TOXICOLOGY OF ORGANIC CATIONS AND REGULATION OF ORGANIC CATION TRANSPORT IN *DROSOPHILA MELANOGASTER*

TOXICOLOGY OF ORGANIC CATIONS AND REGULATION OF ORGANIC CATION TRANSPORT IN *DROSOPHILA MELANOGASTER*

By

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ABSTRACT

Insects accumulate various xenobiotics and toxic molecules through feeding and environmental exposure. This study examines the toxicology and regulation of a class of toxic molecules, organic cations, in *Drosophila melanogaster*.

The results of this thesis demonstrate that transepithelial tetraethylammonium (TEA) secretion across the main segment of the Malpighian tubules is increased in response to diuretic factors. Both cAMP and cGMP, which increase transepithelial potential (TEP), as well as tyramine and LK-I, which decrease TEP, all enhanced TEA secretion. Both increases and decreases of TEP may enhance proton transport into the lumen of the tubule thus increasing the rate of organic cation/proton exchange across the apical membrane. These findings suggest that factors previously referred to as diuretic factors may in fact act primarily or secondarily as stimulants of organic cation excretion.

Haemolymph concentrations of TEA increased linearly with the concentration of TEA in the diet and declined rapidly upon transfer of the larvae to TEA-free diet. The rate of decline was reduced by slowing the metabolic rate or by the addition of cimetidine to a diet containing TEA. Although larvae tolerated high levels of TEA in the diet, mortality increased when TEA was combined with either quinidine or cimetidine. It is suggested that inhibition of TEA transport by cimetidine or quinidine results in prolonged exposure to higher levels of TEA in the haemolymph and a consequent increase in toxicity. Surprisingly, TEA flux and fluid secretion rate were both reduced in Malpighian tubules isolated from adult flies raised on TEA-enriched diet. This suggests

that the high concentration of TEA in the diet produced a non-lethal yet deleterious effect on the Malpighian tubules of *Drosophila*.

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Finally, to my mother, who always supported me in whatever choices I have made with respect to my life and career. Without your love and support this thesis would not have been possible.

THESIS ORGANIZATION AND FORMAT

In consultation with my supervisor, it was decided that this thesis would be organized in the "sandwich thesis" format approved by McMaster University. Accordingly, this thesis comprises two research chapters which are manuscripts that are pending submission to scientific journals for peer review. A third chapter (Chapter 1) provides a general introduction to the thesis research. Chapter 4 integrates the findings of the preceding chapters and discusses the implications of my results for our understanding of the toxicology and regulation of organic cations.

Chapter 1: General Introduction.

Chapter 2: Diuretic factors stimulate secretion of the organic cation TEA by the Malpighian tubules of *Drosophila melanogaster*

Authors: George Bijelic and Michael J. O'Donnell

Comments: Data were generated exclusively by G.B., under the supervision of M.J.O.

Chapter 3: Effects of dietary or injected organic cations on mortality and haemolymph tetraethylammonium levels in larval *Drosophila melanogaster*.

Authors: George Bijelic, Nancy R. Kim and Michael J. O'Donnell

Comments: The major portion of this work was performed by G.B under the supervision of M.J.O. An undergraduate assistant (N.R.K.) provided technical assistance under the supervision of G.B.

Chapter 4: General Discussion.

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Chapter 1

General Introduction

Insects have adapted to an extraordinary variety of niches and the environmental stresses that are associated with each niche. The most common stress faced by insects is the maintenance of water and ion balance due to the combination of their small size and large ratio of surface area to volume. Survival in water-rich versus arid environments depends largely on the osmoregulatory capabilities of the insect. Insects that inhabit an environment where water is scarce will reduce the loss of water from the haemolymph by producing concentrated urine. Conversely, insects feeding on fluids produce copious amounts of urine in order to eliminate excess water.

Ionic balance by insects

Urine composition depends greatly on the diet of the insect. Blood feeders such as *Rhodnius prolixus* secrete Na^+ rich urine, whereas phytophagous insects, such as locusts, secrete K^+ rich urine (O'Donnell, 1997). The Malpighian tubules (MTs) and hindgut form the functional kidney in insects and play key roles in urine formation and osmoregulation (Fig. 1). In contrast to vertebrates, urine formation occurs by means of secretion rather than ultra-filtration. The primary urine is produced by active transepithelial secretion of ions and the consequent passive flow of osmotically obliged

water from the haemolymph into the MTs. Reabsorption of ions and water may occur in a downstream segment of the MTs or in the hindgut.

Rates of fluid secretion by isolated Malpighian tubules can be very dramatic. Maximally stimulated tubules of *Rhodnius* or *Drosophila* can secrete a volume of iso-osmotic fluid equivalent to their own cellular volume within 10 seconds (Dow, Davies and Sözen, 1998). The tubules have a relatively high permeability to a wide variety of small, uncharged molecules. In addition, there may be specific active transporters for the reabsorption of useful molecules such as sugars and amino acids or the secretion of waste products such as uric acid or toxins (Phillips, 1981; Maddrell and O'Donnell, 1992).

Structure of the Malpighian tubules

Malpighian tubules are named after the 17th century physician Marcelo Malpighi who first described them. The number of MTs present varies greatly depending on the species. Coccids contain only 2 tubules whereas locusts contain more than 250. Malpighian tubules are long, blind ended tubes that enter the gut at the junction between the midgut and the hindgut. A single layer of squamous epithelial cells forms the wall of the tubule. Some species contain a single cell type in the secretory segment whereas tubules of other species, such as *Drosophila*, are comprised of principal, or primary, cells and stellate, or secondary, cells (Sözen *et al.*, 1997). Each tubule is surrounded by respiratory trachea and tubules of some species such as crickets and cockroaches also contain muscle fibres. Periodic contractions of the muscles produce writhing movements

that are thought to help mix the haemolymph and minimize unstirred layers around the tubules.

Drosophila melanogaster

Drosophila has long been used in disciplines such as genetics and developmental biology and has more recently become a model for the study of insect renal physiology. An adult female may consume the equivalent of its own body weight in one day whereas a larva may consume more than 3-5 times its own weight in food per day. It has been suggested that adult female *Drosophila* may contain as much as 250 nl of haemolymph which suggests, given the maximal rate of fluid secretion, that the MTs could potentially clear all fluid from the haemolymph in 10 minutes (O'Donnell and Maddrell, 1995).

Drosophila contains 2 pairs of Malpighian tubules. The anterior pair is comprised of lower, main and distal segments. The posterior pair is identical except that it lacks a distal segment. The main segment secretes Na^+, K^+ and Cl^- ions and osmotically obliged water into the lumen of the tubule. Rates of fluid secretion do not differ between the anterior and posterior pairs and can be sustained for up to 5 hours *in vitro* (Dow *et al.*, 1994). Fluid secreted by the main segment consists of 120 mmol l^{-1} KCl and 30 mmol l^{-1} NaCl (O'Donnell and Maddrell, 1995). The lower segment reabsorbs $\approx$ 30% of the K^+ and water secreted by the main segment and also acidifies the urine and secretes Ca^{2+} into the lumen (O'Donnell and Maddrell, 1995). Ca^{2+} ions, but not fluid, are also secreted into the distal segment resulting in accumulation of large numbers of Ca^{2+} rich

concretions which contain as much as 25 – 30% of the whole animal Ca^{2+} content (Dube *et al.*, 2000).

Differences in transepithelial potential (TEP) between the main and lower segments have also been measured. The TEP of the main segment is 30 mV to 80 mV, lumen-positive, whereas the TEP of the lower segment is less positive near the main segment and is lumen-negative close to the ureter (Maddrell and O'Donnell, 1995).

A recently proposed model of fluid secretion by the main segment (Fig. 2) suggests that K^+ and Cl^- ions are actively transported into the principal cells by a Na^+-driven, bumetanide-sensitive Na^+: K^+: $2Cl^-$ cotransporter located in the basolateral membrane (Ianowski and O'Donnell, 2004). A large portion of the Na^+ which enters the cell is recycled back into the haemolymph by a basolateral, ouabain-sensitive Na^+/K^+-ATPase. K^+, and some Na^+, ions are secreted into the lumen of the tubule by a proton/alkali metal cation exchanger (Ianowski and O'Donnell, 2004). A vacuolar type H^+-ATPase creates the necessary proton gradient across the apical membrane. The lumen-positive potential generated by the electrogenic proton pump provides a favourable electrical gradient for the movement of chloride ions from the haemolymph into the tubule lumen, possibly through apical chloride channels (Ianowski and O'Donnell, 2004).

Evidence for V-ATPases in insect Malpighian tubules is based on immunohistochemical, genetic and physiological studies. Klein (1992) showed staining of the apical membrane of the MTs of *Manduca sexta* with monoclonal antibodies to the V-ATPase isolated from the midgut. Several genes encoding for V-ATPases in

Drosophila have been described (Dow, 1999). In all species tested, fluid secretion is inhibited when the Malpighian tubules are treated with micromolar concentrations of the V-ATPase inhibitor bafilomycin A_1 (Dow *et al.*, 1994).

By contrast, addition of the Na^+/K^+-ATPase inhibitor ouabain does not completely abolish fluid secretion (Ianowski and O'Donnell, 2004). Treatment of unstimulated tubules with ouabain results in a slight stimulation of fluid secretion and an enhancement of Na^+ transport. It is suggested that Na^+, which is not transported out of the cell by the Na^+/K^+-ATPase in the presence of ouabain is thus available for transport into the lumen by the apical transporters. Ianowski and O'Donnell (2004) propose that the coordinated action of the Na^+/K^+-ATPase and the apical transporters may be important in determining the ratio of Na^+ to K^+ in the secreted fluid.

Regulation of fluid secretion

Fluid secretion by Malpighian tubules is regulated through the actions of several amines, neuropeptides and intracellular second messengers. There are five endogenous compounds that stimulate fluid secretion by *Drosophila* tubules (Fig. 3). Calcitonin-related and corticotropin-releasing factor like (CRF-like) peptides stimulate fluid and ion secretion through increases in intracellular cAMP (Coast *et al.*, 2001; Cabrero *et al.*, 2002). Cardioacceleratory peptide 2b (CAP_{2b}) increases fluid and ion secretion through nitric oxide and cGMP (Davies *et al.*, 1995). Both cAMP and cGMP stimulate the electrogenic V-type H^+-ATPase. cAMP increases fluid secretion rates in tubules of most species tested (O'Donnell, 1997). Fluid secretion by tubules of some species can also be

stimulated by factors that increase transepithelial chloride permeability. Drosokinin, a recently isolated member of the leucokinin family of peptides, increases stellate cell Cl^- conductance through increases in levels of intracellular Ca^{2+} (Terhzaz *et al.*, 1999).

