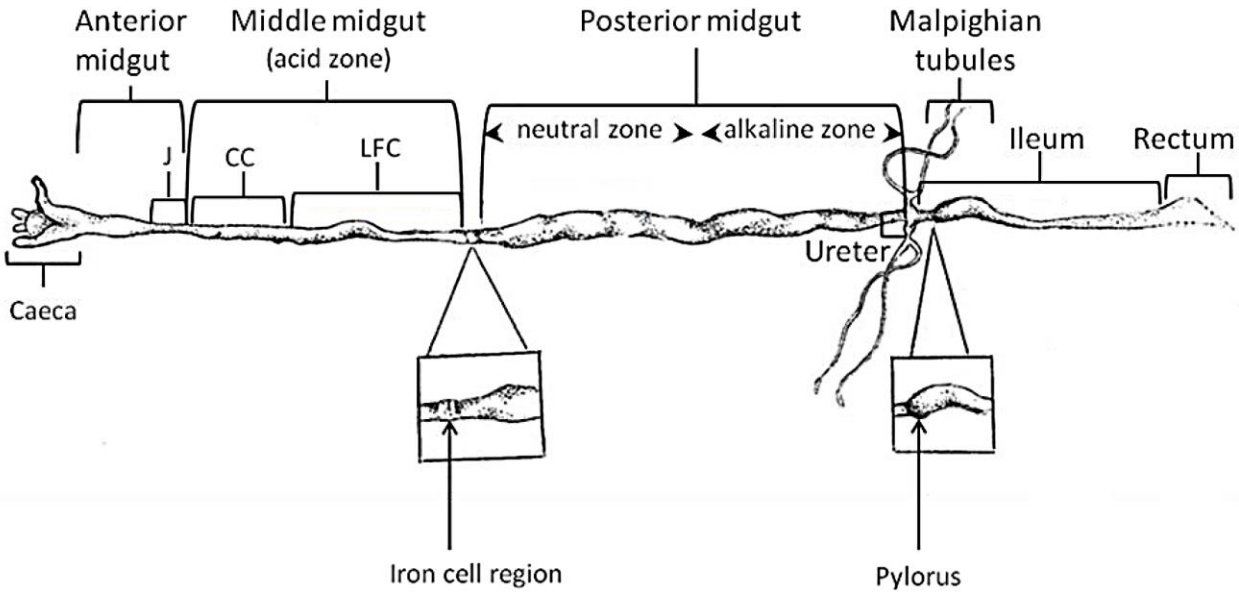
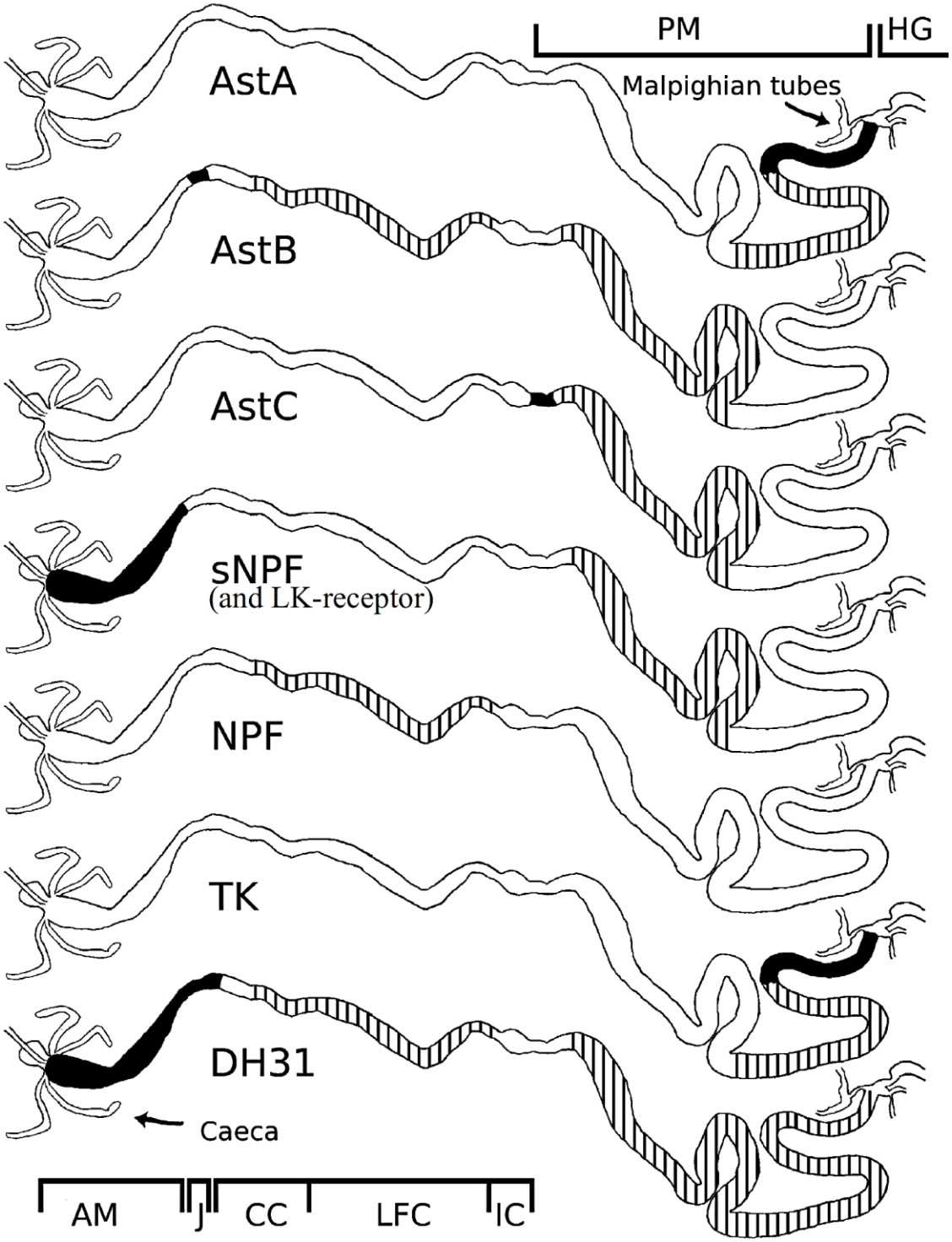

Anterior midgut
Middle midgut (acid zone)
Posterior midgut
Malpighian tubules
neutral zone
alkaline zone
Ileum
Rectum
J
CC
LFC
Ureter
Caeca
Iron cell region
Pylorus

Figure 2. Distribution of peptide expression in the midgut epithelium of third instar *Drosophila* larvae. Blackened and hatched areas are regions of strong and moderate peptide expression, respectively. AstA, allatostatin A; AstB, allatostatin B; AstC, allatostatin C; sNPF, short neuropeptide F; LK, leucokinin; NPF, neuropeptide F; TK, tachykinin; DH31, diuretic hormone 31; AM, anterior midgut; CC, copper cells; HG, hindgut; IC, iron cells; J, junction between anterior and middle midgut; LFC, large flat cells; PM, posterior midgut (Veenstra, 2009).


PM
HG
Malpighian tubes
AstA
AstB
AstC
sNPF
(and LK-receptor)
NPF
TK
DH31
Caeca
AM
J
CC
LFC
IC

Materials and Methods

Experimental organisms

Third instar foraging *Drosophila melanogaster* larvae of the Oregon R strain were used for all experiments. Larvae were raised in laboratory culture at 21-23°C on standard artificial diet described by Roberts and Stander (1998). The diet consisted of a mixture of solutions A and B. Solution A consisted of 0.5 g $CaCl_2$, 0.5 g $MgCl_2$, 0.5 g NaCl, 1 g KH_2PO_4, 8 g KNa tartrate, 18 g agar, 100 g sucrose, and 800 ml tap water. Solution B consisted of 50 g dry active yeast in 200 ml tap water. Each solution was autoclaved then mixed with stirring. The mixture was cooled while stirring to 55°C, at which time 10 ml of acid mixture and 7.45 ml of 10% *p*-hydroxybenzoic acid methyl ester (Tegosept) dissolved in ethanol were added. The acid mixture consisted of 11 parts tap water, 10 parts propionic acid, and 1 part 85% *o*-phosphoric acid.

Dissection

Larvae were dissected under *Drosophila* saline by gripping neighbouring abdominal tergites using forceps then tearing open the cuticle and allowing the gut to uncoil. The saline consisted of (in mM) 10 glutamine, 20 glucose, 15 HEPES, 4.3 NaH_2PO_4, 10.2 $NaHCO_3$, 8.5 $MgCl_2$, 2 $CaCl_2$, 20 KCl, and 117.5 NaCl adjusted to pH 7.0. The dissected gut of each larva was transferred from the dissection dish to a 35 mm Petri dish (BD Biosciences Canada, Mississauga, ON) containing *Drosophila* saline held within a glue enclosure to facilitate the use of small saline volumes. The 35 mm Petri dishes were pre-coated with 70 µl of 62.5 µg ml^{-1} poly-L-lysine (70-150 kDa, Sigma-Aldrich, St. Louis, MO) and air dried before filling with saline. This treatment allowed the dissected tissue to adhere to the bottom of the dish.

Ramsay assay

Larval Malpighian tubules were dissected using forceps as described by Dow et al. (1994) for adult flies. A fine glass probe was used to transfer each pair of tubules joined by a common ureter to a 20 µl droplet of *Drosophila* saline held under paraffin oil. The saline droplet was held within a small well cut into the bottom of a petri dish lined with Sylgard (Dow Corning Corp., Midland, MI). Each tubule pair was arranged such that the end of one tubule was wrapped around a 0.15 mm diameter minuten pin held in the Sylgard while the main segment of the other tubule was bathed in the saline droplet. The common ureter was positioned between the minuten pin and the saline droplet such that a droplet of secreted fluid could accumulate at the ureter opening without fusing with the saline droplet.

A glass probe was used to remove the droplet of secreted fluid from the ureter opening and place it on the bottom of the petri dish. Fluid secretion rate (nl min^{-1}) was calculated based on the diameter of the secreted fluid droplet and the elapsed time. Droplet diameter (d) was measured using an ocular micrometer at 80x magnification and droplet volume was calculated as $\pi d^3/6$. The diameter of the secreted droplet was first measured after 90 minutes, after which either DH31 or drosokinin was added to the bathing droplet to a final concentration of 10 µM. A second droplet of secreted fluid was collected and measured after a subsequent 40-60 minutes.

Ion-selective microelectrodes

Micropipettes were produced by pulling 1.5 mm borosilicate glass capillaries (World Precision Instruments Inc., Sarasota, FL) using a P-97 Flaming/Brown pipette puller (Sutter Instruments Co., Novato, CA). The resulting micropipettes had a tip opening of ~5 µm and were

characterized by a short shank. Micropipettes were silanized with 50 µl of dichlorodimethylsilane applied to the interior of a glass Petri dish which was inverted over a group of approximately 26 micropipettes on the surface of a hot plate at 200°C. Silanized micropipettes were transferred to a desiccator after 30 minutes and stored until use.

Silanized micropipettes were made selective for K^+ ions by backfilling them with 150 mM KCl then tip-filling with a column (~500 µm) of K^+ ionophore I, cocktail B (Fluka, Buchs, Switzerland). Micropipettes were backfilled using a plastic 1 ml syringe pulled out to a fine tip over a low flame (Thomas, 1978).

Scanning ion-selective electrode technique (SIET)

The ion-selective microelectrode was connected to an amplifier headstage whose motion was controlled by three computerized stepper motors. The motors moved the electrode along the X, Y, and Z axes with submicron accuracy and repeatability. Automated Scanning Electrode Technique (ASET) software version 2.0 (Science Wares, Falmouth, MA) and hardware from Applicable Electronics (Forestdale, MA) were used to make SIET measurements and control the motion of the stepper motors. The SIET apparatus is illustrated in Figure 3. Ion-selective microelectrodes were calibrated prior to scanning each tissue. K^+-selective microelectrodes were calibrated in solutions of 150 mM KCl and 15 mM KCl with 135 mM NaCl. The slope for a ten-fold change in K^+ ion concentration was (mean±SEM) 54.6±0.3 mV (N=6). Reference electrodes were produced by pulling 8 cm borosilicate glass filamented capillaries on a vertical electrode puller (Narishige, Tokyo, Japan) to a ~1 µm tip. The micropipette tip was gently broken and enlarged to ~5 µm and was subsequently backfilled with 500 mM KCl.

Measurements of voltage gradients were made by moving the microelectrode tip perpendicularly from the tissue surface between two points separated by 50 μm. Each measurement consisted of a series of computer-controlled motions of the electrode. Initially, the microelectrode tip was positioned within 5 μm of the tissue surface, representing the inner limit of the microelectrode's 50 μm excursion. Positioning was followed by a 4.0 second wait period during which no measurements were made to allow the re-establishment of ion gradients at the tissue surface following the localized disturbance of the microelectrode movement. Naikkhwah and O'Donnell (2012) found that a wait time of at least 3 seconds was sufficient to allow the gradients to fully re-establish. The voltage at the microelectrode tip was recorded for 0.5 second following the wait period. The microelectrode was then moved at 200 μm s^{-1} to a position at the outer limit of the 50 μm range where another wait and sample period was completed. The move, wait, and sample cycle encompassing recordings at both extremes of the microelectrode excursion was completed in ~9.5 seconds and was repeated three times, requiring a total of ~28.5 seconds to measure the voltage gradient at each tissue site.

SIET measurements

Each scan consisted of a measurement of the voltage gradient at five sites on the tissue within the gut region of interest. Sites in the anterior midgut, posterior midgut neutral zone, and the ileum were separated by 200 μm, while sites in the posterior midgut alkaline zone as well as in the middle midgut copper cell and large flat cell zones were separated by 100 μm. The distance separating each site was selected to ensure that the distance between the first and fifth sites encompassed the majority of the length of the region of interest. Preliminary SIET scans measured the voltage gradient at two sites separated by 50 μm within each region of interest in

an effort to reduce variation in K^+ flux among sites. This protocol was discontinued due to concern that the results may not accurately represent the response of the entire gut region if the constituent tissue sites do not respond homogenously (Appendix 1). Regions of the gut were defined by morphological features in accordance with published literature (e.g. Figure 1 in Naikkhwah and O'Donnell, 2012; Figure 2 in Veenstra, 2009; Figure 1 in Shanbhag and Tripathi, 2009).