The presence of multiple diuretic factors raises the possibility that some may have functions other than simply augmenting fluid secretion. For example, Coast (1995) has demonstrated that a CRF-related peptide increases transepithelial Na^+ transport at the expense of K^+ transport in locust tubules. Enhancement of Na^+ secretion may be required to meet the needs of Na^+/K^+ coupled transporters in the tubules or hindgut. The results presented in this thesis raise an additional possibility, namely that some of the diuretic factors may act to augment the excretion of potentially toxic organic cations.

Organic cations and anions

Renal systems of most animals contain mechanisms for the elimination of potentially toxic organic cations and organic anions that may be present in the diet or produced by metabolism. Several transporters have been identified as either organic anion or organic cation transporters. Organic anions (OA) include molecules such as p-aminohippurate (PAH), folates, salicylate and various dyes such as amaranth and indigo carmine. Common organic cations include endogenous compounds such as choline and N-methyl nicotinamide and exogenous compounds such as tetraethylammonium (TEA).

Both invertebrate (Miller and Holliday, 1987; Rheault and O'Donnell, 2004) and vertebrate (Besseghir, Pearce and Rennick, 1981; Dantzler and Brokl, 1988) renal tissues have been shown to transport the prototypical organic cation, TEA. TEA is a small polar

molecule that is an excellent substrate for organic cation transporter family and a poor substrate for the multidrug resistance (MDR) protein, P-glycoprotein (Berkhin and Humphreys, 2001). TEA is not metabolized in vertebrates, suggesting that its clearance is due to excretion therefore making it an ideal substrate for studies of organic cation transport mechanisms (Besseghir, Pearce and Rennick, 1981).

Cellular mechanisms of organic ion transport

A useful starting point for studies of organic ion transport by insect MTs is to consider the mechanisms which have been identified in vertebrate renal tissues. Four organic anion transporters (OATs) with a broad specificity have been identified in a variety of human tissues such as the brain, kidneys, liver and placenta (Lee and Kim, 2004). OATs 1, 2 and 3 are located in the basolateral membrane of the cell whereas OAT4 is found in the apical membrane (Lee and Kim, 2004). A recent model of organic anion transport in vertebrate proximal tubule cells suggests that a Na^+/K^+-ATPase maintains a Na^+ gradient necessary for driving organic anion secretion. An organic anion/dicarboxylate anti-porter drives active uptake of organic anions into the cell. Re-entry of dicarboxylate ions into the cell occurs by a basolateral Na^+/dicarboxylate co-transporter (Berkhin and Humphreys, 2001). Transport of organic anions from cell to lumen is less well characterized but may involve exchange for lumenal anions, a voltage-dependent organic anion transporter (Pritchard and Miller, 1991), organic anion transporting polypeptides (OATPs) or multidrug resistance-associated proteins (MRPs; Lee and Kim, 2004).

In humans, five organic cation transporters (OCTs) have been identified in a range of tissues such as the brain, kidney, liver, placenta and intestines (Lee and Kim, 2004). OCT1 and OCT3 have been immunolocalized to the basolateral membrane whereas OCT2 is found in the apical membrane (Lee and Kim, 2004). Two additional members of the OCT family, OCTN1 and OCTN2, are also present in the apical membrane. It is generally viewed that organic cation transport into the cells is driven by the voltage difference across the basolateral membrane. Extrusion of organic cations into the lumen of the proximal tubule is achieved by an organic cation/proton exchanger in the apical membrane. A Na^+/H^+ exchanger in the apical membrane drives this anti-porter by increasing proton availability in the lumen of the tissue. MDR transporters such as P-glycoprotein may also have a role in organic cation secretion, particularly for larger and more hydrophobic cations.

Organic ion transport by insects

The Malpighian tubules of insects have long been known to transport organic anions such as amaranth and PAH (Maddrell *et al.*, 1974; Bresler *et al.*, 1990; Linton and O'Donnell, 2000). Insect Malpighian tubules actively transport two classes of organic anions; acylimides and sulphonates (Maddrell *et al.*, 1974). Given that competitive inhibition between the two classes is not observed it is believed that two systems exist for the secretion of these compounds. Additionally, it appears that fluid secretion and the secretion of dyes are two separate processes for most species.

Organic anion transport is dependent on the Na^+ gradient as indicated by the inhibition of PAH transport by ouabain. However, in contrast to OA transport by vertebrate cells, PAH transport by *Drosophila* is not affected by low concentrations of alpha-keto acids suggesting that the Na^+/dicarboxylate co-transporter is not required (O'Donnell *et al.*, 2003). Recent research has also shown transport of high levels of the organic anion salicylate by the Malpighian tubules and gut of *Drosophila* (M. O'Donnell and M. Rheault, personal communication).

Whereas organic anion transport by insect MTs was first described more than 60 years ago (Lison, 1937), transport of the prototypical organic cation TEA has only recently been demonstrated (Rheault and O'Donnell, 2004). TEA transport is via a potential driven mechanism across the lower and main segments of the MTs and the posterior midgut of *Drosophila*. TEA flux across the main segment and also the posterior midgut is $\approx$ 1 pmol cm^{-2} s^{-1} whereas that across the lower segment is $\approx$ 6 pmol cm^{-2} s^{-1}. Hyperpolarization of the basolateral membrane elevates TEA influx across the lower and main segments. TEA flux is inhibited 70% and 84% across the same epithelia by the organic cations quinidine and cimetidine.

Thesis Objectives

The first objective of this thesis is to identify whether factors that modulate inorganic ion transport and fluid secretion by the Malpighian tubules of *Drosophila* also modulate organic cation secretion. There is some evidence of regulation of organic cation transport in vertebrate systems. cGMP inhibits organic cation secretion by rat

organic cation transporter 1 (rOCT1) and human organic cation transporter 2 (hOCT2) (Schlatter *et al.*, 2002). Conversely, organic cation transport by rOCT1 can be increased by stimulation of protein kinase C (PKC; Mehrens *et al.*, 2000). Identifying the effects intracellular second messengers, peptides and amines produce upon organic cation transport may indicate whether any of these factors serve functions additional to stimulation of fluid secretion.

The second objective of this thesis is to identify the effects of maintaining larvae on TEA-enriched diet. Transport of TEA by isolated tubules and the posterior midgut of *Drosophila* raises the question of whether such transport can serve to eliminate organic cations which are contained in the diet. Nothing is known about the toxicity of small organic cations such as TEA to insects that may consume many times their body weight per day. In this thesis I have correlated larval mortality with the concentration of TEA in the diet and in the haemolymph. I have measured changes in haemolymph TEA concentration after *Drosophila* larvae have been maintained on a TEA-enriched diet or after the injection of TEA into the haemocoel. Finally, this thesis aims to identify whether prior exposure to dietary TEA alters the rates of TEA secretion by isolated MTs of *Drosophila*. This line of investigation was suggested by past studies which have shown that ingestion of a protein-rich meal induces higher rates of transport of both PAH and uric acid by the isolated tubules of the blood feeder, *Rhodnius prolixus* (Maddrell and Gardiner, 1975; O'Donnell, Maddrell and Gardiner, 1983). It is suggested that catabolism of the blood cells leads to elevation of circulating levels of organic anions and

uric acid. By analogy, increased levels of TEA in the haemolymph might be expected to up-regulate the rates of transport of organic cations by the Malpighian tubules.

Figure 1. Structure of the Malpighian tubules of *Drosophila melanogaster* (From Maddrell and O'Donnell, 1995)

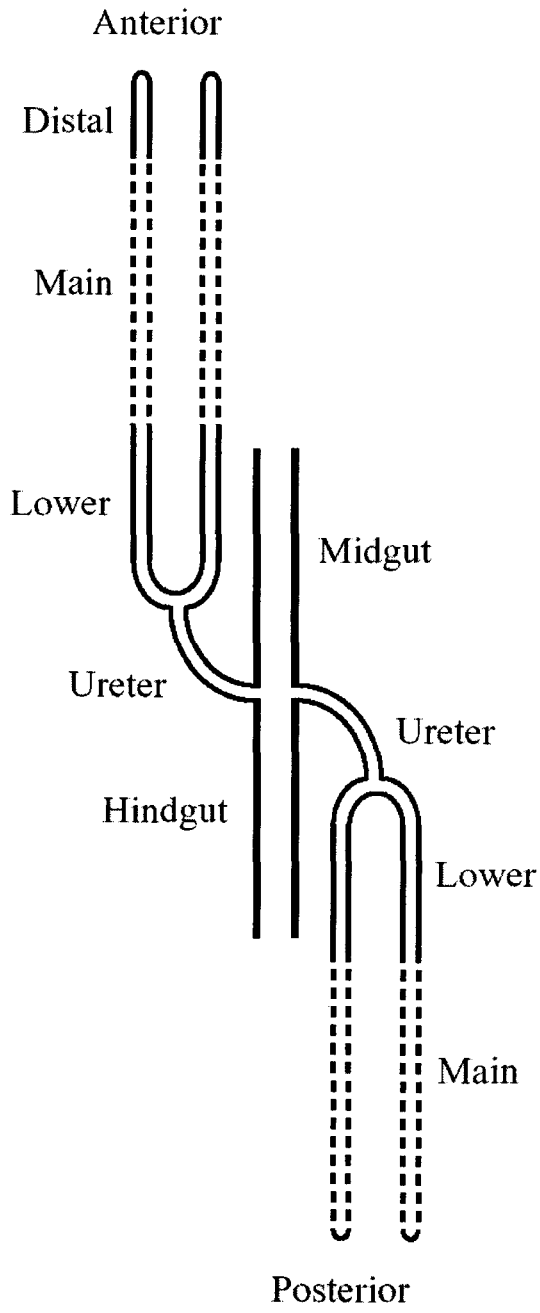

Anterior
Distal
Main
Lower
Midgut
Ureter
Ureter
Hindgut
Lower
Main
Posterior

Figure 2. A proposed model of ion transport in the principal (left) and stellate (right) cells of the main segment of *Drosophila*. (Modified from Ianowski and O'Donnell, 2004).

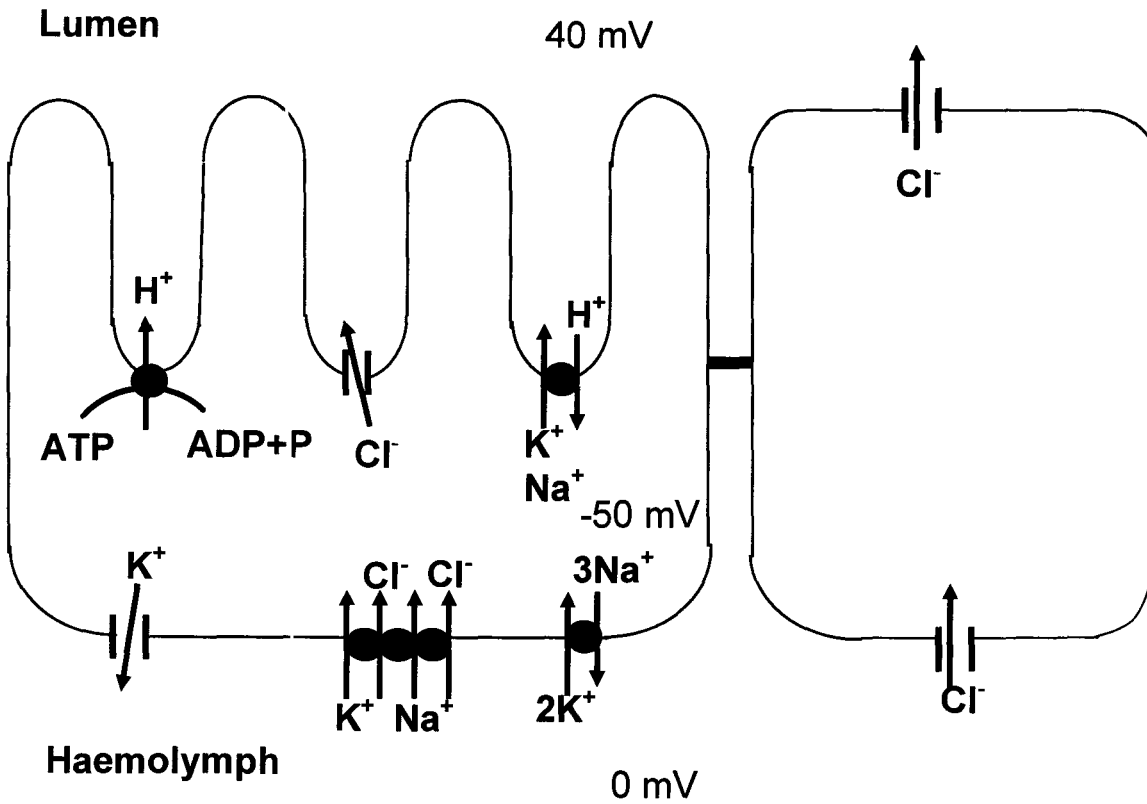
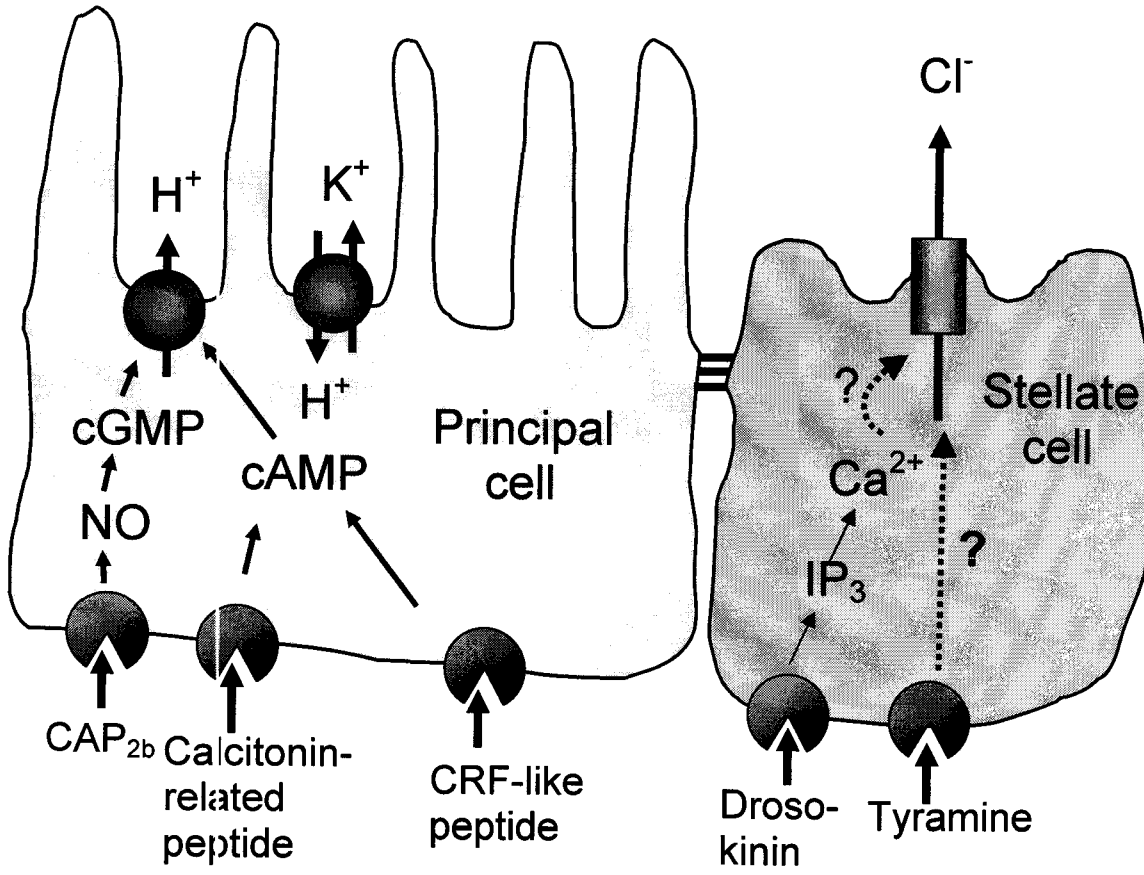

Lumen
40 mV
H+
ATP
ADP+P
Cl-
H+
K+
Na+
-50 mV
Cl-
K+
Cl- Cl-
3Na+
K+ Na+
2K+
Haemolymph
0 mV
Cl-
Cl-

Figure 3. Proposed model identifying factors which stimulate fluid and transepithelial inorganic ion secretion by the main segment of the Malpighian tubules of *Drosophila*.


Cl-
H+
K+
H+
cGMP
cAMP
Principal cell
NO
?
Stellate cell
Ca2+
IP3
?
CAP2b
Calcitonin-related peptide
CRF-like peptide
Droso-kinin
Tyramine

Chapter 2

Diuretic factors stimulate secretion of the organic cation TEA by the Malpighian tubules of *Drosophila melanogaster*

Abstract

This study showed that 4 diuretic factors which stimulate fluid secretion and transepithelial inorganic ion transport across the main segment of the Malpighian tubules of *Drosophila melanogaster* also stimulate transepithelial secretion of the prototypical organic cation tetraethylammonium (TEA). TEA fluxes across the Malpighian tubules and gut were measured using a TEA-selective self-referencing (TEA-SeR) microelectrode. TEA flux across isolated Malpighian tubules was also measured using a TEA-selective microelectrode positioned in droplets of fluid secreted by tubules set up in a modified Ramsay assay. TEA flux was stimulated by cAMP and cGMP, which increase the lumen-positive transepithelial potential (TEP), and also by tyramine and leucokinin-I (LK-I), which decrease TEP. The largest increase was measured in response to LK-I which increased transepithelial TEA flux by 72%. TEA flux in the lower tubule was stimulated slightly (13%) by tyramine but not by any of the other factors. TEA flux across the midgut was also unchanged by any of the diuretic factors. We propose that these diuretic factors enhance proton/organic cation exchange across the apical membrane by increasing proton availability in the lumen of the tubule. This is the first paper to demonstrate the effects of insect diuretic factors on excretion of organic cations.

Introduction

All animals must eliminate potentially toxic molecules that they produce through metabolism or take in from their environment. Systems for the excretion of xenobiotics include those for the transport of organic cations such as choline, N-methyl nicotinamide and tetraethylammonium (TEA) as well as organic anions such as p-aminohippurate (PAH), benzyl penicillin and various acidic dyes such as amaranth. Both vertebrate (Dantzler and Brokl, 1988; Smith, Pritchard and Miller, 1988) and invertebrate (Miller and Holliday, 1987) renal tissues transport the prototypical organic cation TEA. TEA transport has been studied in the mammalian and teleost kidney (Pritchard and Miller, 1991), human embryonic kidney (HEK) 293 cells (Yabuuchi *et al.*, 1999), and *Xenopus* oocytes (Sweet and Pritchard, 1999), as well as in the crustacean antennal gland (Miller and Holliday, 1987). A recent study has demonstrated that the Malpighian tubules and gut of *Drosophila melanogaster* transport TEA (Rheault and O'Donnell, 2004).

The Malpighian tubules (MTs) and hindgut comprise the functional kidney of insects. The tubules contribute to osmoregulation by secretion of a near iso-osmotic fluid. Water and/or ions are reabsorbed downstream either by a lower segment of the tubule or the hindgut (Maddrell, 1981; O'Donnell and Maddrell, 1995). There are two pairs of tubules in *Drosophila*; each pair is joined by a common ureter to the junction of the midgut and hindgut. The anterior pair of MTs consists of lower, main and distal segments whereas the posterior pair lacks distal segments. The distal segment does not secrete fluid but transports calcium ions at high rates (Dube *et al.*, 2000). Fluid containing 120 mmol l^{-1} KCl and 30 mmol l^{-1} NaCl is secreted by the main segment

(O'Donnell and Maddrell, 1995), which is comprised of two distinct cell types, principal and stellate cells (Sözen *et al.*, 1997). The lower segment consists of a single morphological cell type which reabsorbs approximately 30% of the KCl and fluid secreted by the main segment (O'Donnell and Maddrell, 1995).

At least 5 diuretic factors that augment fluid secretion by *Drosophila* tubules act upon either the principal or stellate cells in the main segment. Secretion is stimulated by corticotropin releasing factor-like (CRF-like; Cabrero *et al.*, 2002) and calcitonin related peptides (Coast *et al.*, 2001), both of which act through intracellular cAMP to stimulate the activity of the apical vacuolar H^+-ATPase in the principal cells. Cardioacceleratory peptide 2b (CAP_{2b}) also stimulates this ATPase but does so through increases in nitric oxide (NO) and cGMP (Davies *et al.*, 1995). Fluid secretion is also stimulated by the leucokinin-like peptide drosokinin and the amine tyramine. Drosokinin enhances transepithelial chloride permeability through increases in intracellular calcium levels in the stellate cells (Rosay *et al.*, 1997; Terhzaz *et al.*, 1999). A similar mechanism is proposed to mediate the actions of tyramine (Blumenthal, 2002).