A total of four scans were performed on each tissue. The first scan was performed in 196 µl of *Drosophila* saline. Following completion of the initial scan, 4 µl of DH31 (GenScript, Piscataway, NJ), drosokinin (GenScript, Piscataway, NJ), or allatostatin A (AnaSpec, Fremont, CA) were added to the bathing saline to a final concentration of 1 µM. Tissues simultaneously exposed to DH31 and allatostatin A were bathed in 192 µl of *Drosophila* saline during the first scan, after which an 8 µl mixture of both peptides was added such that each was at a final concentration of 1 µM. An equal volume of *Drosophila* saline was added instead of the peptides to the bathing saline of control samples following completion of the first scan. The second, third, and fourth scans consisted of measurements of the voltage gradients at the same five tissue sites as the initial scan. A total of ~20 minutes was required to complete all SIET measurements on each tissue sample, with the initial measurement being made within 5 minutes of dissection. Stability of apical and basal membrane potentials as well as transepithelial potential of isolated midgut segments over a ~25 minute period suggests that preparations remain physiologically viable for a time period of at least this length post dissection (e.g. Figure 5 in Shanbhag and Tripathi, 2005; Figure 14 in Shanbhag and Tripathi, 2009). DH31 and drosokinin dissolved in *Drosophila* saline were stored in 50 µl aliquots at a concentration of 50 µM. Preliminary

experiments conducted with DH31 and drosokinin dissolved in a chromatography solvent consisting of 5.7 mM ACN and 13 μM TFA indicated that the solvent unacceptably altered epithelial K^+ flux relative to *Drosophila* saline (Appendix 2). Allatostatin A dissolved in water was stored in 25 μl aliquots at a concentration of 50 μM. All peptides were stored at -20°C until use.

The microelectrode tip was moved several hundred micrometers perpendicularly away from the tissue surface to a reference site following completion of the fourth scan. At this site, which is sufficiently distant from the tissue that the influence of epithelial K^+ flux is negligible, the voltage gradient was recorded across the microelectrode's 50 μm excursion in the same manner as when making measurements at the tissue surface. Although this voltage gradient should, in theory, be negligible, it is subtracted from the voltage gradients at the measurement sites in the calculation of epithelial K^+ flux to correct for microelectrode noise and any voltage drift during scanning.

Calculation of K^+ flux

The voltages recorded at the ends of the 50 μm range were amplified 1000-fold and then the voltage difference between the two points was calculated. A standard microelectrode calibration curve relating voltage to K^+ activity was used to convert the voltage difference across the 50 μm excursion to K^+ activity. The equation $[\Delta C=C_B10^{(\Delta V/S)}-C_B]$ was used to convert the voltage gradient into a K^+ concentration gradient. ΔC represents the concentration gradient in μmol cm^{-3}; C_B represents the background ion concentration (the average of the concentrations at all points) in mM; ΔV represents the voltage gradient measured at the tissue surface less the voltage

gradient at the reference site in μV; S is the slope of the electrode in μV. Although ion-selective microelectrodes measure ion activity rather than concentration, data can be expressed in units of concentration if the activity coefficient for both the calibration and experimental solutions is assumed to be similar. This assumption is valid because the saline and calibration solutions are of similar ionic strength.

Fick's first law of diffusion was used to calculate the K^+ flux based on the concentration gradient through the equation [$J_I=(D_I\Delta C)\Delta x^{-1}$]. J_I represents the net flux in pmol cm^{-2} s^{-1}; D_I is the diffusion coefficient of K^+ ($1.92*10^{-5}$ cm^2 s^{-1}; Lide, 2002); ΔC represents the concentration gradient in pmol cm^{-3}; Δx represents the excursion distance expressed in centimeters separating the points where voltage was recorded by the microelectrode. The calculation is diagrammed in Figure 4.

Measurement of contraction frequency

Muscle contractions in the anterior midgut and the pyloric sphincter were counted visually. Peller et al. (2009) used a similar technique to count contractions of the crop in adult *Drosophila*. In the current study, counting was performed either in real-time via a microscope-mounted video camera displayed on an external monitor or in video recordings of the monitor made with an Xacti VPC-PD2BK video camera (Sanyo Electric Co., Osaka, Japan). Counts were manually recorded on paper. Visual counting of tissue contractions was advantageous due to the ability to observe and count independent contractions over most of the gut region of interest. Preliminary attempts to measure gut contractions using a force transducer indicated that the tissue was too delicate for the successful application of this technique. Visual counting was also more time-

efficient than alternative techniques such as impedance measurements, which were limited to recording contractions of a small area of the tissue and were highly variable (Appendix 3).

Visual counting of contractions began within five minutes of tissue dissection. Recordings were made over four consecutive periods each lasting two minutes, requiring a total of ~8 minutes per preparation. Counts made during the initial two minute recording period were conducted with the preparation bathed in 196 µl of *Drosophila* saline. Upon completion of the first counting period, 4 µl of DH31 or drosokinin were added to the bathing saline to a final concentration of 1 µM. Preparations exposed to both DH31 and drosokinin simultaneously were bathed in 192 µl of *Drosophila* saline during the initial counting period, after which 8 µl of a mixture of the peptides was added to the bath such that both DH31 and drosokinin were present at concentrations of 1 µM. A contraction was defined as a momentary, simultaneous, and localized inward movement of the gut wall on both sides of the preparation with an amplitude equivalent to at least ~20% of the gut diameter.

Graphing and statistics

GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) was used to conduct one-way ANOVA with Dunnett's post-hoc tests. Values are expressed as mean+SEM. for the given number of samples (*N*). The significance level is $P<0.05$.

K^+ flux values measured at the five sites on each preparation were pooled to minimize error associated with slight changes in microelectrode position between SIET scans. The Control bar (white) in the figures represents the pooled K^+ flux from the initial SIET scan conducted on each

preparation in all treatments. The black bars represent the pooled K^+ flux from the third and fourth SIET scans conducted on each preparation in its respective treatment group (Saline, DH31, DK, AST-A, DH31+AST-A). Measurements from the second scan, which were made immediately following the addition of the test factor, were omitted. Given that the physiological response of the tissue may not immediately manifest following the addition of the test factor, omission of this data prevents its masking of a more robust response that becomes apparent after several minutes of exposure.

Contraction frequency data are similarly displayed, with the white bar representing the pooled contraction counts from the initial two minute counting period in all treatments. The black bars represent the pooled contraction counts for the third and fourth counting periods of each preparation in its respective treatment group (Saline, DH31, DK, DH31+DK). Counts made during the second counting period were omitted for the reason mentioned above.

Figure 3. Schematic diagram of the SIET apparatus. ISM, ion-selective microelectrode.

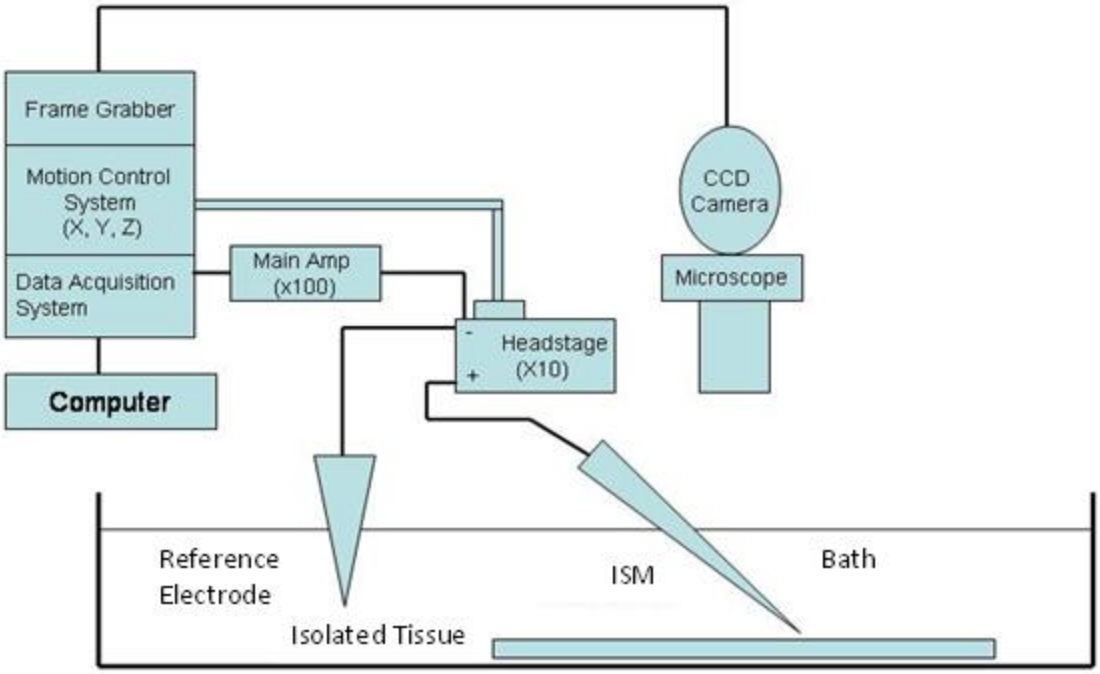
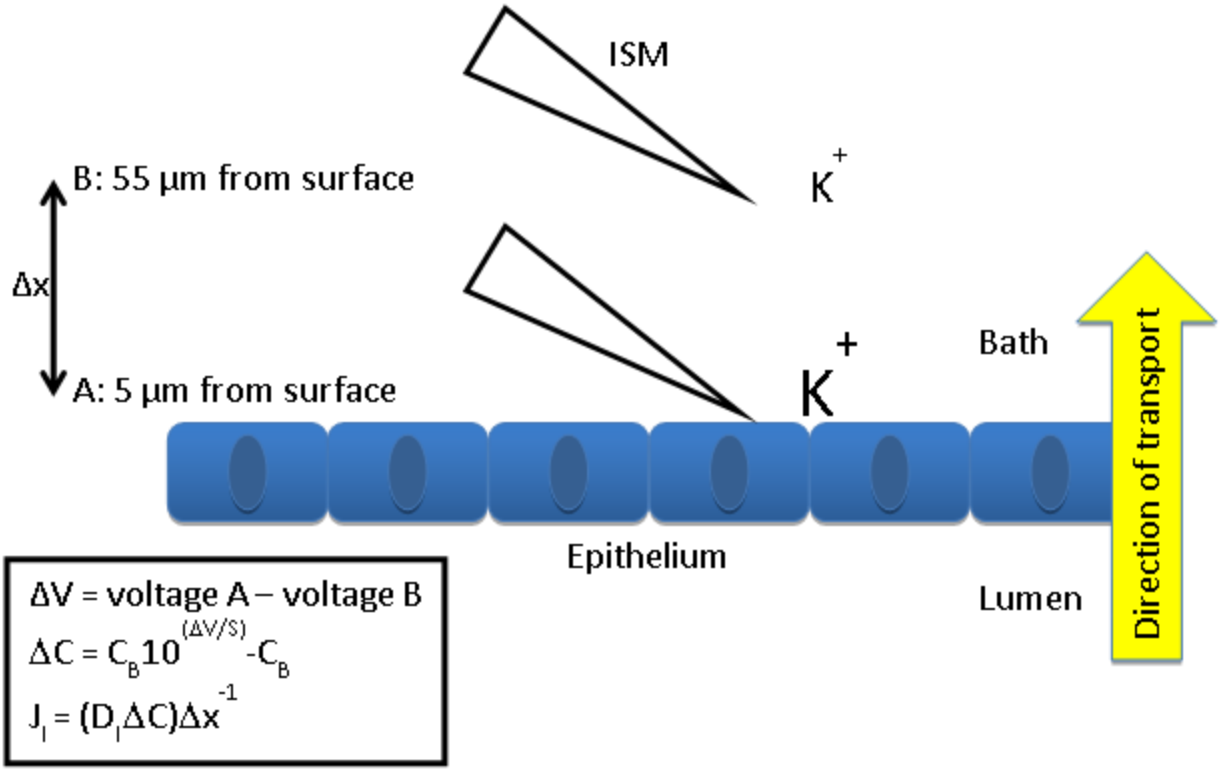

Frame Grabber
Motion Control System (X, Y, Z)
Data Acquisition System
Computer
Main Amp (x100)
Headstage (X10)
CCD Camera
Microscope
Reference Electrode
Isolated Tissue
ISM
Bath

Figure 4. Sample calculation of K^+ flux from SIET measurements. K^+ is being transported from the gut lumen into the bath (absorption), indicated by the greater K^+ potential at point A relative to point B. See text for a description of the calculation. ISM, ion-selective microelectrode.


ISM
B: 55 µm from surface
K+
Δx
K+
Bath
A: 5 µm from surface
Direction of transport
Epithelium
Lumen
ΔV = voltage A – voltage B
ΔC = C_B 10^(ΔV/S) - C_B
J_I = (D_I ΔC)Δx^-1

Results

Verification of peptide efficacy

The efficacy of the DH31 and drosokinin stocks were confirmed using the Ramsay assay before SIET measurements were made on the gut. The fluid secretion rates of isolated Malpighian tubules increased ~2-fold upon exposure to 10 µM DH31 or drosokinin for 40-60 minutes, consistent with the findings of Coast et al. (2001) and Terhzaz et al. (1999), respectively (Figure 5).