The Malpighian tubules of *Drosophila* secrete TEA across both the lower and main segments but not the distal segment (Rheault and O'Donnell, 2004). The posterior midgut also secretes TEA at a rate similar to that of the main segment (Rheault and O'Donnell, 2004). TEA transport across the anterior midgut is negligible and there is a small efflux across the hindgut and rectum. Flux values from bath to lumen as large as 6 pmol cm^{-2} s^{-1} are found across the lower segment and ureter while bath to lumen TEA fluxes across the main segment and the posterior midgut are $\approx$ 1 pmol cm^{-2} s^{-1} (Rheault

and O'Donnell, 2004). The transepithelial potential (TEP) is in the range of 30mV to 80 mV lumen-positive and lumen concentrations of TEA may exceed those of the bath by a factor of 12 or more, indicating that TEA is actively transported across the main segment against an opposing electrochemical gradient. TEA transport is enhanced by hyperpolarization of the basolateral membrane potential and is competitively inhibited by organic cations such as cimetidine and quinidine (Rheault and O'Donnell, 2004).

Studies of vertebrate renal organs show that rates of organic cation transport can be modulated by several intracellular second messengers and protein kinases. Stimulation of protein kinase C increases organic cation transport in rabbit proximal tubules expressing rat organic cation transporter 1 (rOCT1; Hohage *et al.*, 1994). TEA transport is also stimulated by a calcium-calmodulin dependent pathway in HEK293 cells expressing human organic cation transporter 2 (hOCT2; Çetinkaya *et al.*, 2003). Conversely, cGMP inhibits organic cation transport by human proximal tubules and HEK293 cells expressing rOCT1 (Pietig *et al.*, 2001; Schlatter *et al.*, 2002). Stimulation of protein kinase A with the adenylyl cyclase activator forskolin inhibits transport of TEA in HEK293 cells expressing hOCT2 (Çetinkaya *et al.*, 2003) but stimulates transport of TEA in the same cells expressing rOCT1 (Mehrens *et al.*, 2000).

It is unknown whether intracellular second messengers such as cAMP, cGMP and calcium can modulate the rate of organic cation secretion across the Malpighian tubules and gut of *Drosophila*. Many recent studies (O'Donnell *et al.*, 1996; Dow, Davies and Sözen 1998; O'Donnell and Spring, 2000) have addressed the modulation of fluid secretion and inorganic ion transport by *Drosophila* MTs; however control of organic

cation secretion has not been studied. The presence of multiple diuretic factors raises the possibility that some may have functions other than stimulating fluid secretion. For example, some may act as clearance factors stimulating both secretion by the tubules and reabsorption of water and ions by the lower tubule and hindgut so as to passively clear the haemolymph of toxic molecules or waste products. Alternatively, factors that stimulate inorganic ion transport and fluid secretion may also stimulate the mechanisms involved in excretion of specific classes of toxic molecules. In this paper, we examine the effects of the cyclic nucleotides cAMP and cGMP, the amine tyramine and the peptide leucokinin-I (LK-I) on transport of TEA by the gut and Malpighian tubules of *Drosophila melanogaster*.

TEA transport has been measured by two techniques previously described by Rheault and O'Donnell (2004). The self-referencing ion selective microelectrode technique permits non-invasive measurement of the difference in ion concentration between two sites within the unstirred layer near the surface of a cell or tissue (Fig. 1). Corresponding ion fluxes can be calculated using measured concentration differences and the Fick equation. This technique has been used extensively for measuring proton flux in the midgut of larval *Aedes aegypti* (Boudko *et al.*, 2001), potassium flux in *Drosophila* Malpighian tubules (Rheault and O'Donnell, 2001) and chloride flux in foetal lung epithelial cells (Land and Collett, 2001). TEA-selective self-referencing (TEA-SeR) microelectrodes have recently been developed to permit analysis of TEA flux by the Malpighian tubules and midgut of *Drosophila* (Rheault and O'Donnell, 2004).

In the second technique both the rates of fluid secretion by isolated Malpighian tubules set up in a Ramsay secretion assay and the concentration of TEA in droplets of secreted fluid were measured. TEA flux was then calculated as the product of fluid secretion rate and secreted fluid TEA concentration.

Materials and Methods

Animals and dissection procedures

We used 2-3 day old females of the Oregon R strain of *Drosophila melanogaster* in all experiments. Flies were raised on standard yeast media using protocols described by Ashburner (1989). Experiments were performed at room temperatures (22 - 25 °C) and ambient humidity. The alimentary canal and MTs were isolated from the animals in *Drosophila* saline containing (in mmol l^{-1}): NaCl (117.5), KCl (20), $CaCl_2$ (2), $MgCl_2 6H_2O$ (8.5), $NaHCO_3$ (10.2), NaH_2PO_4 (4.3), HEPES (8.6), L-glutamine (10), and glucose (20). The saline was titrated with NaOH to pH 7.0. Dissection procedures have been described by Dow *et al.* (1994).

Chemicals

cAMP, cGMP and tyramine were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). LK- I was obtained from Bachem California Inc. (Torrance, CA, USA). Both cAMP and cGMP were applied at 1 mmol l^{-1} in *Drosophila* saline and both LK-I and tyramine were applied at 1 μmol l^{-1}. In each case, the concentration

applied has been shown to stimulate fluid secretion by isolated *Drosophila* MTs (O'Donnell *et al.*, 1996; Terhzaz *et al.*, 1999; Blumenthal, 2002).

TEA-SeR microelectrode measurements of TEA flux

Procedures for dissection and scanning of the tubules have been described by Rheault and O'Donnell (2001, 2004). Briefly, a pair of tubules was placed in a 35 mm Petri dish pre-coated with poly-L-lysine to facilitate adherence of tissues and bathed in saline containing 0.1 mmol l^{-1} TEA. This concentration is close to the value for half-maximal transport (K_t) of TEA (Rheault and O'Donnell, 2004). When measuring TEA flux across the midgut, the entire alimentary canal and both pairs of tubules were kept intact. TEA-selective microelectrodes were constructed as described previously (Rheault and O'Donnell, 2004). In saline containing 20 mmol l^{-1} K^+, the electrode slope for a change from 0.1 to 1 mmol l^{-1} TEA was 59.8 ± 0.3 mV (n = 65 microelectrodes). Microelectrodes were positioned with a CMC-4 computerized 3-D motion control system and voltages were measured using an IPA-2 amplifier (Applicable Electronics, Forrestdale, MA, USA). The system was controlled using Automated Scanning Electrode Technique software (ASET; Sciencewares , East Falmouth, MA, USA).

Tissues were scanned with TEA-SeR microelectrodes for periods up to 40 minutes. At each measurement site, the electrode was moved perpendicular to the tissue surface between two positions separated by 50 μm. The TEA-SeR microelectrode was viewed using an inverted microscope equipped with a video camera and the "*move, wait and sample*" protocol at each measurement site was controlled through the ASET

software. The TEA-SeR microelectrode tip was first *moved* to a site 5 μm from the tissue surface. The microelectrode then remained stationary during the 3 s *wait* period to allow ion gradients near the tubule to re-establish after the localized stirring during the movement period. No data was collected during the wait period. The microelectrode voltage was recorded and averaged for 0.5 s during the *sample* period. The TEA-SeR microelectrode was then moved perpendicular to the tissue surface to the other extreme of the 50 μm excursion, followed by another wait and sample period. Each move, wait and sample cycle at each extreme of microelectrode excursion was complete in 3.5 s. Each flux determination required measurement of the concentration difference between the two extremes of the excursion, for a total of 7 s. Fluxes are reported as the mean of 3 repetitive measurements at each site.

TEA-specific signal differences (ΔV; measured in μV) obtained across the excursion distance of the TEA-SeR microelectrode were converted to TEA concentration differences and corresponding flux calculations were performed as previously described (Rheault and O'Donnell, 2004). Briefly, TEA-specific voltage differences were converted to TEA concentration differences (ΔC) using the equation:

$$\Delta C = 2.3(\Delta V C_B)/S, \quad (1)$$

where ΔV is the signal difference measured between the two extremes of the microelectrode excursion, C_B is the background TEA concentration in the bathing medium (0.1 μmol cm^{-3} in all experiments) and S is the slope of the electrode (μV).

Values of ΔC were converted into corresponding fluxes by substitution into the Fick equation:

$$J_{TEA} = D(\Delta C/\Delta r), \quad (2)$$

where J_{TEA} is the flux of TEA (pmol cm^{-2} s^{-1}), D is the diffusion coefficient of TEA at 25°C (0.868 x 10^{-5} cm^2 s^{-1}) and Δr is the excursion distance of the microelectrode (cm).

Measurement of TEA flux in secreted fluid

Modification of the Ramsay assay for studies of *Drosophila* MTs has been described by Dow *et al.* (1994). Briefly, a pair of tubules was dissected and transferred to a droplet of saline under paraffin oil. One tubule from the pair was immersed in the bathing saline and the other was pulled out of the saline and wrapped around a steel pin so as to position the ureter halfway between the bathing droplet and the pin. A droplet of secreted fluid formed at the ureter and was collected using a fine glass rod. Droplets were collected at 30 minute intervals over a span of 90 minutes. A droplet of secreted fluid was collected in the absence of the TEA in the bathing saline to determine the background signal for the TEA-selective microelectrode. TEA was then added to the bath to a final concentration of 0.1 mmol l^{-1}. A stimulator of fluid secretion was added to the bath after a second droplet of secreted fluid had been collected. A third and final droplet was collected 30 minutes after the addition of the stimulant. Under this procedure each tubule was used as its own control. The concentration of TEA in the secreted fluid ($[TEA]_{sf}$) was measured using a TEA-selective microelectrode and a calibration curve relating voltage to concentration. Calibration solutions contained 0.05, 0.5 and 5 mmol l^{-1} TEA in saline. Tips of TEA-selective microelectrodes were coated with poly-vinyl chloride to permit measurements of droplets under oil, as described by Rheault and

O'Donnell (2004). Voltage differences between the TEA-selective microelectrode and a 500 mmol l^{-1} KCl-filled reference microelectrode were measured with a high-impedance differential electrometer (AM Systems, Carlsborg, WA) and recorded using a PC-based data acquisition system (Axotape, Axon Systems, Union City, CA). TEA flux (pmol min^{-1} $tubule^{-1}$) was calculated by multiplying the fluid secretion rate (nl min^{-1}) by the $[TEA]_{sf}$ (mmol l^{-1}).

Statistics

Bar charts are presented as means + SEM. Significance of differences between control and experimental groups were assessed using a paired students *t*-test (two-tailed) with $P < 0.05$ as the critical value.

Results

TEA-SeR microelectrode measurements

TEA influx across the main segment of the Malpighian tubule was stimulated 15% by cAMP, 18% by cGMP, 58% by tyramine and 32% by LK-I (Fig. 2). By contrast, there was no significant effect of cAMP and cGMP on TEA flux across either the lower MT or the midgut (Fig. 2). TEA flux was also not significantly affected across the midgut in response to tyramine. The stimulation of TEA influx across the lower MT in response to tyramine (13%) may relate to the stimulation of fluid secretion in the main segment as discussed below.

Secreted fluid measurements

Fluid secretion rates by the main segment were significantly increased by all five treatments (Fig. 3A). Fluid secretion rates were increased 72% by cAMP, 39% by cGMP, 107% by tyramine and 75% by LK-I. The largest increase in fluid secretion rate (126%) was measured after addition of cAMP and LK-I together.

Significant decreases in $[TEA]_{sf}$ were produced by cAMP, tyramine and the combination of cAMP and LK-I but not by cGMP or LK-I alone. The largest decrease in $[TEA]_{sf}$ was caused by tyramine which lowered the $[TEA]_{sf}$ by 38% (Fig. 3B). Decreases in TEA concentrations of 18% and 34% were observed after addition of cAMP alone and cAMP plus LK-I, respectively.

Transepithelial TEA flux increased significantly in response to each of the stimulants of fluid secretion (Fig. 3C). Flux increased by 41%, 35% and 31% in response to cAMP, cGMP and tyramine, respectively. The greatest increases in TEA flux were in response to LK-I (72%) followed by cAMP plus LK-I (48%).

Discussion

We utilized both the TEA-SeR technique and the Ramsay fluid secretion assay to assess the effects of cAMP, cGMP, tyramine and LK-I on TEA flux across the Malpighian tubules and posterior midgut of *Drosophila.* The two approaches are complimentary and yield similar values for TEA flux in unstimulated MTs when fluxes measured by the Ramsay technique (pmol min^{-1}) are divided by tubule surface area x 60 to yield the same units as the TEA-SeR technique (pmol cm^{-2} s^{-1}; Rheault and O'Donnell,

2004). The TEA-SeR technique can be used to measure flux across specific regions of an epithelium such as the lower segment of the MTs. It is also suitable for tissues, such as the midgut or the distal segment of the MT, that do not secrete fluid. However, the TEA-SeR technique does not allow discrimination between basolateral uptake followed by subsequent intracellular sequestration and true transepithelial flux from bath to lumen. By contrast, the Ramsay approach measures only transepithelial flux and also provides additional information in the form of fluid secretion rate and secreted fluid TEA concentration. Changes in fluid secretion rate confirm the effectiveness of the four stimulants on cell function at the concentrations used in this study. Intracellular second messengers could potentially increase TEA flux through stimulation of intracellular sequestration or through stimulation of transepithelial flux. The increases in TEA flux in response to cAMP, cGMP, LK-I and tyramine were similar in measurements made by either technique, consistent with stimulation of net transepithelial flux.

Current models of ion transport by the main segment propose that fluid secretion involves the flow of osmotically obliged water driven by active transepithelial ion transport (Maddrell and O'Donnell, 1992; O'Donnell and Spring, 2000; Ianowski and O'Donnell, 2004). Our results suggest a link between stimulation of fluid secretion rate and transepithelial TEA transport. Two explanations for this link can be proposed. Increased fluid secretion dilutes TEA concentrations in the tubule lumen, and transepithelial TEA flux may thus increase in response to lower backflux of TEA from lumen to cell. Alternatively, enhanced transport of ions in response to a stimulant may lead to higher rates of TEA transport if the movement of TEA across the cell membrane

is coupled to the movement of another ion. In this context, it is worth noting that stimulation of the electrogenic apical H^+-ATPase of the Malpighian tubules by cAMP or cGMP increases the rate of proton transport into the lumen and shifts both apical membrane potential and TEP to a more lumen positive value (O'Donnell *et al.*, 1996). The increased proton availability will thus drive higher rates of Na^+/H^+ and K^+/H^+ exchange and the more lumen positive TEP will drive higher transepithelial chloride transport. The net effect is stimulation of transepithelial Na^+, K^+, and Cl^- flux and a consequent increase in the flow of osmotically obliged water. By contrast, tyramine and LK-I act to increase transepithelial chloride permeability with a consequent shift of TEP to a less positive value (Blumenthal, 2002; O'Donnell and Spring, 2000). This creates a more favourable electrical gradient for the movement of protons from cell to lumen and thereby stimulates transepithelial transport of Na^+ and K^+ as well as Cl^-.

Our working hypothesis for the mechanism of TEA secretion by the main segment is based upon the finding that treatments which stimulate active transepithelial transport of Na^+ and K^+ also stimulate TEA secretion. Given that transport of Na^+ and K^+ across the apical membrane is proposed to involve an alkali cation/H^+ exchanger (O'Donnell and Spring, 2000), we suggest that the movement of TEA from cell to lumen involves a process of TEA/H^+ exchange. It is worth noting in this context that current models of organic cation transport for vertebrate renal epithelia (Dantzler and Brokl, 1988; Pritchard and Miller, 1991; Ito, 1999) propose that organic cation movement across the apical membrane also involves organic cation/proton exchange. In vertebrate renal cells it is proposed that the necessary proton gradient is driven by an exchange of H^+ for

luminal Na^+ (Pritchard and Miller, 1991; Berkhin and Humphreys, 2001). By contrast, in *Drosophila* protons are actively secreted into the lumen of the Malpighian tubules by the vacuolar type H^+-ATPase (O'Donnell *et al.*, 1996). Our data are consistent with some form of electroneutral transport of TEA from cell to lumen. In the lepidopteran midgut, there is evidence for the movement of K^+ from cell to lumen by an electrogenic $2H^+/K^+$ apical exchanger that is driven by the electrical gradient created by an apical electrogenic H^+-ATPase (Wieczorek, 1992). Electrogenic mechanisms of TEA movement (e.g. $2H^+/TEA^+$) are unlikely in *Drosophila* Malpighian tubules because treatments which increase or decrease the lumen positive TEP were nonetheless associated with stimulation of transepithelial TEA secretion.

The highest rates of TEA transport are found in the lower segment of the Malpighian tubule (Rheault and O'Donnell, 2004) which is also involved in acidification of the urine (O'Donnell and Maddrell, 1995). An apical TEA/H^+ exchanger may therefore be involved in TEA transport across the apical membrane of the lower tubule as well, although additional mechanisms may be present. However, with the exception of tyramine, none of the factors that stimulated TEA secretion by the main segment had a significant stimulatory effect upon TEA flux across the lower tubule. It's important to point out that the apical and basolateral ion transporters in the lower tubule of *Drosophila* have not yet been identified. The lower tubule acidifies the urine, reabsorbs K^+, Cl^- and water and secretes Ca^{2+} into the lumen (O'Donnell and Maddrell, 1995). Moreover, the TEP in the lower tubule is less positive than that of the main segment and is lumen negative in the region closest to the ureter (O'Donnell and Maddrell, 1995). Factors that

stimulate fluid and ion transport in the main segment would not be expected *a priori* to modulate ion transport by the lower MT, given the fundamentally different ion transport processes in the two segments. In particular the proposal that cAMP, cGMP, LK-I and tyramine stimulate organic cation transport in the main segment by increasing proton availability in the lumen may not apply to the lower tubule. This may explain the lack of effect of these compounds on TEA transport across the lower tubule, even if the basal rate of H^+ secretion is sufficient to drive high rates of the putative H^+/TEA exchange process. TEA secretion by the midgut was also unaffected by cAMP, cGMP, LK-I and tyramine. To date, relatively few factors that modulate ion transport across the insect midgut have been identified, although allototropin has been shown to inhibit ion transport across the posterior midgut of *Manduca* larvae (Lee, Horodyski and Chamberlin, 1998).

As noted above, stimulants of fluid secretion will dilute TEA in the lumen and may thus reduce passive backflux of TEA from lumen to bath. Two lines of evidence argue against the possibility of increasing net TEA secretion by such a reduction in backflux. First, there is not tight correlation between the percentage increase in fluid secretion rate and the percentage increase in TEA secretion. For example, fluid secretion was increased to the greatest extent by the combination of cAMP and LK-I but the corresponding increase in TEA flux was similar to that produced by cAMP or LK-I alone. Secondly, the lower tubule does not show any corresponding increase in TEA flux when fluid secretion rate is increased in the main segment upstream. It is also worth noting that a significant paracellular backflux of TEA from lumen to bath seems unlikely

given that treatments which produce opposite effects on TEP in the main segment stimulate TEA secretion to similar extents.

One of the challenges facing insect physiologists who study osmoregulation is to provide a rational explanation for the apparent redundancy of diuretic factors in particular species. There are five diuretic factors described to date for *Drosophila melanogaster*, and at least 9 peptidergic and aminergic compounds which alter MT fluid secretion rate in *Manduca sexta* (Skaer *et al.*, 2002). Our results raise the possibility that some of the diuretic factors in *Drosophila* may serve not only osmoregulatory functions, but may also act to stimulate excretion of potentially toxic molecules such as organic cations.

Figure 1. A representative scan of TEA flux at two sites along the main segment of the Malpighian tubule. Thin arrows indicate TEA-specific signal differences prior to the addition of 1 μmol l^{-1} tyramine to the bathing saline. Thick arrows indicate the differential signal after the addition of tyramine. The leftmost vector of each group is positioned at the scan origin site whereas subsequent vectors are offset to the right to avoid overlap.