Effects of peptides on K^+-selective microelectrode measurements

Comparison of voltage gradients measured at reference sites that were greater than 1000 µm distant from the preparation suggests that none of the three peptides interfered with voltages recorded by K^+-selective microelectrodes during SIET measurements (Figure 6). The average of the voltage gradients measured across the microelectrode's 50 µm excursion at the tissue surface during the third and fourth SIET scans were (mean±SEM, in µV) 114.9±22.7 for preparations bathed in *Drosophila* saline, 74.0±6.6 for preparations bathed in 1 µM DH31, 70.3±10.0 for preparations bathed in 1 µM drosokinin, and 107.4±9.4 for preparations bathed in 1 µM allatostatin A (*N*=12). The average reference site voltage gradient for each treatment was (mean±SEM, in µV) 0.2±1.9, -0.3±1.2, 4.1±1.1, and 2.6±1.8, respectively (*N*=12). Each of these averages is comprised of the pooled data from two preparations from each the six gut regions in their respective treatments. Voltage gradients at the tissue surface and the reference site were compiled from data from the same twelve preparations. Non-zero voltage gradients at the reference site typically result from slow drift of the voltage of either the K^+-selective or reference microelectrodes, which can be caused by slow evaporation of the backfilling solution. The ratio

between the voltage gradient recorded at the tissue surface and at the reference site suggests the degree of confidence with which the voltage signal can be attributed to K^+ transport across the epithelium. Given the average voltage gradients at the tissue and at the reference site, the signal:noise ratio was 589:1 for the saline treatment, 256:1 for the DH31 treatment, 17:1 for the drosokinin treatment, and 41:1 for the allatostatin A treatment. Small peristaltic contractions of the preparation during SIET measurements may have disturbed the unstirred layer during some SIET scans, leading to an underestimation of voltage gradients at the tissue surface by K^+-selective microelectrodes.

Anterior midgut

All three peptides stimulated significant increases in K^+ absorption across the anterior midgut epithelium. DH31 was associated with a 56% increase in K^+ absorption whereas drosokinin and allatostatin A were associated with increases of 42% and 69%, respectively (Figure 7A). The involvement of an epithelial V-ATPase in driving K^+ transport in the anterior midgut was probed by measuring H^+ gradients with SIET (Appendix 4). The application of DH31 stimulated H^+ absorption across the epithelium, however the data represent the responses of two closely spaced sites on the tissue. Thus, the data may not adequately represent the entire region and is susceptible to variation because of ion-selective microelectrode positioning error.

The frequency of anterior midgut contractions was significantly higher after the addition of DH31 and drosokinin. The peptides stimulated increases in contraction frequency of 741% and 329%, respectively, when they were present in the bathing saline individually. When added

simultaneously, a 505% increase in contraction frequency was observed (Figure 8A). The increase elicited by the addition of *Drosophila* saline to the bath was not statistically significant.

Middle midgut

DH31 and allatostatin A produced significant but opposite effects on K^+ flux across the copper cell (anterior) portion of the middle midgut. DH31 reduced K^+ absorption by 70% compared to the control while allatostatin A exposure resulted in a 64% increase (Figure 7B). The decreases in K^+ flux in the *Drosophila* saline and drosokinin treatments were not statistically significant. In the large flat cell (posterior) portion of the middle midgut, K^+ absorption was reduced 65% by exposure to allatostatin A (Figure 7C). The trends towards lower fluxes in response to the other peptides were not statistically significant.

Posterior midgut

Only drosokinin exposure produced a significant change in the magnitude of K^+ flux in the neutral zone within the posterior midgut, increasing K^+ absorption by 60% relative to the control level. The effects of DH31 and allatostatin A were negligible (Figure 7D). In the alkaline zone within this region, K^+ absorption was stimulated by adding saline or any of the test factors except drosokinin. Addition of *Drosophila* saline, DH31, and allatostatin A elicited increases in K^+ flux of 52%, 87%, and 58%, respectively. Simultaneous addition of DH31 and allatostatin A produced a 64% increase in K^+ absorption over the control (Figure 7E). However, the increases attributed to DH31 and/or allatostatin A could not be statistically differentiated from the increase produced by the addition of *Drosophila* saline (one-way ANOVA with Tukey's post-hoc test, N=6-33, P<0.05, data not shown).

Ileum

The addition of *Drosophila* saline and DH31 caused statistically significant reversals of the direction of K^+ flux in the ileum. Both of these test factors resulted in weak absorption of K^+ across the anterior ileum epithelium, in contrast to this ion's secretion during the control scans (Figure 7F). Although the responses to saline and DH31 differed from the control value indicated by the white bar, there were no significant differences between the treatments indicated by the black bars in Figure 7F (one-way ANOVA with Tukey's post-hoc test, N=6-25, P<0.05, data not shown).

The frequency of pyloric sphincter contractions increased by 172% upon the simultaneous addition of DH31 and drosokinin to the bath (Figure 8B). The small increases in contraction frequency recorded following the addition of *Drosophila* saline, DH31, or drosokinin were not statistically significant, although qualitative observations indicated that drosokinin increased the amplitude of pyloric sphincter contractions.

Figure 5. Fluid secretion rate of isolated Malpighian tubules bathed in *Drosophila* saline, 10 μM DH31, or 10 μM drosokinin. An asterisk indicates statistical significance between the saline (white) and peptide treatment (black) bars ($P<0.05$, $N=10\text{-}14$). DH31, diuretic hormone 31; DK, drosokinin.

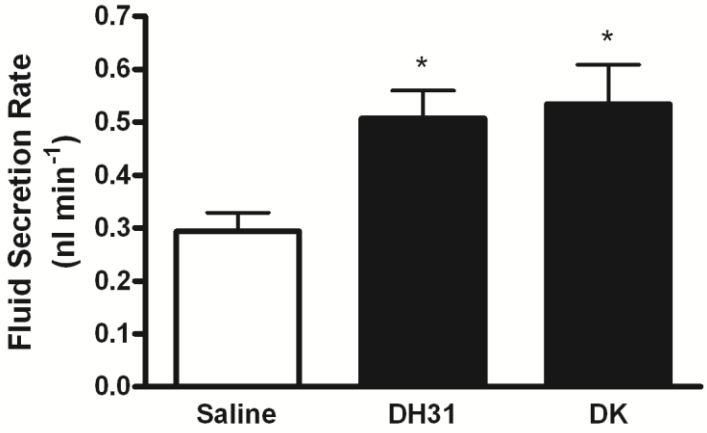

Fluid Secretion Rate
(nl min-1)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
Saline
DH31
DK

Figure 6. Voltage gradient recorded at the reference site for preparations bathed in *Drosophila* saline or 1 μM DH31, drosokinin, or allatostatin A. An asterisk indicates statistical significance between the saline (white) and peptide treatment (black) bars ($P<0.05$, $N=12$). DH31, diuretic hormone 31; DK, drosokinin; AST-A, allatostatin A.

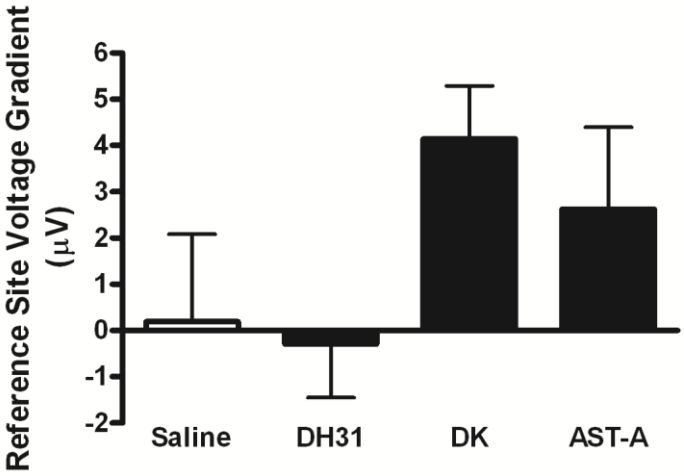

Reference Site Voltage Gradient
(μV)
6
5
4
3
2
1
0
-1
-2
Saline
DH31
DK
AST-A

Figure 7. K^+ flux across the anterior midgut (A), middle midgut copper cells (B) and large flat cells (C), posterior midgut neutral zone (D) and alkaline zone (E), and ileum (F) epithelia of preparations bathed in *Drosophila* saline or 1 μM DH31, drosokinin, or allatostatin A. Preparations were bathed in *Drosophila* saline during control measurements (white bars). Subsequent measurements were made following the addition of one of the test factors (*Drosophila* saline, DH31, DK, AST-A, or DH31+AST-A) and are represented by black bars. Peptides were present at a concentration of 1 μM in the bathing saline. An asterisk indicates statistical significance between the control and each treatment ($P<0.05$, $N=6$-33). AMG, anterior midgut; MMG, middle midgut; PMG, posterior midgut; DH31, diuretic hormone 31; DK, drosokinin; AST-A, allatostatin A.

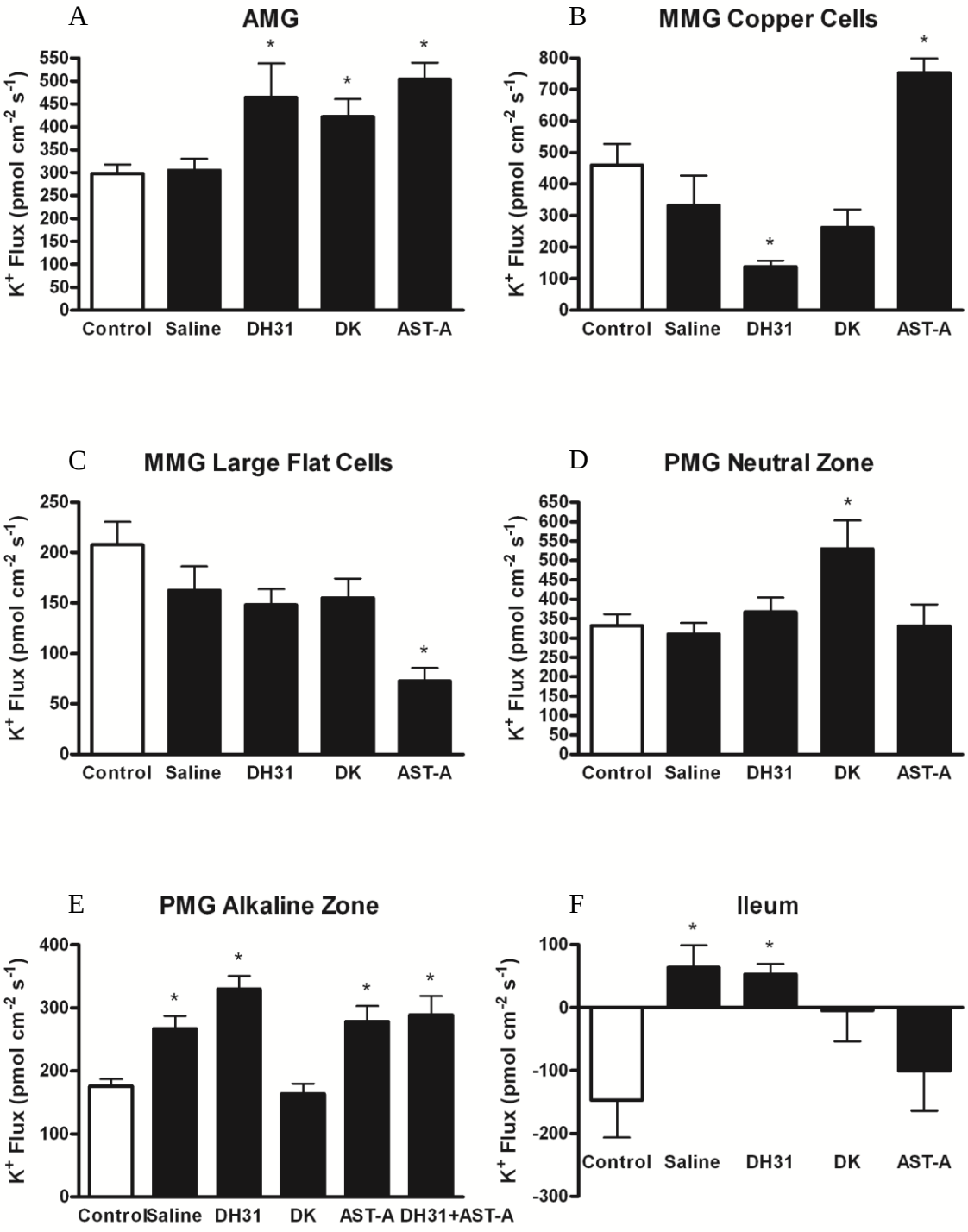
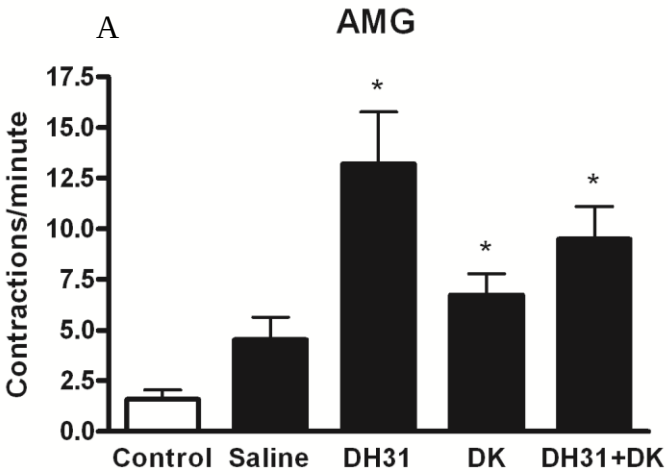
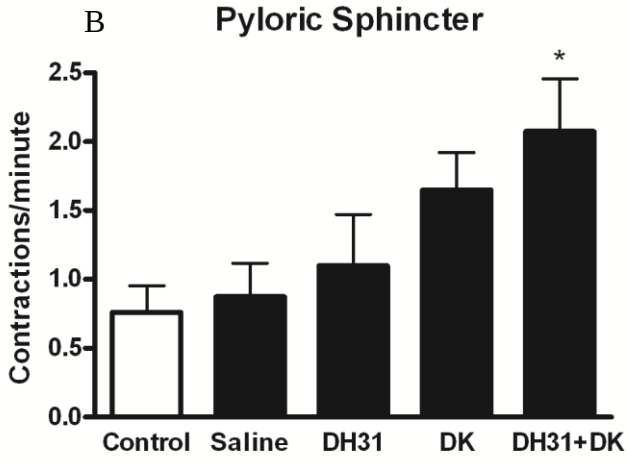

A
AMG
B
MMG Copper Cells
C
MMG Large Flat Cells
D
PMG Neutral Zone
E
PMG Alkaline Zone
F
Ileum
K+ Flux (pmol cm-2 s-1)
Control
Saline
DH31
DK
AST-A
DH31+AST-A

Figure 8. Contraction frequency of the anterior midgut (A) and pyloric sphincter (B) of preparations bathed in *Drosophila* saline or 1 μM DH31, drosokinin, or both DH31 and drosokinin. Preparations were bathed in *Drosophila* saline during control measurements (white bars). Subsequent measurements were made following the addition of one of the test factors (*Drosophila* saline, DH31, DK, or DH31+DK) and are represented by black bars. Peptides were present at a concentration of 1 μM in the bathing saline. An asterisk indicates statistical significance between the control and each treatment ($P<0.05$, $N=10\text{-}52$). AMG, anterior midgut; DH31, diuretic hormone 31; DK, drosokinin.