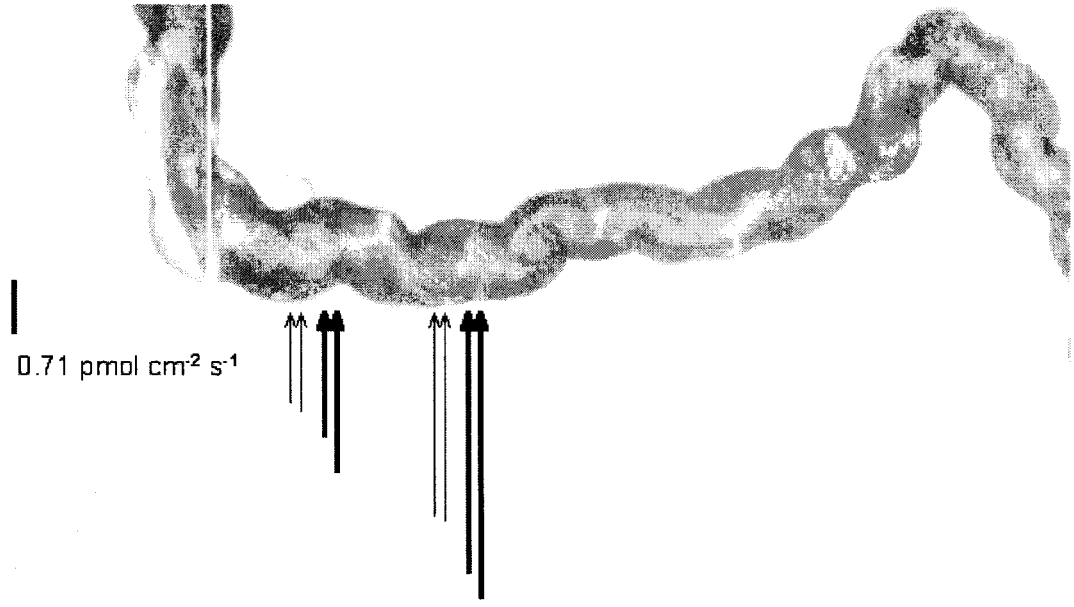
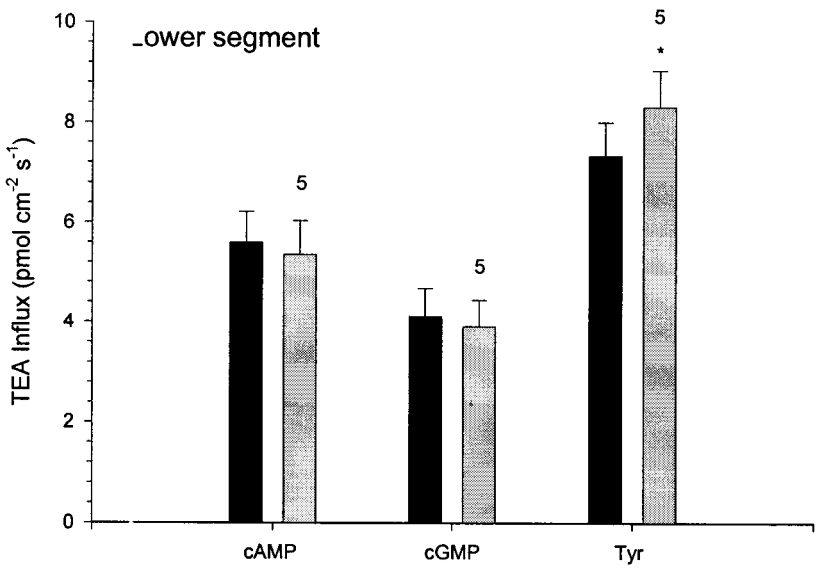
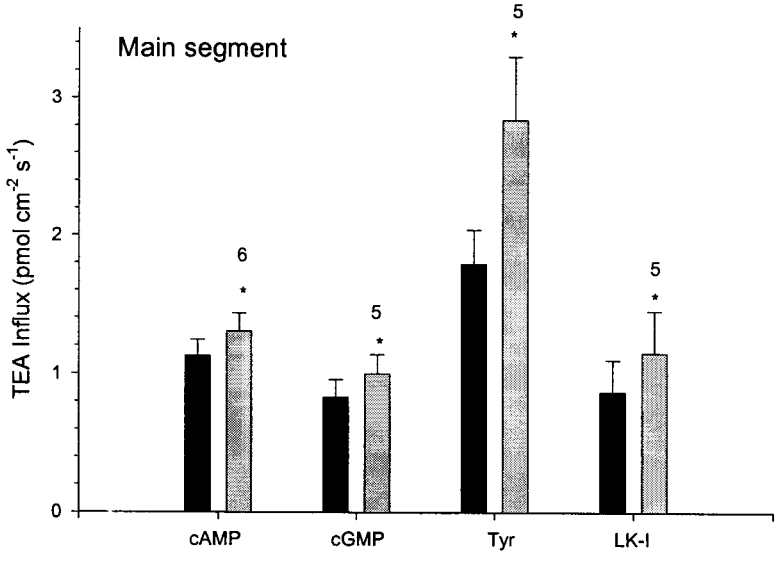

0.71 pmol cm-2 s-1

Figure 2. TEA-SeR measurements of TEA influx across the lower and main segments of the Malpighian tubule and the posterior midgut of *Drosophila*. Black and grey bars indicate control and experimental values respectively. N values are indicated above the bars. Significant differences between control and experimental values (paired *t*-test, $P < 0.05$) are indicated by asterisks.


Lower segment
TEA Influx (pmol cm-2 s-1)
0
2
4
6
8
10
5
5
5
*
cAMP
cGMP
Tyr


Main segment
TEA Influx (pmol cm-2 s-1)
0
1
2
3
6
*
5
*
5
*
5
*
cAMP
cGMP
Tyr
LK-I

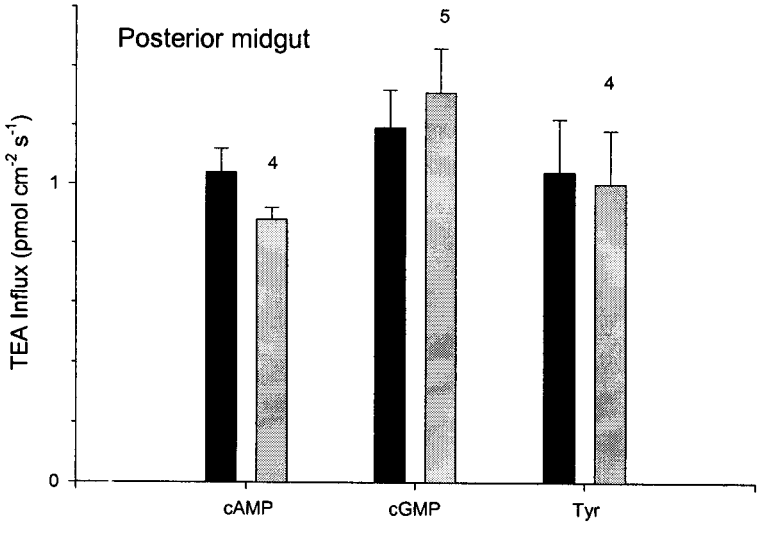
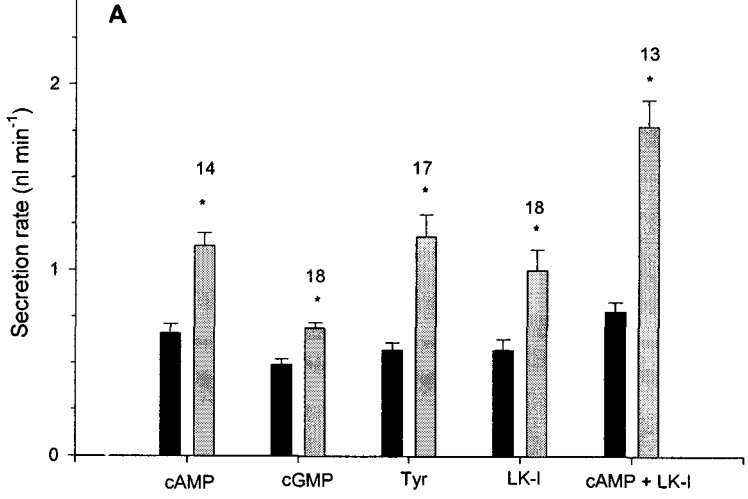
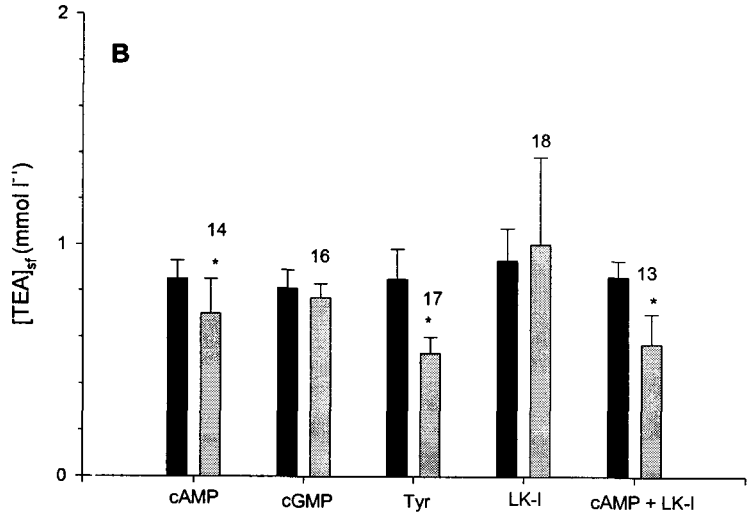
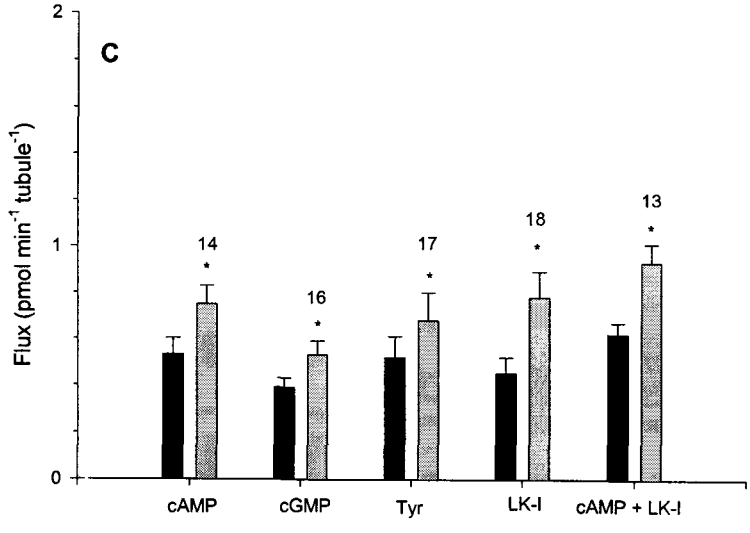

Posterior midgut
TEA Influx (pmol cm-2 s-1)
0
1
4
5
4
cAMP
cGMP
Tyr

Figure 3. Effects of diuretic factors on (A) secretion rate, (B) $[TEA]_{sf}$ and (C) TEA flux by the main segments of isolated Malpighian tubules set up in a modified Ramsay assay. Black and grey bars indicate control and experimental values respectively. N values are indicated above the bars. Significant differences between control and experimental values (paired *t*-test, $P < 0.05$) are indicated by asterisks.