A


AMG
Contractions/minute
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
*
*
Control
Saline
DH31
DK
DH31+DK

B


Pyloric Sphincter
Contractions/minute
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Saline
DH31
DK
DH31+DK

Discussion

A possible role for DH31 and drosokinin in modulating gut ion transport or peristalsis was suggested by previous studies indicating the presence of the peptides and their receptors in specific regions of the larval gut. Endocrine cells expressing DH31 are present throughout the midgut and the peptide is also expressed in the hindgut and CNS (Veenstra, 2009; Chintapalli et al., 2007). The DH31 receptor is expressed in the midgut and hindgut as well as in the Malpighian tubules (Chintapalli et al., 2007). Drosokinin is strongly expressed by the larval CNS in abdominal leucokinergic neurons (de Haro et al., 2010), and its receptor is expressed in the anterior midgut and posterior midgut neutral zone (Veenstra, 2009). Although expression of drosokinin in the hindgut is weak, its receptor is strongly expressed in this region and in the Malpighian tubules (Chintapalli et al., 2007). Diuretic activity has been ascribed to both DH31 and drosokinin in *Drosophila* Malpighian tubules (Coast et al., 2001; Terhzaz et al., 1999). The current study is the first to demonstrate changes in epithelial K^+ flux in the gut in response to these peptides. With the assumption that epithelial K^+ gradients drive the flow of osmotic water, these changes are consistent with the promotion of diuresis. Additionally, this study has demonstrated a role for allatostatin A in altered epithelial K^+ transport. This peptide is known to be expressed by midgut endocrine cells and its receptor is present in the larval *Drosophila* midgut, hindgut, and Malpighian tubules. Lastly, DH31 and drosokinin increase the frequency of contractions in the larval gut. Figure 9 summarizes all of these results. This study reports changes in epithelial K^+ flux in the gut that are evidence for the presence of functional receptors for DH31, drosokinin, and allatostatin A in the midgut and for the coordination of diuretic activity between the midgut and Malpighian tubules.

DH31 stimulates K^+ absorption across the midgut epithelium

DH31 stimulated K^+ flux in the anterior midgut and inhibited K^+ flux in the middle midgut copper cell zone (Figure 9). The spatial distribution of DH31 receptor expression among the distinct regions of the midgut has not been previously reported. However, the presence of functional DH31 receptors can be inferred in the anterior midgut and the copper cell zone based on the changes in epithelial K^+ transport elicited by DH31 in these regions. Since this peptide is known to be expressed by endocrine cells in these regions, locally-produced DH31 might act as a paracrine factor. Given that DH31 did not significantly affect K^+ flux in either zone of the posterior midgut or in the ileum, this peptide's receptors may not be expressed in these regions or the peptide may influence processes that are unrelated to K^+ transport. Interestingly, the results suggest that addition of saline may enhance epithelial transporter activity in the alkaline zone and the ileum, possibly by transient stirring and oxygenation of the bath.

The physiological significance to diuresis of the effects of DH31 on midgut K^+ flux depends on the net movement of osmotically-obliged water across the anterior midgut epithelium, where DH31 was stimulatory, relative to that of the copper cell zone, where the peptide was inhibitory. While the fluid absorption assay described by Te Brugge et al. (2009) for the *Rhodnius* anterior midgut is ideally suited for such measurements, the general trend can be elucidated from the net transport of K^+ across the epithelia. Comparison of net K^+ transport in the anterior midgut and the copper cell zone requires the calculation of tissue surface area in the manner described by Naikkhwah and O'Donnell (2012). Qualitatively, however, the circumference and length of the anterior midgut far exceed those of the copper cell zone. It follows that the modest increase in K^+ absorption elicited by DH31 in the anterior midgut likely more than compensates for the

decrease in the copper cell zone owing to the former region's larger surface area. If K^+ transport drives the flow of osmotically-obliged water, net stimulation of K^+ absorption across the midgut epithelium by DH31 suggests that this peptide elicits a net increase in fluid uptake from the midgut lumen. Stimulation of K^+ transport by DH31 across both the midgut and the Malpighian tubule epithelia is consistent with a coordinated diuretic response to the peptide. Such a response would facilitate the preservation of haemolymph volume and osmolarity during diuresis.

Although DH31's reduction of K^+ absorption in the copper cell zone may seem anomalous in the context of diuresis, it is paralleled by the peptide's lack of stimulation of water absorption in the anterior midgut epithelium of *Rhodnius*, a species in which DH31 also stimulates Malpighian tubule fluid secretion (Te Brugge et al., 2009, 2005). Moreover, DH31 does not affect the short-circuit current, transepithelial voltage, or resistance of the *Rhodnius* anterior midgut, suggesting that the peptide has little or no effect on epithelial ion transport in this tissue (Te Brugge et al., 2009). In *Drosophila*, the reduction in K^+ absorption in the copper cell zone in response to DH31 may relate to a redistribution of fluid uptake among the regions of the gut. DH31 could reduce K^+ absorption in the copper cell zone concomitant with increases elsewhere to prevent the water content of the lumen from falling below an optimal level for nutrient absorption, digestive enzyme activity, or gut motility. Altering the water content of the midgut lumen via changes in epithelial K^+ transport may also be an indirect mechanism of pH homeostasis by changing the lumenal volume requiring acidification. This effect might be supplementary to the control of the activity of the basolateral V-ATPase that was reported in midgut epithelial cells by Shanbhag and Tripathi (2005). Alternatively, the reduction in K^+ absorption in the copper cell zone in response to DH31 may minimize increases in K^+ concentration in the haemolymph, thereby

maintaining optimal concentrations of ions for physiological functions. For example, MacVicker et al. (1993) found that a K^+:Na^+ ratio of 1:5.5 was optimal for the activity of a Na^+/K^+-ATPase in the basolateral membrane of midgut epithelial cells in *Anopheles.*

DH31 also stimulated a significant increase in the contraction frequency of the anterior midgut (Figure 9). This peptide has myostimulatory activity in the *Rhodnius* hindgut (Te Brugge et al., 2008), and also stimulates peristaltic contractions of the junction region of the midgut in larval *Drosophila* (LaJeunesse et al., 2010). Te Brugge et al. (2009) suggested that stimulation of contractions of the *Rhodnius* anterior midgut by diuretic neuropeptides contributes to mixing of the lumen and haemolymph to reduce unstirred layers and facilitate hormone circulation. In the *Drosophila* anterior midgut, the stimulation of contractions by DH31 likely supplements the increase in K^+ absorption elicited by this peptide by preventing both the depletion of K^+ ions in the unstirred layer adjacent to the epithelium's apical membrane and the accumulation of K^+ adjacent to the basolateral side. In addition to optimizing conditions for K^+ absorption, the contractions might facilitate the diffusion of endocrine factors through the haemolymph. Thus, the stimulation of contractions by DH31 likely augments its diuretic activity.

Drosokinin stimulates K^+ absorption across the midgut epithelium

Drosokinin stimulated K^+ absorption in the anterior midgut and the posterior midgut neutral zone (Figure 9). These findings are in agreement with Veenstra (2009), who reported that the anterior midgut and the neutral zone were the only regions of the midgut showing expression of the drosokinin receptor. Drosokinin is not expressed by midgut endocrine cells but is released from

nerve endings in the caudal abdomen, suggesting that it may diffuse through the haemolymph as an endocrine factor to bind its receptors in the midgut epithelium (de Haro et al., 2010).

Drosokinin stimulates Malpighian tubule fluid secretion in larval *Drosophila* (Terhzaz et al., 1999). In order to preserve haemolymph volume and osmolarity while increasing the organism's rate of fluid excretion, this peptide was expected to promote fluid uptake across the midgut epithelium. With the assumption that altered K^+ gradients drive the osmotic flow of water, the peptide's stimulatory effect on K^+ absorption in the midgut is consistent with the coordination of its diuretic activities between this tissue and the Malpighian tubules.

The lack of a significant effect attributable to drosokinin in the hindgut was unexpected given the very strong expression of this peptide's receptor in this region (Chintapalli et al., 2007). It is possible that this peptide's effect in the ileum is unrelated to its effects on fluid transport in other tissues, or it may alter the osmotic flow of water by affecting the epithelial transport of an unmeasured ion, such as Na^+; the direction of water flow across the ileal epithelium would be largely dependent on the relative strengths of the osmotic gradients of both K^+ and Na^+. Previous studies have demonstrated Na^+ absorption by the ileum (Naikkhwah and O'Donnell, 2012). An enhancement of Na^+ absorption by drosokinin would be expected to produce an osmotic gradient favouring water uptake from the ileal lumen to the haemolymph, and therefore promote antidiuresis. Alternatively, drosokinin might be released only in response to an environmental challenge not presented in the current study, such as highly water-saturated food. This response could be mediated by reduced sensitivity of drosokinin receptors in the hindgut, thereby preventing their activation at lower peptide concentrations that are associated with stimulation of

ion transport across the Malpighian tubule and midgut epithelia. Terhzaz et al. (1999) noted drosokinin's bimodal stimulation of Malpighian tubule fluid secretion at 10^{-11}-10^{-10} M and 10^{-7} M, as well as the especially wide concentration range over which its effects are evident. This bimodal response is reminiscent of the differing potencies of the three *Aedes* leucokinins in their stimulation of stellate cell Cl^- conductance and fluid secretion (Veenstra et al., 1997), although only one leucokinin is known in *Drosophila*. Reduced sensitivity of drosokinin receptors in the hindgut compared to those in the midgut and Malpighian tubules would allow for graded responses based on drosokinin's concentration in the haemolymph.

Like DH31, drosokinin increased the contraction frequency of the anterior midgut (Figure 9). The *Aedes* leucokinins have been reported to stimulate hindgut contractions in that species (Veenstra et al., 1997), but this is the first time that myostimulatory activity has been attributed to a leucokinin in *Drosophila*. In addition to facilitating epithelial fluid transport by minimizing unstirred layers, mixing of the haemolymph through tissue contractions would be particularly relevant to the diffusion of drosokinin through the haemolymph as an endocrine factor. Contractions of the anterior midgut and of the pyloric sphincter were also stimulated by the simultaneous presence of DH31 and drosokinin the bathing saline (Figure 9). In both of these regions, the stimulation of contractions was neither additive nor synergistic, suggesting that unlike in the Malpighian tubules, these peptides may activate a common second messenger in the midgut epithelium. In this case, the second messenger signal would be fully activated by the binding of one of the peptides to its cognate receptor in the tissue, preventing further stimulation of contractions even in the presence of another myostimulatory peptide. Given that both of these peptides increase Malpighian tubule fluid secretion rate (Coast et al., 2001; Terhzaz et al., 1999),

the stimulation of pyloric sphincter contractions may facilitate the entry of an increased volume of lumenal contents into the hindgut.

Allatostatin A stimulates K^+ absorption across the midgut epithelium

Allatostatin A stimulated K^+ absorption in the anterior midgut and in the middle midgut copper cell zone while inhibiting K^+ absorption in the large flat cell zone (Figure 9). Interpretation of the physiological significance of the changes in K^+ flux elicited by allatostatin A is hampered by the lack of published information on this peptide's effects on water balance in the insect gut and renal system. Specifically, the effect of this peptide on epithelial fluid transport by the gut cannot be definitively classified as being consistent with diuresis or antidiuresis without knowing its effect, if any, on fluid secretion by the Malpighian tubules. The effects of allatostatin A on the anterior midgut are similar to those of DH31 and drosokinin, suggesting that this peptide may also play a role in a diuretic strategy, particularly if Malpighian tubule fluid secretion is concomitantly enhanced. The lack of a significant effect on K^+ flux in the posterior midgut or in the ileum suggests either that allatostatin A receptors are not expressed in these regions or that the peptide's effects are unrelated to the rate of epithelial K^+ transport. Although the specific locations of expression of the allatostatin A receptor (DAR-2) in the midgut have not been previously reported, functional receptors can be inferred to be present in the anterior midgut and both zones of the middle midgut where allatostatin A had significant effects. Allatostatin A is produced by endocrine cells only in the posterior midgut alkaline zone (Veenstra, 2009), so it may diffuse through the haemolymph to reach its targets as a paracrine factor. Interestingly, all three of the studied peptides stimulated K^+ absorption in the anterior midgut, suggesting that this region may be particularly involved in fluid uptake upon ingestion of a dilute meal;

concentration of the lumenal contents might facilitate efficient digestion in subsequent gut regions.

The functions of increased K^+ absorption in the copper cell zone of the middle midgut and the reduction of K^+ absorption in the large flat cell zone in response to allatostatin A are unclear. These changes may relate to a redistribution of fluid uptake among the gut regions in the manner postulated for DH31; allatostatin A may reduce absorption of K^+ and osmotically-obliged water in the large flat cell zone to prevent excessive dehydration of the food bolus and over-acidification of the lumen. Alternatively, the decrease could reflect an attempt to limit the concentration of K^+ in the haemolymph while instead driving epithelial fluid uptake with enhanced Na^+ absorption. If the contributions of each midgut region are considered, the short length and small diameter of the large flat cell zone mean that reduced fluid uptake in this region has a small impact upon the net stimulation of fluid absorption by allatostatin A in the midgut as a whole. The possibility remains that the observed changes in epithelial K^+ flux elicited by allatostatin A are not related to the stimulation of fluid uptake but are rather correlates of another function in the midgut. Allatostatin A has been implicated in the mediation of satiety signals and feeding behaviour by its stimulation of food aversion in adult *Drosophila* (Hergarden et al., 2012). Although the effect of this peptide on larval feeding habits is unknown, stimulation of midgut K^+ absorption could indicate that allatostatin A is involved in mediating physiological responses to an ingested meal. Lepidopteran larvae utilize K^+ gradients to drive cotransport of amino acids across the lumenal membrane of intestinal enterocytes (Hanozet et al., 1980; Sacchi et al., 1990; see Giordana et al., 1989). If amino acid absorption in the anterior midgut and copper cell zone also involves cotransport with K^+, then an increase in the absorption of this

cation may indicate that allatostatin A plays a role in stimulating absorption of amino acids in *Drosophila*.