A
Secretion rate (nl min-1)
0
1
2
14
18
17
18
13
cAMP
cGMP
Tyr
LK-I
cAMP + LK-I


B
[TEA]sf (mmol l-1)
0
1
2
14
16
17
18
13
cAMP
cGMP
Tyr
LK-I
cAMP + LK-I


C
Flux (pmol min-1 tubule-1)
0
1
2
14
16
17
18
13
cAMP
cGMP
Tyr
LK-I
cAMP + LK-I

Chapter 3

Effects of dietary or injected organic cations on mortality and haemolymph tetraethylammonium levels in larval *Drosophila melanogaster.*

Abstract

Larval *Drosophila melanogaster* tolerate high levels of the prototypical organic cation tetraethylammonium (TEA) in the diet and in the haemolymph. The LD_{50} value for dietary TEA was 158.4 mmol l^{-1}. Mortality increased if two organic cations were presented in the diet. The combination of 100 mmol l^{-1} TEA and either 10 mmol l^{-1} quinidine or 100 mmol l^{-1} cimetidine in the diet increased mortality to 83% and 67%, respectively, relative to the level of 24% when the diet contained 100 mmol l^{-1} TEA alone. TEA-selective microelectrode measurements indicated that the haemolymph TEA concentration was ≈ 3% of that in the diet for larvae maintained on TEA-enriched diet for 24 hours. Haemolymph TEA concentration declined at rates of 0.67, 0.62, 0.74 and 1.72 mmol l^{-1} hr^{-1} when larvae previously maintained on diet containing 100, 150, 200 and 300 mmol l^{-1}, respectively, were transferred to TEA-free diet. The rate of decline was temperature dependent and was reduced by previous exposure to diet containing both TEA and cimetidine. Haemolymph TEA concentration declined at a rate of 3.40 mmol l^{-1} hr^{-1} after haemolymph TEA levels were elevated to 10.9 mmol l^{-1} by injection of TEA into the haemocoel. Fluid secretion rates and transepithelial TEA flux by isolated MTs from adult flies raised on 100 mmol l^{-1} TEA-enriched diet were reduced 40% relative to flies raised on TEA-free diet. We propose that TEA concentrations in the haemolymph

are reduced both by active transport across the MTs and gut, and also by passive diffusion into the gut. The latter component is particularly important when larvae previously maintained upon TEA-enriched diet are transferred to a TEA-free diet. The ingestion of TEA-free food not only clears the gut lumen, but also creates a TEA-free compartment which TEA can passively diffuse into from the haemolymph.

Introduction

Animals are continuously exposed to a variety of environmental toxins and metabolic waste products. Detoxification mechanisms such as metabolizing enzymes and transport proteins mediate the inactivation and excretion of such compounds. For excretion, a plethora of transmembrane transport proteins are present in the renal tissues, gut and organs of intermediary metabolism. These transporters are responsible for elimination of organic cations, organic anions, neutral and cationic hydrophobic compounds and anionic conjugates.

Organic cations are chemically heterogeneous compounds possessing a carbon backbone, a hydrophobic moiety and a net positive charge at physiological pH. Numerous endogenous and exogenous molecules, many of which are harmful, can be classified as organic cations, and their elimination is therefore essential for the maintenance of homeostasis. Transport of the prototypical organic cation tetraethylammonium (TEA) has been studied extensively in both vertebrate (Dantzler and Brokl, 1988), and invertebrate (Miller and Holliday, 1987), tissues. *In vitro* studies have

shown active transport of TEA across isolated Malpighian tubules (MTs) and the posterior midgut of *Drosophila melanogaster* (Rheault and O'Donnell, 2004).

The four MTs in *Drosophila* are arranged in two pairs. The anterior pair contains a non-secretory distal segment, a secretory main segment and a reabsorptive lower segment. Tubules in the posterior pair lack the distal segment but are otherwise identical. The lower segment transports TEA at rates as high as 6 pmol cm^{-2} s^{-1}, whereas the main segment and posterior midgut each transport TEA at lower rates ($\approx$ 1 pmol cm^{-2} s^{-1}; Rheault and O'Donnell, 2004).

Current models of organic cation transport in vertebrate renal systems propose that organic cations enter the cell across the basolateral membrane through a potential dependent mechanism and are subsequently transported across the apical membrane into the lumen of the tubule through an organic cation/proton exchanger or by the ATP-dependent efflux pump P-glycoprotein (P-gp; Lee and Kim, 2004). P-gp is a membrane bound protein involved in multidrug resistance (MDR). Many alkaloids and large organic cations are transported via P-gp however TEA is not an ideal P-gp substrate (Berkhin and Humphreys, 2001).

Transport of potentially toxic molecules may be upregulated in response to exposure in the diet or the environment. For example, exposure of the marine snail, *Monodonta turbinata*, to diesel oil is associated with an induction of P-gp activity (Kurelec, 1992). Similar induction is found in the mussel, *Mytilus californianus* in response to P-gp substrates such as pentachlorophenol and chlorthal (Eufemia and Epel, 2000). Transport of the organic anion para-aminohippurate (PAH) is upregulated in the

Malpighian tubules of the blood-feeding insect *Rhodnius prolixus* after consumption of a protein rich meal (Maddrell and Gardiner, 1975). By contrast, nicotine is transported at high rates by Malpighian tubules of larvae of *Manduca sexta*, *Pieris brassicae* and *Rhodnius prolixus* and the transport persists even when the alkaloid is not present in the diet (Maddrell and Gardiner, 1976). Bijelic and O'Donnell (2004) have demonstrated that the rates of TEA transport across the main segment of the Malpighian tubules of *Drosophila* are increased by factors which stimulate transepithelial transport of inorganic ions and fluid (tyramine, leucokinin-I, cAMP and cGMP). Rates of TEA transport by the lower segment of the tubule or the posterior midgut are unaffected by the same factors.

The goals of this study were to address several questions arising from previous studies of organic cation and organic anion transport both in insects and in other animals: 1) Is TEA absorbed from the diet by *Drosophila* larvae and does it appear in the haemolymph? 2) What levels of dietary TEA are associated with increased larval mortality? 3) Does the presence of other organic cations such as quinidine or cimetidine in the diet affect the rate of TEA decline from the haemolymph and the mortality associated with a given dietary concentration of TEA? The organic cations cimetidine and quinidine inhibit TEA transport by *Xenopus* oocytes (Okuda *et al.*, 1996), rat renal cortical slices (Sambasava and Arvind, 1994) and isolated Malpighian tubules of *Drosophila* (Rheault and O'Donnell, 2004) raising the possibility that addition of these compounds to the diet may diminish the ability of the larvae to excrete TEA. Previous studies in the mosquito *Culex pipiens* have identified an interaction between insecticide toxicity and P-gp blockers. The P-gp inhibitor verapamil increases toxicity to

insecticides such as endosulfan and ivermectin that are known to be good P-gp substrates (Buss *et al.*, 2002). 4) Does exposure of larvae to TEA in the diet alter the capacity of the Malpighian tubules to transport TEA?

Materials and Methods

Animals and Dissection Procedures

Experiments on the Oregon R strain of *Drosophila melanogaster* were performed at room temperature (22-25 °C) and ambient humidity. The effects of dietary TEA on haemolymph levels of TEA and larval mortality were studied in second and third instar larvae. Ramsay fluid secretion assays were performed with Malpighian tubules from 2-3 day old adult females dissected under saline following procedures described by Dow *et al.* (1994). *Drosophila* saline contained (in mmol l^{-1}): NaCl (117.5), KCl (20), $CaCl_2$ (2), $MgCl_2 \cdot 6H_2O$ (8.5), $NaHCO_3$ (10.2), NaH_2PO_4 (4.3), HEPES (8.6), L-glutamine (10), and glucose (20). Saline was titrated with NaOH to pH 7.0.

Diet preparation

Drosophila yeast media was prepared from two solutions as described by Roberts and Stander (1998):

Solution A: 800 mL tap water, 100 g sucrose, 18 g agar, 1 g KH_2PO_4, 8 g KNa tartrate, 0.5 g NaCl, 0.5 g $MgCl_2$, 0.5 g $CaCl_2$, and 0.5 g ferric sulphate. Solution B: 200 mL tap water and 50 g dry active yeast. The two solutions were autoclaved, then combined and stirred. After cooling to < 60 °C, 7.45 mL of 10% p-hydroxybenzoic acid methyl ester

(Tegosept) dissolved in ethanol and 10 mL of an acid mix (11 parts tap water, 10 parts propionic acid and 1 part 85% o-phosphoric acid) were added to the mixture. This control diet contained 36.6 mmol l^{-1} Na^+ and 14.5 mmol l^{-1} Cl^-. Experimental diets were prepared by addition of appropriate volumes of stock solutions to produce diet concentrations of 10, 50, 100, 150, 200 or 300 mmol l^{-1} TEA, 10 or 100 mmol l^{-1} cimetidine, 1 or 10 mmol l^{-1} quinidine, and 100, 200 or 300 mmol l^{-1} NaCl. Fly culture vials (28.5 mm X 95 mm) were filled with ≈ 10 mL of medium and stored at 4 °C until required.

Larval mortality studies

Given that larvae feed at higher rates relative to adults, these studies were aimed at determining mortality in larvae rather than adults. A minimum of 90 second instar larvae were maintained on a TEA-enriched diet with or without quinidine or cimetidine. Ten larvae were placed in each 35 mm Petri dish containing fly media and 9 - 32 dishes were used for each diet. Six of the 35 mm dishes were then placed into a 140 mm Petri dish lined with moist filter paper to maintain high humidity.

Puparia were monitored daily for successful completion of metamorphosis as evidenced by formation of a distinct head, thorax and abdomen. Larval mortality rates were determined using the equation:

$$\text{Percent mortality} = ((L-A)/L)*100 \quad (1)$$

where L is the initial number of larvae and A is the number of adults. We scored the adults prior to emergence and subsequent feeding because of concerns that the toxicity of a particular diet might vary between larvae and adults.