The literature concerning the effect of allatostatin A on midgut ion transport is limited to a study by Onken et al. (2004) in larval *Aedes*. These authors found that the five *Aedes* isoforms of the peptide stimulated a 10-15% depolarization in transepithelial voltage of the anterior stomach, which the authors attribute to a decrease in 5-HT-stimulated epithelial HCO_3^- secretion. Lumenal alkalization in larval *Aedes* is driven by basolateral H^+ V-ATPases (Boudko et al., 2001), so the depolarization of transepithelial voltage may reflect a reduction of this enzyme's activity attributable to allatostatin A. Shanbhag and Tripathi (2009) proposed that a basolateral V-ATPase energizes the transepithelial transport of HCO_3^- and other solutes in the larval *Drosophila* midgut, and this enzyme may also be located apically. It is not known if the V-ATPase is linked to K^+ absorption in the *Drosophila* midgut. Elucidation of the presence and location of K^+/H^+ antiporters, the Na^+/K^+-ATPase, and other transport proteins in the larval *Drosophila* midgut may clarify the cellular targets of allatostatin A and provide insight into its effects.

The absence of allatostatin A-mediated stimulation of K^+ secretion in the ileal epithelium would appear to rule out an antidiuretic role for this peptide, although changes in the secretion of other ions such as Na^+ could result in either diuretic or antidiuretic effects. In the locust, allatostatin A inhibits epithelial K^+ transport and peristaltic contractions in the ileum (L. Robertson, A.B. Lange, A. Donini, personal communication). Unlike *Drosophila*, the locust ileum absorbs K^+

ions (Phillips and Audsley, 1995), so the effect of allatostatin A in this insect is presumably to limit fluid reabsorption from the ileal lumen.

Future Experiments

Further functional characterization of allatostatin A is of interest, particularly with respect to its effects on Malpighian tubule fluid secretion and gut motility in *Drosophila*. These investigations seem particularly promising given the known expression of this peptide and its receptor in the gut, Malpighian tubules, and CNS, and may further clarify the physiological role of allatostatin A as a factor with a putative role in regulating water balance in insects. Yoon and Stay (1995) found innervation of the outer muscle layer of the larval *Drosophila* hindgut by axons of allatostatin A-immunoreactive nerves extending from the last abdominal neuromere. Based on a similar innervation pattern in *Calliphora* and the known myoinhibitory activity of allatostatin A in the hindgut of that species (Duve and Thorpe, 1994), these authors propose the peptide to inhibit ileal peristalsis in larval *Drosophila* as well. The allatostatin A receptor DAR-2, which is expressed in the hindgut, may be primarily expressed in visceral muscle, allowing allatostatin A to bind as a paracrine factor.

It would also be appealing to study the effects of DH31, drosokinin, and allatostatin A on epithelial Na^+ transport in the gut. As previously stated, the peptides could alter the flux of this ion instead of K^+ to drive the osmotic flow of water. Like K^+, Na^+ flux across the midgut and Malpighian tubule epithelia is affected by dietary salt stress in larval *Drosophila*, and the magnitude and direction of Na^+ flux has been reported for most of the larval gut by Naikkhwah and O'Donnell (2011, 2012).

One of the inherent challenges of this study was to separate the effects of externally applied hormones from those produced endogenously. Making paired measurements, in which each preparation served as its own control, was one accommodation that was made to minimize variations in response between samples. Such variation may be due to slight differences in age or feeding state that alter endogenous hormone production or receptivity, thereby influencing the response to exogenous hormones. In a further effort to reduce variation among preparations, endogenous hormones could be cleared from the dissected preparation before scanning. Temporary immersion of the dissected gut in a high K^+ *Drosophila* saline would be expected to stimulate hormone release from exposed nerve endings by depolarizing neurohaemal areas; such a procedure has been shown to release diuretic hormones in *Rhodnius* and the tsetse fly *Glossina* as well as 5-HT in the blowfly *Calliphora* (Trimmer, 1985; Maddrell and Gee, 1974). In the latter species, a 60 mM K^+ saline was used (Trimmer, 1985). In *Rhodnius*, the largest volumes of secreted hormone were produced within four minutes of exposure to saline containing 70 mM K^+ (Maddrell and Gee, 1974). Brief exposure of preparations to high K^+ saline immediately followed by restoration of physiological K^+ levels in the saline and SIET measurements might reveal if factors that are released upon depolarization alter the transport of ions such as K^+. Alternatively, longer exposure to high K^+ saline prior to the restoration of physiological levels could be used to deplete endogenous stores of peptides in the midgut. In this case, the effects of the subsequent application of exogenous peptides such as DH31 might be more apparent if the basal rate of release of endogenous peptides has been diminished.

Another avenue of inquiry may be the investigation of the involvement of the H^+ V-ATPase in the peptide-mediated changes in epithelial K^+ flux reported in the current study. The electrogenic

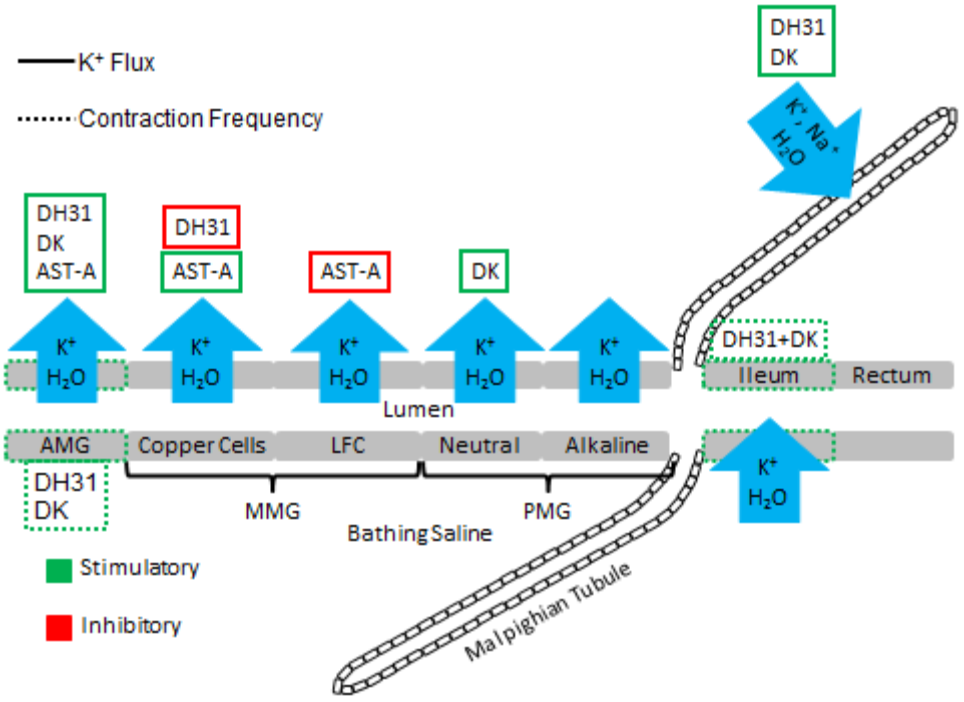
V-ATPase is known to drive the secondary active transport of Na^+ and K^+ ions into the Malpighian tubule lumen, and DH31 has been proposed to stimulate this enzyme's activity in this tissue (Beyenbach, 2001; Coast et al., 2001). The V-ATPase is also present in the basal and possibly the apical membranes of the *Drosophila* midgut epithelium (Shanbhag and Tripathi, 2005, 2009). A V-ATPase inhibitor, bafilomycin A_1, could be used to assess the involvement of this enzyme in peptide-mediated changes in ion transport by the gut epithelium. Shanbhag and Tripathi (2005) demonstrated a depolarization of transepithelial potential in the larval midgut epithelium in response to 3.5 μM bafilomycin A_1.

Finally, it would be interesting to explore the cellular signals underlying the effects of DH31 and drosokinin on epithelial K^+ transport in the gut. Coast et al. (2001) showed that 8-bromo-cAMP increased Malpighian tubule fluid secretion rate to the same extent as DH31 and that the effects of DH31 and 8-bromo-cAMP were not additive, suggesting that both factors act on the same transport mechanism. It is tempting to assume DH31 acts via cAMP in the gut, as well. Drosokinin increases the chloride permeability of the Malpighian tubule epithelium via a rise in intracellular Ca^{2+} (O'Donnell et al., 1998), and Radford et al. (2002) reported that this peptide increased intracellular Ca^{2+} in several *Drosophila* tissues including the adult hindgut and larval CNS. It would be interesting to probe the involvement of intracellular Ca^{2+} signalling in drosokinin's effects on the larval gut by exposing the tissue to the Ca^{2+}-mobilizing agent thapsigargin. Exposure of *Drosophila* Malpighian tubules to leucokinin does not increase the fluid secretion rate above that produced by exposure to thapsigargin, consistent with leucokinin acting through Ca^{2+} as an intracellular second messenger (Davies et al., 1995).

Significance of studies of the effects of peptides on epithelial ion transport in insects

The results of this work implicate DH31, drosokinin, and allatostatin A in the regulation of midgut K^+ transport in larval *Drosophila*. Such findings are foundational to the development of novel pesticides that are species-specific and environmentally benign. The terrestrial adults of the major insect pests are particularly vulnerable to the disruption of their ability to limit excretory water loss; fatal dehydration could be induced by exposure to diuretic hormone agonists or antidiuretic hormone antagonists (Coast et al., 2002). Insects are also susceptible to the impairment of excretory pathways by antidiuretic compounds that reduce their capacity to clear toxic wastes (Coast et al., 2002). The development of neuropeptide-based pesticides is challenged, however, by the necessity of developing compounds that act on target insect species without detrimentally impacting the survival of others. A recent study suggests that stable biomimetic analogues of leucokinin retain the efficacy of the native peptide and could be used as the basis for novel compounds that disrupt the critical processes of pest insects (Nachman et al., 2012).

Figure 9. Summary of peptide effects on epithelial K^+ flux and tissue contractions in the larval *Drosophila* gut and Malpighian tubules. Blue arrows represent the direction of K^+ transport and water flow across epithelia. The simplified diagram depicts two of four Malpighian tubules. AMG, anterior midgut; MMG, middle midgut; LFC, large flat cells; PMG, posterior midgut; DH31, diuretic hormone 31; DK, drosokinin; AST-A, allatostatin A.


K+ Flux
Contraction Frequency
DH31
DK
K+, Na+
H2O
DH31
DK
AST-A
DH31
AST-A
AST-A
DK
K+
H2O
DH31+DK
Ileum
Rectum
Lumen
AMG
Copper Cells
LFC
Neutral
Alkaline
DH31
DK
MMG
PMG
Bathing Saline
Stimulatory
Inhibitory
Malpighian Tubule

References

Aguilar, R., Maestro, J.L., Vilaplana, L., Pascual, N., Piulachs, M.D., Belles, X. (2003). Allatostatin gene expression in brain and midgut, and activity of synthetic allatostatins on feeding-related processes in the cockroach *Blattella germanica*. *Regulatory Peptides*. 115: 171-177.

Al-Anzi, B., Armand, E., Nagamei, P. Olszewski, M., Sapin, V., Waters, C., Zinn, K., Wyman, R.J., Benzer, S. (2010). The leucokinin pathway and its neurons regulate meal size in *Drosophila*. *Current Biology*. 20: 969-978.

Audsley, N., Weaver, R.J., Edwards, J.P. (2001). In vivo effects of *Manduca sexta* allatostatin and allatotropin on larvae of the tomato moth, *Lacanobia oleracea*. *Physiological Entomology*. 26: 181-188.

Audsley, N. and Weaver, R.J. (2009). Neuropeptides associated with the regulation of feeding in insects. *General and Comparative Endocrinology*. 162: 93-104.

Audsley, N., Kay, I., Hayes, T.K., Coast, G.M. (1995). Cross reactivity studies of CRF-related peptides on insect Malpighian tubules. *Comp. Biochem. Physiol.* 110A: 87-93.

Beyenbach, K.W. (2001). Energizing epithelial transport with the vacuolar H^+-ATPase. *News Physiol. Sci.* 16: 145-151.

Boudko, D.Y., Moroz, L.L., Harvey, W.R., Linser, P.J. (2001). Alkalinization by chloride/bicarbonate pathway in larval mosquito midgut. *PNAS*. 98: 15354-15359.

Chintapalli, V.R., Wang, J., Dow, J.A.T. (2007). Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nature Genetics*. 39: 715–720.

Coast, G.M., Holman, G.M., Nachman, R.J. (1990). The diuretic activity of a series of cephalomyotropic neuropeptides, the achetakinins, on isolated Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect. Physiol.* 36: 481-488.

Coast, G.M. and Schooley, D.A. (2011). Toward a consensus nomenclature for insect neuropeptides and peptide hormones. *Peptides*. 32: 620-631.

Coast, G.M. (2009). Neuroendocrine control of ionic homeostasis in blood-sucking insects. *J. Exp. Biol.* 212: 378-386.

Coast, G.M., Webster, S.G., Schegg, K.M., Tobe, S.S., Schooley, D.A. (2001). The *Drosophila melanogaster* homologue of an insect calcitonin-like diuretic peptide stimulates V-ATPase activity in fruit fly Malpighian tubules. *J. Exp. Biol.* 204: 1795-1804.

Coast, G.M., Orchard, I., Phillips, J.E., Schooley, D.A. (2002). Insect diuretic and antidiuretic hormones. *Advances in Insect Physiology*. 29: 279-409.

Dadd, R.H. (1975). Alkalinity within the midgut of mosquito larvae with alkaline-active digestive enzymes. *J. Insect. Physiol.* 21: 1847-1853.

Dames, P., Zimmermann, B., Schmidt, R., Rein, J., Voss, M., Schewe, B., Walz, B., Baumann, O. (2006). cAMP regulates plasma membrane vacuolar-type H^+-ATPase assembly and activity in blowfly salivary glands. *PNAS*. 103: 3926-3931.

Davies, S.A., Huesmann, G.R., Maddrell, S.H., O'Donnell. M.J., Skaer, N.J., Dow, J.A., Tublitz, N.J. (1995). CAP_{2b}, a cardioacceleratory peptide, is present in *Drosophila* and stimulates tubule fluid secretion via cGMP. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 269: R1321-R1326.

Davis, N.T., Veenstra, J.A., Feyereisen, R., Hildebrand, J.G. (1997). Allatostatin-like-immunoreactive neurons of the tobacco hornworm, *Manduca sexta*, and isolation and identification of a new neuropeptide related to cockroach allatostatins. *The Journal of Comparative Neurology*. 385: 265-284.

de Haro, M., Al-Ramahi, I., Benito-Sipos, J., López-Arias, B., Dorado, B., Veenstra, J.A., Herrero, P. (2010). Detailed analysis of leucokinin-expressing neurons and their candidate functions in the *Drosophil*a nervous system. *Cell Tissue Res.* 339: 321-336.

Dow, J.A.T. and Davies, S.A. (2003). Integrative physiology and functional genomics of epithelial function in a genetic model organism. *Physiol. Rev.* 83: 687-729.

Dow, J.A.T., Maddrell, S.H.P., Gortz, A., Skaer, N.J.V., Brogan, S., Kaiser, K. (1994). The Malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. *J. Exp. Biol.* 197: 421-428.

Duve, H. and Thorpe, A. (1994). Distribution and functional significance of Leu-callatostatins in the blowfly *Calliphora vomitoria*. *Cell Tissue Res.* 276: 367-379.

Duve, H., Wren, P., Thorpe, A. (1995). Innervation of the foregut of the cockroach *Leucophaea maderae* and inhibition of spontaneous contractile activity by callatostatin neuropeptides. *Physiological Entomology*. 20: 33-44.

Duve, H., East, P.D., Thorpe, A. (1999). Regulation of lepidopteran foregut movement by allatostatins and allatotropin from the frontal ganglion. *The Journal of Comparative Neurology*. 413: 405-416.

Ejaz, A. and Lange, A.B. (2008). Peptidergic control of the heart of the stick insect, *Baculum extradentatum*. *Peptides*. 29: 214-225.

Furuya, K., Milchak, R.J., Schegg, K.M., Zhang,J., Tobe, S.S., Coast, G.M., Schooley, D.A. (2000). Cockroach diuretic hormones: characterization of a calcitonin-like peptide in insects. *Proc. Natl. Acad. Sci. USA.* 97: 6469–6474.

Fusé, M., Zhang, J.R., Partridge, E., Nachman, R.J., Orchard, I., Bendena, W.G., Tobe, S.S. (1999). Effects of an allatostatin and a myosuppressin on midgut carbohydrate enzyme activity in the cockroach *Diploptera punctata*. *Peptides*. 20: 1285-1293.

Giordana, B., Sacchi, V.F., Parenti, P., Hanozet, G.M. (1989). Amino acid transport systems in intestinal brush-border membranes from lepidopteran larvae. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 257: R494-R500.

Gupta, B.L., Wall, B.J., Oschman, J.L., Hall, T.A. (1980). Direct microprobe evidence of local concentration gradients and recycling of electrolytes during fluid absorption in the rectal papillae of *Calliphora*. *J. Exp. Biol.* 88: 21-47.

Hanozet, G.M., Giordana, B., Sacchi, V.F. (1980). K^+-dependent phenylalanine uptake in membrane vesicels isolated from the midgut of *Philosamia cynthia* larvae. *Biochimica et Biophysica Acta*. 596: 481-486.

Hayes, T.K., Pannabecker, T.L., Hinckley, D.J., Holman, G.M., Nachman, R.J., Petzel, D.H., Beyenbach, K.W. (1989). Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sciences.* 44: 1259-1266.

Hergarden, A.C., Tayler, T.D., Anderson, D.J. (2012). Allatostatin-A neurons inhibit feeding behavior in adult *Drosophila*. *PNAS*. 109: 3967-3972.

Holman, G.M., Cook, B.J., Nachman, R.J. (1987). Isolation, primary structure and synthesis of leucokinins VII and VIII: the final members of this new family of cephalomyotropic peptides isolated from head extracts of *Leucophaea maderae*. *Comp. Biochem. Physiol.* 88C: 31-34.

Ianowski, J.P. and O'Donnell, M.J. (2004). Basolateral ion transport mechanisms during fluid secretion by *Drosophila* Malpighian tubules: Na^+ recycling, Na^+:K^+:2Cl^- cotransport and Cl^- conductance. *J. Exp. Biol.* 207: 2599-2609.

Inoue, T. and Forgac, M. (2005). Cysteine-mediated cross-linking indicates that subunit C of the V-ATPase is in close proximity to subunits E and G of the V_1 domain and subunit a of the V_0 domain. *The Journal of Biological Chemistry*. 280: 27896-27903.

Janzen, W.P., Menold, M., Granger, N.A. (1991). Effects of endogenous esterases and an allatostatin on the products of *Manduca sexta* larval corpora allata *in vitro.* 16: 283-293.

Johnson, E.C., Shafer, O.T., Trigg, J.S., Park, J., Schooley, D.A., Dow, J.A.T., Taghert, P.H. (2005). A novel diuretic hormone receptor in *Drosophila*: evidence for conservation of CGRP signaling. *J. Exp. Biol*. 208: 1239-1246.

LaJeunesse, D.R., Johnson, B., Presnell, J.S., Catignas, K.K., Zapotoczny, G. (2010). Peristalsis in the junction region of the *Drosophila* larval midgut is modulated by DH31 expressing enteroendocrine cells. *BMC Physiology.* 10: 14-27.

Lange, A.B., Chan, K.K., Stay, B. (1993). Effect of allatostatin and proctolin on antennal pulsatile organ and hindgut muscle in the cockroach, *Diploptera punctata*. *Archives of Insect Biochemistry and Physiology*. 24: 79-92.

Lange, A.B., Bendena, W.G., Tobe, S.S. (1995). The effect of the thirteen Dip-allatostatins on myogenic and induced contractions of the cockroach (*Diploptera punctata*) hindgut. *J. Insect. Physiol.* 41: 581-588.

Lechleitner, R.A., Audsley, N., Phillips, J.E. (1989). Antidiuretic action of cyclic AMP, corpus cardiacum, and ventral ganglia on fluid absorption across locust ileum in vitro. *Can. J. Zool.* 67: 2655-2661.

Lenz, C., Williamson, M., Hansen, G.N., Grimmelikhuijzen, C.J.P. (2001). Identification of four *Drosophila* allatostatins as the cognate ligands for the *Drosophila* orphan receptor DAR-2. *Biochemical and Biophysical Research Communications*. 286: 1117-1122.

Lide, D.R. (2002). Handbook of Chemistry and Physics. 83rd Edition. CRC Press, Boca Raton, FL.

Linton, S.M. and O'Donnell, M.J. (1999). Contributions of K^+:Cl^- cotransport and Na^+/K^+-ATPase to basolateral ion transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* 202: 1561-1570.

López-Arias, B., Dorado, B., Herrero, P. (2011). Blockade of the release of the neuropeptide leucokinin to determine its possible functions in fly behavior: chemoreception assays. *Peptides*. 32: 545-552.

Loveridge, J.P. (1975). Studies on the water balance of adult locusts. III. The water balance of non-flying locusts. *Zool. Afr*. 10: l-28.

Kramer, S.J., Toschi, A., Miller, C.A., Kataoka, H., Quistad, G.B., Li, J.P., Carney, R.L., Schooley, D.A. (1991). Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. *Proc. Natl. Acad. Sci. USA*. 88: 9458-9462.

Machin, J. (1976). Passive exchanges during water vapour absorption in mealworms (*Tenebrio molitor*): a new approach to studying the phenomenon. *J. Exp. Biol.* 65: 603-615.

MacVicker, J.A.K., Billingsley, P.F., Djamgoz, M.B.A. (1993). ATPase activity in the midgut of the mosquito, *Anopheles stephensi*: biochemical characterisation of ouabain-sensitive and ouabain-insensitive activities. *J. Exp. Biol.* 174: 167-183.

Maddrell, S.H.P. (1969). Secretion by the Malpighian tubules of *Rhodnius*, the movements of ions and water. *J. Exp. Biol.* 51: 71-97.

Maddrell, S.H.P. and Gee, J.D. (1974). Potassium-induced release of the diuretic hormones of *Rhodnius prolixus* and *Glossina austeni:* Ca dependence, time course and localization of neurohaemal areas. *J. Exp. Biol.* 61: 155-171.

Nachman, R.J., Coast, G.M., Holman, G.M., Beier, R.C. (1995). Diuretic activity of C-terminal group analogues of the insect kinins in *Acheta domesticus*. *Peptides.* 16: 809-813.

Nachman, R.J., Kaczmarek, K., Zabrocki, J., Coast, G.M. (2012). Active diuretic peptidomimetic insect kinin analogs that contain β-turn mimetic motif 4-aminopyroglutamate and lack native peptide bonds. *Peptides*. 34: 262-265.

Naikkhwah, W. and O'Donnell, M.J. (2011). Salt stress alters fluid and ion transport by Malpighian tubules of *Drosophila melanogaster*: evidence for phenotypic plasticity. *J. Exp. Biol.* 214: 3443-3454.

Naikkhwah, W. and O'Donnell, M.J. (2012). Phenotypic plasticity in response to dietary salt stress: Na^+ and K^+ transport by the gut of *Drosophila melanogaster* larvae. *J. Exp. Biol.* 215: 461-470.

Nicolson, S.W. (1991). Diuresis or clearance: Is there a physiological role for the "diuretic hormone" of the desert beetle *Onymacris*? *J. Insect Physiol.* 37: 447-452.

O'Donnell, M.J., Aldis, G.K., Maddrell, S.H.P. (1982). Measurements of osmotic permeability in the Malpiphian tubules of an insect, *Rhodnius prolixus* Stal. *Proc. R. Soc.* B216: 267-277.

O'Donnell, M.J., Dow, J.A.T., Huesmann, G.R., Tublitz, N.J., Maddrell, S.H.P. (1996). Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* 199: 1163-1175.

O'Donnell, M.J., Rheault, M.R., Davies, S.A., Rosay, P., Harvey, B.J., Maddrell, S.H.P., Kaiser, K., Dow, J.A.T. (1998). Hormonally controlled chloride movement across *Drosophila* tubules is via ion channels in stellate cells. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 274: R1039-R1049.

Onken, H., Moffett, S.B., Moffett, D.F. (2004). The anterior stomach of larval mosquitoes (*Aedes aegypti*): effects of neuropeptides on transepithelial ion transport and muscular motility. *J. Exp. Biol.* 207: 3731-3739.

Pannabecker, T.L., Hayes, T.K., Beyenbach, K.W. (1993). Regulation of epithelial shunt conductance by the peptide leucokinin. *J. Membrane Biol.* 132: 63-76.

Patrick, M.L., Aimanova, K., Sanders, H.R., Gill, S.S. (2006). P-type Na^+/K^+-ATPase and V-type H^+-ATPase expression patterns in the osmoregulatory organs of larval and adult mosquito *Aedes aegypti*. *J. Exp. Biol.* 209: 4638-4651.

Peller, C.R., Bacon, E.M., Bucheger, J.A., Blumenthal, E.M. (2009). Defective gut function in *drop-dead* mutant *Drosophila*. *Journal of Insect Physiology*. 55: 834-839.

Phillips, J.E. and Audsley, N. (1995). Neuropeptide control of ion and fluid transport across locust hindgut. *Amer. Zool.* 35: 503-514.

Radford, J.C., Davies, S.A., Dow, J.A.T. (2002). Systematic G-protein-coupled receptor analysis in *Drosophila melanogaster* identifies a leucokinin receptor with novel roles. *J. Biol. Chem.* 277: 38810-38817.

Reynolds, S.E. and Bellward, K. (1989). Water balance in *Manduca sexta* caterpillars: water recycling from the rectum. *J. Exp. Biol.* 141: 33-45.

Roberts, D.B. and Stander, G.N. (1998). *Drosophila*: *A Practical Approach*, Oxford University Press, UK.

Robertson, L., Lange, A.B., Donini, A. (personal communication). Allatostatin inhibits locust gut contraction and ileal ion transport.

Sacchi, V.F., Giordana, B., Campanini, F., Bonfanti, P., Hanozet, G.M. (1990). Leucine uptake in brush-border membrane vesicles from the midgut of a lepidopteran larva, *Philosamia cynthia*. *J. Exp. Biol.* 149: 207-221.

Shanbhag, S. and Tripathi, S. (2005). Electrogenic H^+ transport and pH gradients generated by a V-H^+-ATPase in the isolated perfused larval *Drosophila* midgut. *J. Membrane Biol.* 206: 61-72.

Shanbhag, S. and Tripathi, S. (2009). Epithelial ultrastructure and cellular mechanisms of acid and base transport in the *Drosophila* midgut. *J. Exp. Biol.* 212: 1731-1744.

Stay, B., Fairbairn, S., Yu, C.G. (1996). Role of allatostatins in the regulation of juvenile hormone synthesis. Arch. *Insect. Biochem. Physiol.* 32: 287–297.

Stobbart, R.H. (1971). Factors affecting the control of body volume in the larvae of the mosquitoes *Aedes aegypti* (L.) and *Aedes detritus* EDW. *J. Exp. Biol.* 54: 67-82.

Te Brugge, V.A., Lombardi, V.C., Schooley, D.A., Orchard, I. (2005). Presence and activity of a Dippu-DH_{31}-like peptide in the blood-feeding bug, *Rhodnius prolixus*. *Peptides*. 26: 29-42.

Te Brugge, V.A., Schooley, D.A., Orchard, I. (2008). Amino acid sequence and biological activity of a calcitonin-like diuretic hormone (DH_{31}) from *Rhodnius prolixus*. *J. Exp. Biol.* 211: 382-390.

Te Brugge, V., Ianowski, J.P., Orchard, I. (2009). Biological activity of diuretic factors on the anterior midgut of the blood-feeding bug, *Rhodnius prolixus*. *General and Comparative Endocrinology*. 162: 105-112.

Terhzaz, S., O'Connell, F.C., Pollock, V.P., Kean, L., Davies, S.A., Veenstra, J.A., Dow, J.A. (1999*).* Isolation and characterization of a leucokinin-like peptide of Drosophila melanogaster. *J Exp Biol*. 202: 3667-3676.

Thomas R.C. (1978). *Ion-Sensitive Intracellular Microelectrodes, How to Make and Use Them*. Academic Press, London, UK.

Trimmer, B.A. (1985). Serotonin and the control of salivation in the blowfly *Calliphora*. *J. Exp. Biol.* 114: 307-328.

Veelaert, D., Devreese, B., Schoofs, L., Van Beeumen, J., Vanden Broeck, J., Tobe, S.S., De Loof, A. (1996). Isolation and characterization of eight myoinhibiting peptides from the desert locust, *Schistocerca gregaria*: new members of the cockroach allatostatin family. *Molecular and Cellular Endocrinology*. 122: 183-190.

Veenstra, J.A. (2009). Peptidergic paracrine and endocrine cells in the midgut of the fruit fly maggot. *Cell Tissue Res.* 336: 309-323.

Veenstra, J.A., Pattillo, J.M., Petzel, D.H. (1997). A single cDNA encodes all three *Aedes* leucokinins, which stimulate both fluid secretion by the Malpighian tubules and hindgut contractions. *The Journal of Biological Chemistry*. 272: 10402-10407.

Veenstra, J.A., Agricola, J-H., Sellami, A. (2008). Regulatory peptides in fruit fly midgut. *Cell Tissue Res.* 334: 499-516.

Voss, M., Vitavska, O., Walz, B., Wieczorek, H., Baumann, O. (2007). Stimulus-induced phosphorylation of vacuolar H^+-ATPase by protein kinase A. *The Journal of Biological Chemistry*. 282: 33735-33742.

Wigglesworth, V.B. (1933). The function of the anal gills of the mosquito larva. *J. Exp. Biol.* 10: 16-26.

Yoon, J.G. and Stay, B. (1995). Immunocytochemical localization of *Diploptera punctata* allatostatin-like peptide in *Drosophila melanogaster*. *The Journal of Comparative Neurology*. 363: 475-488.

Zitnan, D., Sehnal, F., Bryant, P.J. (1993). Neurons producing specific neuropeptides in the central nervous system of normal and pupariation-delayed *Drosophila*. *Developmental Biology.* 156: 117-135.

Appendix 1: Effects of DH31 and drosokinin on K^+ transport across the midgut epithelium measured at two sites within each region

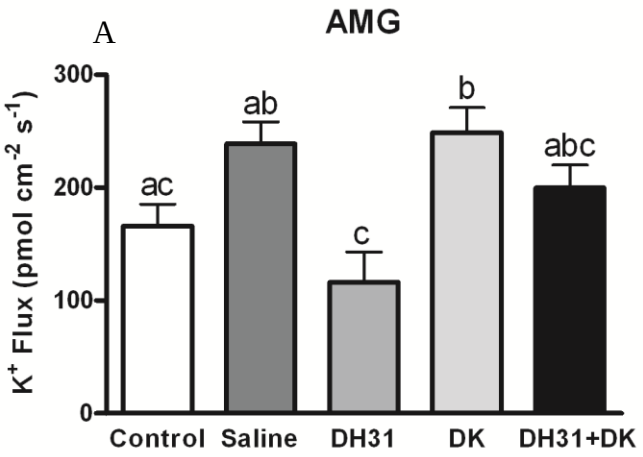
The individual and combined effects of DH31 and drosokinin on K^+ flux were assessed through SIET measurements in the anterior midgut, and in the anterior and posterior halves of the ileum (Figure A1). These measurements were conducted as described in Materials and Methods, with the exception that the voltage gradient was measured at only two sites along the surface of each preparation. The two sites were separated by 50 μm and were centrally located within the gut region of interest. A total of ~9 minutes was required to complete all SIET measurements on each preparation, with the initial measurement being made within 5 minutes of dissection. This protocol was selected in an effort to reduce variation in response among tissue sites within each region, however it was ultimately discontinued due to concern that the results may not be representative of the entire region, some of which approach 1000 μm in total length. Variation in overall size between preparations made it difficult to measure K^+ flux at the same two sites from one preparation to another. This inherent positioning error may have contributed to variation in the measured voltage gradients between preparations.

Interpretation of the data is complicated by statistical significance between the Control and Saline bars in each region. In the anterior midgut, only drosokinin stimulates K^+ absorption to a level that is significantly greater than the control. This result may be interpreted as being consistent with the presence of drosokinin receptors in this region and with the stimulation of fluid uptake into the haemolymph associated with the known diuretic activity of this peptide (Veenstra, 2009; Terhzaz et al., 1999). The larval hindgut, comprised of the ileum and rectum, is known to strongly express the drosokinin receptor (Chintapalli et al., 2007). In the anterior half

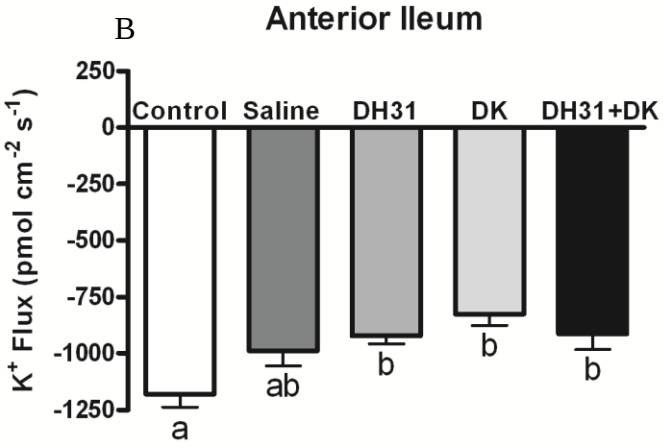
of the ileum, DH31 and drosokinin reduced the magnitude of K^+ secretion relative to the control when present individually and in combination. This result is consistent with a clearance hormone function, where the peptides promote excretion through the Malpighian tubules but stimulate reabsorption or inhibit further secretion of ions and water in the hindgut (Nicolson, 1991). The same interpretation can be applied to the stimulation of K^+ uptake by both peptides in the posterior half of the ileum.

Figure A1. K^+ flux across the anterior midgut (A), and anterior (B) and posterior (C) ileum epithelia measured at two sites within each gut region. Preparations were bathed in *Drosophila* saline during control measurements (white bar). Subsequent measurements were made following the addition of one of the test factors (*Drosophila* saline, DH31, DK, or DH31+DK). Peptides were present at a concentration of 1 µM in the bathing saline. Data are expressed as mean+SEM with letters indicating statistical significance between treatments (one-way ANOVA with Tukey's post-hoc test, $P<0.05$, $N=7\text{-}33$). AMG, anterior midgut; DH31, diuretic hormone 31; DK, drosokinin.

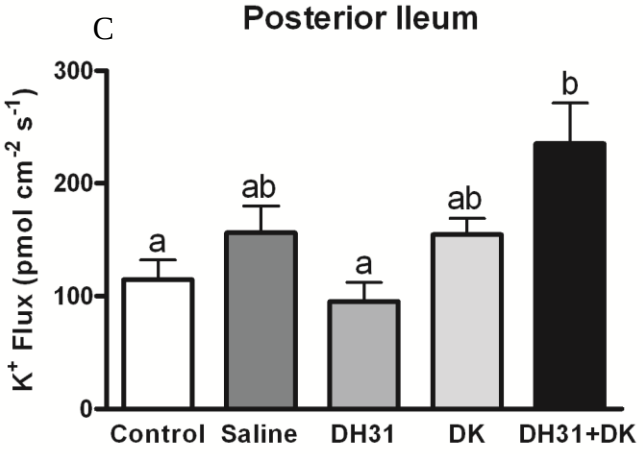
A


AMG
K+ Flux (pmol cm-2 s-1)
300
200
100
0
ac
ab
c
b
abc
Control
Saline
DH31
DK
DH31+DK

B


Anterior Ileum
K+ Flux (pmol cm-2 s-1)
250
0
-250
-500
-750
-1000
-1250
Control
Saline
DH31
DK
DH31+DK
a
ab
b
b
b

C


Posterior Ileum
K+ Flux (pmol cm-2 s-1)
300
200
100
0
a
ab
a
ab
b
Control
Saline
DH31
DK
DH31+DK

Appendix 2: Dissolution of DH31 and drosokinin in an acetonitrile, trifluoroacetic acid solvent

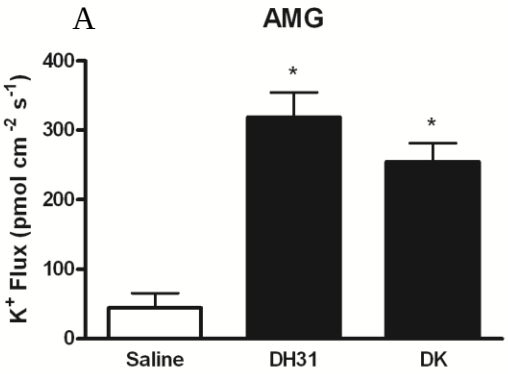
A total of three SIET scans were performed on each preparation. Each scan consisted of a measurement of the voltage gradient at five sites along the dissected tissue. Sites were separated by 50 µm and measurements were made at the same five sites for all scans. All scans were performed in a 200 µl bath consisting of 198 µl of *Drosophila* saline and 2 µl of the test factor. The test factor was either *Drosophila* saline, DH31 dissolved in an acetonitrile (ACN) and trifluoroacetic acid (TFA) solvent, or drosokinin dissolved in an ACN and TFA solvent. DH31 and drosokinin dissolved in the ACN, TFA solvent were stored at -20°C in 1 µl aliquots at a concentration of 1 mM. 9 µl of *Drosophila* saline was added to the stored peptide aliquot immediately prior to use, bringing the peptide concentration to 0.1 mM in the 2 µl test factor droplet that was added to the *Drosophila* saline bath. The final concentration of DH31 or drosokinin in the bathing saline was 1 µM and the final concentrations of the solvent components were 5.7 mM ACN and 13 µM TFA. A total of ~17 minutes was required to complete all SIET measurements on each preparation, with the initial measurement being made within 5 minutes of dissection. The voltage gradients at the reference site were measured in the manner described in Materials and Methods.

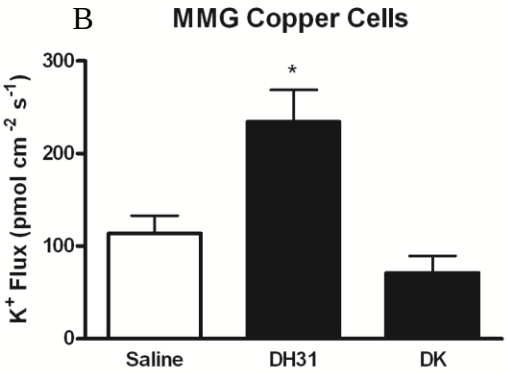
All experiments performed from September 2011 through March 2012 measured changes in K^+ flux by preparations bathed in DH31 or drosokinin dissolved in the ACN, TFA solvent. The data suggests that DH31 stimulates K^+ absorption across the epithelia of the anterior midgut and the middle midgut copper cell zone, while it inhibits K^+ absorption in both the large flat cell zone and the posterior midgut neutral zone (Figure A2). Like DH31, drosokinin stimulated K^+

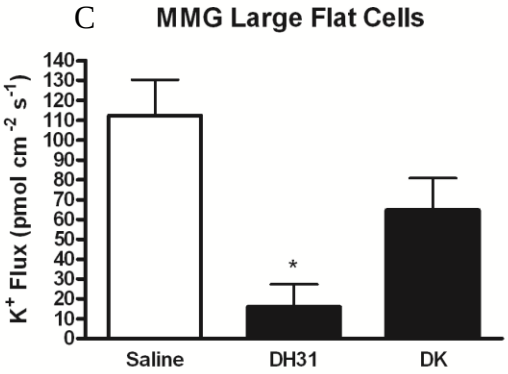
absorption in the anterior midgut (Figure A2). The anterior midgut was shown by Veenstra (2009) to strongly express DH31-producing endocrine cells and the drosokinin receptor, so the stimulation of absorption in this region suggests that these peptides may stimulate water uptake associated with diuresis. A similar correlation can be drawn in the copper cell zone, where DH31-producing cells are also located. The inhibition of K^+ flux by DH31 in the large flat cell zone and the neutral zone is unexpected given the known diuretic activity of this peptide in the Malpighian tubules (Coast et al., 2001), however the effect is consistent with the presence of DH31-producing endocrine cells in these regions (Veenstra, 2009).

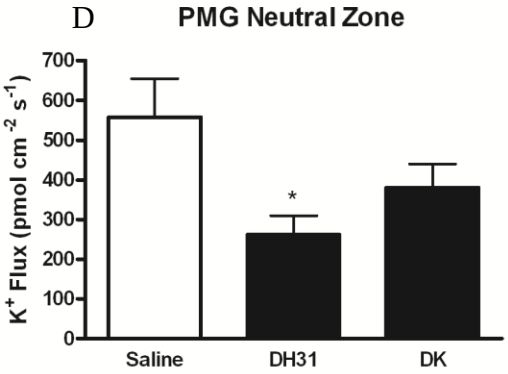
SIET measurements in March 2012 showed unexpectedly large differences in K^+ flux attributable to the presence of the ACN, TFA solvent in the bathing saline (Figure A3). The use of the solvent was discontinued from this point. Data collected during the use of the ACN, TFA solvent is potentially invalid due to an inability to distinguish its effects from those of the peptides of interest.

Figure A2. K^+ flux across the anterior midgut (A), middle midgut copper cells (B) and large flat cells (C), posterior midgut neutral zone (D) and alkaline zone (E) epithelia of preparations bathed in *Drosophila* saline, 1 µM DH31, or 1 µM drosokinin dissolved in an ACN, TFA solvent. The bathing saline of preparations exposed to either of the peptides also contained 5.7 mM ACN and 13 µM TFA. Data are expressed as mean+SEM with an asterisk indicating statistical significance between the control (saline) and each peptide treatment (one-way ANOVA with Dunnett's post-hoc test, $P<0.05$, $N=5\text{-}19$). AMG, anterior midgut; MMG, middle midgut; PMG, posterior midgut; DH31, diuretic hormone 31; DK, drosokinin.


A
AMG
K+ Flux (pmol cm-2 s-1)
400
300
200
100
0
*
*
Saline
DH31
DK


B
MMG Copper Cells
K+ Flux (pmol cm-2 s-1)
300
200
100
0
*
Saline
DH31
DK


C
MMG Large Flat Cells
K+ Flux (pmol cm-2 s-1)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
*
Saline
DH31
DK


D
PMG Neutral Zone
K+ Flux (pmol cm-2 s-1)
700
600
500
400
300
200
100
0
*
Saline
DH31
DK

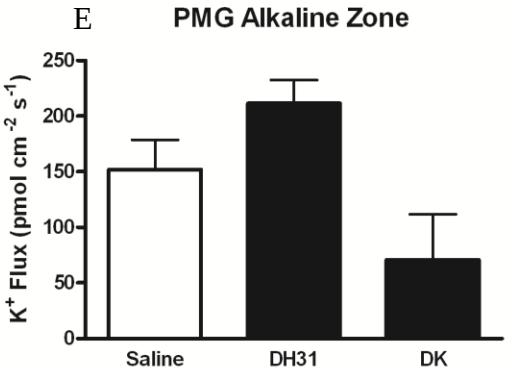
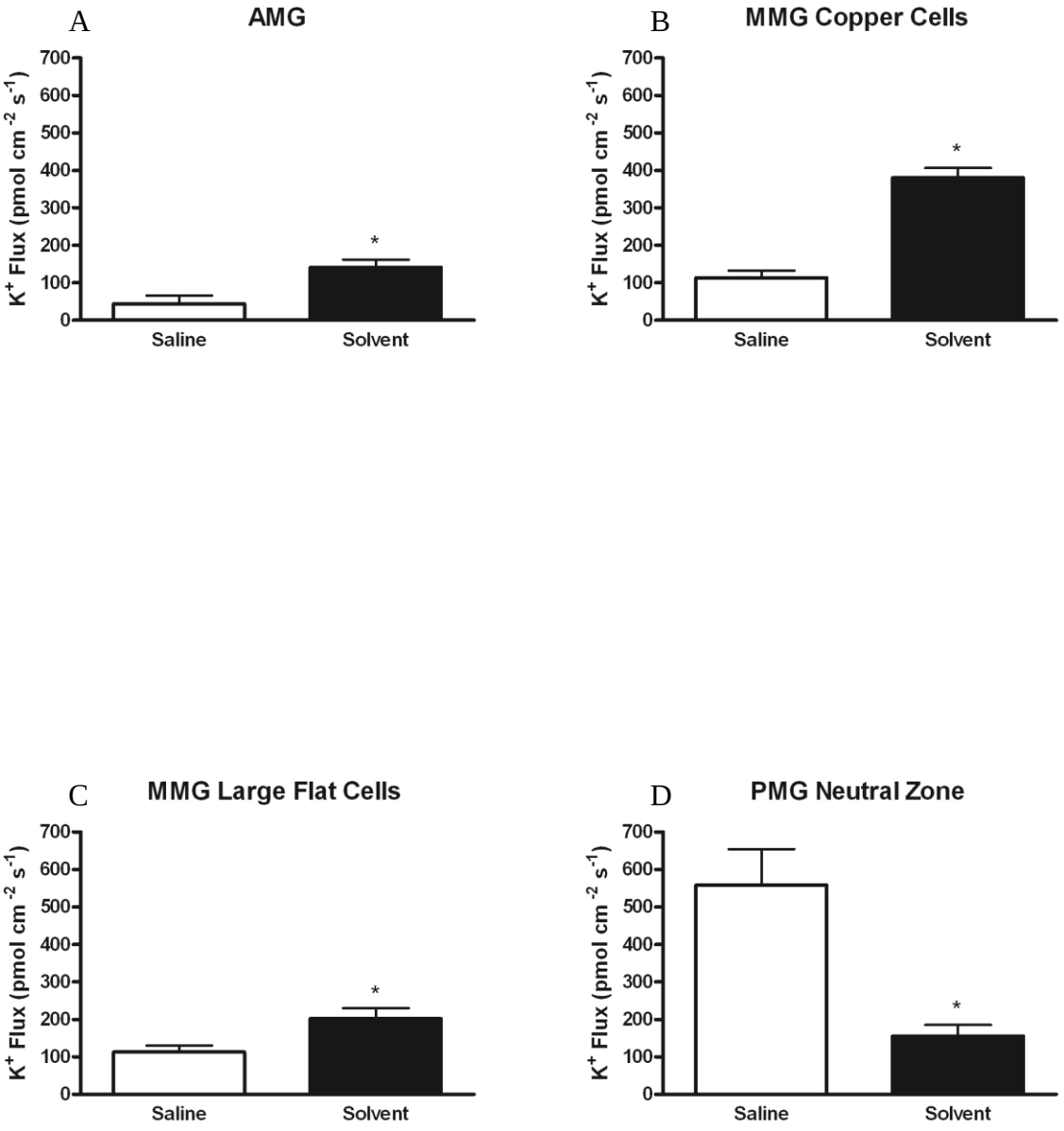

E
PMG Alkaline Zone
K+ Flux (pmol cm-2 s-1)
250
200
150
100
50
0
Saline
DH31
DK

Figure A3. K^+ flux across the anterior midgut (A), middle midgut copper cells (B) and large flat cells (C), and the posterior midgut neutral zone (D) epithelia of preparations bathed in *Drosophila* saline or a 5.7 mM ACN, 13 μM TFA solvent. Data are expressed as mean+SEM with an asterisk indicating statistical significance between treatments (unpaired t-test, $P<0.05$, $N=5\text{-}19$). AMG, anterior midgut; MMG, middle midgut; PMG, posterior midgut.


A
AMG
B
MMG Copper Cells
C
MMG Large Flat Cells
D
PMG Neutral Zone
K+ Flux (pmol cm-2 s-1)
0
100
200
300
400
500
600
700
*
Saline
Solvent

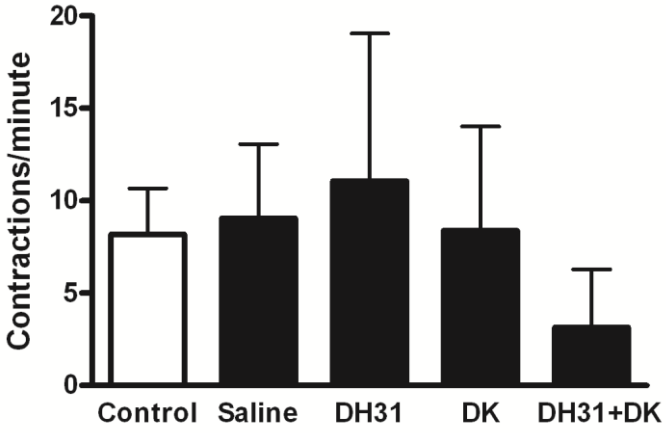
Appendix 3: Impedance recording of contractions in the anterior midgut

Silver wire electrodes attached to an impedance converter (Model 2991, UFI, Morro Bay, CA) were used to record gut contractions in the anterior midgut. The impedance converter (set to alternating current, short) was connected to a data acquisition system (Power Lab, AD Instruments, Sydney, Australia) and measurements were recorded with Chart 5 software. Initial visually observed tissue contractions of each preparation were marked to provide a threshold amplitude that was applied to the impedance recording to isolate tissue contractions from background interference. Measurements began within 5 minutes of tissue dissection. Recordings were made over four consecutive periods each lasting two minutes, requiring a total of ~8 minutes per preparation. Measurements during the initial two minute recording period were made with the preparation bathed in 196 µl of *Drosophila* saline. Upon completion of the first recording period, 4 µl of DH31 or drosokinin were added to the bathing saline to a final concentration of 1 µM. Preparations exposed to both DH31 and drosokinin simultaneously were bathed in 192 µl of *Drosophila* saline during the initial recording period, after which 8 µl of a mixture of the peptides were added to the bath such that both DH31 and drosokinin were present at concentrations of 1 µM. Data are displayed in the manner described in Materials and Methods, however the black bars represent the pooled contraction counts for the second through fourth counting periods of each preparation in its respective treatment group (Saline, DH31, DK, DH31+DK). No treatment produced a statistically significant change in contraction frequency of the anterior midgut (Figure A4).

Impedance measurements are limited to recording contractions within the small cross-section of the tissue located between the electrodes, making this technique ideal for the recording of static

contractions. The technique has previously been used to record contractions of the heart of the stick insect *Baculum*, and the antennal pulsatile organ in *Diploptera* (Ejaz and Lange, 2008; Lange et al., 1993). With independent contractions occurring over a range of locations within the region of interest in *Drosophila* preparations, impedance measurements may not be representative of the frequency of contractions over the whole region. This technique is also disadvantaged by its sensitivity to minute lateral and longitudinal movements of the preparation between the electrodes while recording. Such movement can produce erroneous signals on the trace that obscure signals produced by true contractions and increase the variability of the data.

Figure A4. Frequency of anterior midgut contractions measured by an impedance coverter. Preparations were bathed in *Drosophila* saline during control measurements (white bar). Subsequent measurements were made following the addition of one of the test factors (*Drosophila* saline, DH31, DK, or DH31+DK) and are represented by black bars. Peptides were present at a concentration of 1 μM in the bathing saline. Data are expressed as mean+SEM with an asterisk indicating statistical significance between the control and each treatment (one-way ANOVA with Dunnett's post-hoc test, $P<0.05$, $N=5\text{-}20$). DH31, diuretic hormone 31; DK, drosokinin.


Contractions/minute
20
15
10
5
0
Control
Saline
DH31
DK
DH31+DK

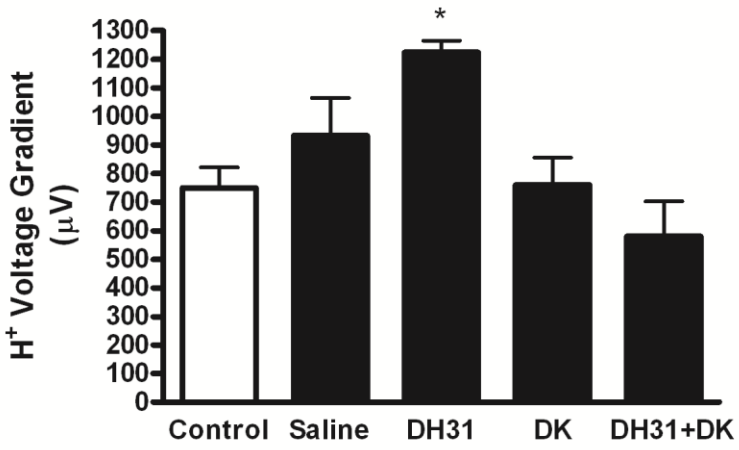
Appendix 4: Effect of DH31 and DK on H^+ transport across the anterior midgut epithelium

H^+-selective microelectrodes were produced by backfilling silanized microelectrodes with a solution of NaCl and sodium citrate each at a concentration of 100 mM and titrated to pH 6.0. The microelectrodes were then tip-filled with a column (~500 μm) of H^+ ionophore I, cocktail B (Fluka, Buchs, Switzerland). H^+-selective microelectrodes were calibrated in solutions of *Drosophila* saline adjusted to pH 6.5 and 7.5. The slope for a ten-fold change in H^+ concentration was (mean±SEM) 63.4±0.7 mV (*N*=6). The buffering activities of HEPES and $NaHCO_3$ in *Drosophila* saline prevent the accurate calculation of H^+ flux. H^+ measurements are thus expressed as a voltage gradient (μV) reflective of the direction and magnitude of H^+ movement across the midgut epithelium. SIET measurements were made at two sites on the tissue surface in the manner described in Appendix 1, and are thus vulnerable to the criticism of representing the response of a very small area within the region of interest.

The significance of the observed increase in H^+ absorption elicited by DH31 in the anterior midgut (Figure A5) is unclear, however it may reflect a stimulation of V-ATPase activity in the midgut epithelium to drive the secondary active transport of K^+ as it does in the Malpighian tubules (Coast et al., 2001). Indeed, Shanbhag and Tripathi (2005) have confirmed the presence of an electrogenic V-ATPase on the basal membrane of the larval midgut epithelium, and suggest that this enzyme may also be located apically (Shanbhag and Tripathi, 2009). The V-ATPase could also be involved in lumenal alkalization of the anterior midgut, which is known to be alkaline in both *Drosophila* and *Aedes* larvae (Shanbhag and Tripathi, 2005; Dadd, 1975). In the latter species, high lumenal pH is optimal for the activity of amylases and proteases (Dadd,

1975). Alternatively, the increased H^+ gradient may reflect an increase in epithelial cell metabolism and the consequent release of more metabolic CO_2.

Figure A5. H^+ voltage gradient adjacent to the anterior midgut epithelium as an indication of the direction and magnitude of H^+ transport. Preparations were bathed in *Drosophila* saline during control measurements (white bar). Subsequent measurements were made following the addition of one of the test factors (*Drosophila* saline, DH31, DK, or DH31+DK) and are represented by black bars. Peptides were present at a concentration of 1 µM in the bathing saline. Data are expressed as mean+SEM with an asterisk indicating statistical significance between the control and each treatment (one-way ANOVA with Dunnett's post-hoc test, $P<0.05$, $N=7\text{-}31$). DH31, diuretic hormone 31; DK, drosokinin.


H+ Voltage Gradient (μV)
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
*
Control
Saline
DH31
DK
DH31+DK