Measurement of haemolymph TEA concentration ($[TEA]_{haem}$) in larvae maintained on a TEA-enriched diet with or without cimetidine

Fifteen 3rd instar larvae were placed in a 35 mm Petri dish containing TEA-enriched diet with or without cimetidine. Larvae were maintained on the diet for 24 hours before being removed from the Petri dish, briefly rinsed for 5 – 10 seconds in 5 mL distilled water and dried on tissue paper. Larvae were then transferred to a 100 mm Petri dish containing paraffin oil. The cuticle was torn with forceps to permit the escape of haemolymph. Care was taken to avoid damaging the gut. A sample of the haemolymph which exuded from the wound was collected using a 2 μL pipette. Haemolymph was also collected from larvae which had been maintained for 24 hours on TEA-enriched diet with or without cimetidine, then transferred for 4, 8, or 24 hours to Petri dishes containing TEA-free diet.

The concentration of TEA in droplets of haemolymph under paraffin oil was measured using TEA-selective microelectrodes which were constructed as described previously (Rheault and O'Donnell, 2004). Briefly, micropipettes were backfilled with 100 mmol l^{-1} TEA Cl and then tip filled with a short column (≈ 100 μm) of Corning ionophore 477317. This ionophore is 10^7 times more selective for TEA than for K^+, the major interfering inorganic ion (Oehme and Simon, 1976). To permit measurements in

fluid samples under oil, the tip of the microelectrode was coated with a thin layer of poly-vinyl chloride as described previously (Rheault and O'Donnell, 2004). Voltage differences between the TEA-selective microelectrode and a 500 mmol l^{-1} KCl-filled reference microelectrode were measured with a high-impedance differential electrometer (AM Systems, Carlsborg, WA) and recorded using a PC-based data acquisition system (Axotape, Axon Systems, Union City, CA). TEA concentrations were calculated using a calibration curve relating voltage to concentration. Calibration solutions contained 0.05, 0.5 and 5 mmol l^{-1} TEA in *Drosophila* saline.

Effects of temperature on the rate of decline of $[TEA]_{haem}$

Third instar larvae were placed into a 35 mm Petri dish containing 150 mmol l^{-1} TEA-enriched diet. After four hours, larvae were transferred into one of two 35 mm Petri dishes containing TEA-free diet. One dish was maintained at room temperature, whereas the other was maintained at 4°C. Haemolymph samples were collected immediately prior to, and 4 hours after, the transfer to TEA-free diet.

Injection of TEA into the haemocoel

Third instar larvae were secured to a Petri dish with double-sided tape. A 1 mL plastic syringe, pulled out to a fine tip over a low flame, was used to collect 0.1 µL of 100 mmol l^{-1} TEA from a 2 µL micropipette and expel the solution into the tapered shank of a glass micropipette pulled to a tip diameter of ≈ 3 µm. The micropipette was mounted on a micromanipulator and the tip was advanced through the cuticle and into the haemocoel

of the larva. Air pressure supplied by a 60 mL syringe connected to the back of the micropipette through PE tubing was used to expel the TEA solution into the haemocoel. Two haemolymph samples were collected with glass micropipettes, the first at 3-18 minutes and the second at 70-157 minutes, after injection of TEA. Haemolymph samples were expelled under paraffin oil and $[TEA]_{haem}$ was measured as described above.

Influence of TEA-enriched diet on Malpighian tubule fluid secretion rate and TEA flux

Fluid secretion rates, secreted fluid TEA concentrations and transepithelial TEA flux were measured for Malpighian tubules isolated from adult flies raised on either TEA-free or TEA-enriched diets. 2nd instar larvae were placed into fly vials with diet containing 100 mmol l^{-1} TEA. Larvae placed in similar vials containing TEA-free diet served as controls. Malpighian tubules were dissected from adult female flies 2-3 days after emergence as described above and set up in a modified Ramsay assay (Dow *et al.*, 1994). Briefly, a pair of tubules were dissected and transferred to a droplet of saline under paraffin oil. One tubule from the pair was immersed in the bathing saline whereas the other was pulled out of the saline and wrapped around a steel pin so as to position the ureter halfway between the bathing droplet and the pin. A droplet of secreted fluid formed at the ureter end was collected using a fine glass rod. A secreted fluid droplet was collected in the absence of TEA in the bathing saline to determine a background signal for the TEA-selective microelectrode. TEA was then added to the bathing saline to a final concentration of 0.1 mmol l^{-1}. Subsequent droplets were collected 30 and 60

minutes after the addition of TEA to the bathing saline. TEA concentration in the secreted fluid was measured using TEA-selective microelectrodes as described above.

Statistical Analysis

Values are expressed as mean ± S.E.M. for the indicated number of measurements. Where appropriate, two-sample *F*-tests were used to compare the variance of the control and experimental groups. Depending on the outcome of the *F*-test, mean values were compared using unpaired student's *t*-tests assuming either equal or unequal variances (SigmaPlot 6; SPSS Inc., Chicago, IL). Where appropriate, arcsine transformations of data were used prior to performing a *t*-test (GraphPad Instat 3.0; GraphPad Software, San Diego, CA). Differences were considered significant if $P < 0.05$.

Error bars for mortality studies of larvae maintained on diets enriched with TEA alone were determined using the equation:

$$(p(100-p)/n)^{1/2} \qquad (2)$$

where p is the percentage of larval mortality and n denotes the number of larvae. Error bars are only visible when greater than the diameter of the symbol used in the figure. Plots of mortality versus TEA concentration were fitted to sigmoidal curves using non-linear regression (SigmaPlot 6). Values of LD_{50} and the associated 95% confidence intervals were determined using Probit analysis (SigmaPlot 6). Significance of differences in mortality for larvae maintained on different diets was determined using the z-statistic (Mendenhall, 1971):

$$z = (P_1 - P_2) / ((P_p (100-P_p) (1/N_1 + 1/N_2))^{1/2} \quad (3)$$

where N_1 and N_2 were the number of larvae in each group, P_1 and P_2 were the corresponding percentages of larval mortality. P_p was the pooled estimate of the percentage of larval mortality and was calculated as:

$$P_p = (N_1P_1 + N_2P_2) / (N_1 + N_2) \quad (4)$$

Differences were considered significant when $z > 1.96$, corresponding to $P < 0.05$.

Results

Larval mortality

Larval mortality increased sigmoidally with the concentration of TEA in the diet (Fig. 1). The LD_{50} TEA concentration and 95% confidence intervals were 158.4 mmol l^{-1} and (126.7, 193.3) respectively. Control experiments indicated that increases in mortality with higher concentrations of TEA in the diet were not due to associated increases in ionic strength and/or osmolality. Mortality levels for larvae maintained on diets containing 100, 200 and 300 mmol l^{-1} NaCl were 12%, 12% and 10% respectively ($N > 90$). These values were not significantly different from mortality of larvae maintained on the control diet (6%, $P > 0.05$).

Mortality associated with maintenance on diets containing both TEA and either quinidine or cimetidine was significantly greater than the sum of the mortalities observed when larvae were maintained on diets containing either TEA, quinidine or cimetidine alone (*t*-test, $P < 0.05$). For example, mortality of larvae maintained on diet containing 100 mmol l^{-1} TEA and 10 mmol l^{-1} quinidine was 83% (Fig. 2) which is nearly double the

sum of the mortality levels observed in separate exposure to 100 mmol l^{-1} TEA and 10 mmol l^{-1} quinidine indicating a synergistic interaction between 100 mmol l^{-1} TEA and 10 mmol l^{-1} quinidine. Synergism was also observed for the combination of 100 mmol l^{-1} TEA and 100 mmol l^{-1} cimetidine (Fig. 2).

Haemolymph TEA concentration ($[TEA]_{haem}$) in larvae maintained on a TEA-enriched diet with or without cimetidine

$[TEA]_{haem}$ of 3rd instar larvae maintained on TEA-enriched diet for 24 hours varied linearly with the concentration of TEA in the diet. The level of TEA in the haemolymph was ≈ 3% of the concentration in the diet (Fig. 3). The background signal measured in haemolymph collected from larvae maintained on TEA-free diet was equivalent to 0.04 mmol l^{-1}. This background level presumably represents the interfering effects of other endogenous organic or inorganic cations on the TEA-selective microelectrodes.

$[TEA]_{haem}$ declined after larvae were transferred from TEA-enriched to TEA-free diet. The rate of decline during the first 4 hours after transfer to TEA-free diet was 0.67, 0.62, 0.74 and 1.72 mmol l^{-1} hr^{-1} for larvae previously maintained on diet containing 100, 150, 200 and 300 mmol l^{-1} TEA respectively. By 8 hours after the transfer to TEA-free diet, $[TEA]_{haem}$ of larvae previously maintained on each of the diets had declined to less than 12% of the concentration measured before transfer (Fig. 4A).

$[TEA]_{haem}$ of larvae maintained for 24 hours on diet containing 100 mmol l^{-1} TEA did not differ significantly from that of larvae maintained for the same period on diet

containing 100 mmol l^{-1} TEA and either 10 or 100 mmol l^{-1} cimetidine (Fig. 3). However, exposure to both TEA and cimetidine significantly reduced the rate of decline of $[TEA]_{haem}$ relative to larvae exposed to TEA alone (Fig. 4B). During the first 4 hours after transfer, $[TEA]_{haem}$ of larvae maintained on diet containing 100 mmol l^{-1} TEA plus either 10 or 100 mmol l^{-1} cimetidine declined at rates of 0.28 mmol l^{-1} hr^{-1} and 0.44 mmol l^{-1} hr^{-1} respectively. These rates are significantly lower than that of larvae previously maintained on diet containing 100 mmol l^{-1} alone (0.67 mmol l^{-1} hr^{-1}).

Effects of temperature on the rate of decline of $[TEA]_{haem}$

Mean $[TEA]_{haem}$ was reduced 15-fold in larvae maintained for 4 hours on TEA-free diet at room temperature after being maintained on diet containing 150 mmol l^{-1} TEA for the same period of time (Fig. 6). The rate of decline of $[TEA]_{haem}$ at room temperature was 0.71 mmol l^{-1} hr^{-1}. By contrast, there was no significant change in $[TEA]_{haem}$ 4 hours after larvae were transferred from diet containing 150 mmol l^{-1} TEA to TEA-free diet and maintained at 4° C.

Injection of TEA into the haemocoel

Concentrations of TEA in the haemolymph were measured at two intervals after each larva was injected with 0.1 µl of 100 mmol l^{-1} TEA (Fig. 6). Mean $[TEA]_{haem}$ within 18 minutes of injection was 10.9 ± 1.07 mmol l^{-1} and the mean rate of decline was 3.4 ± 0.29 mmol l^{-1} hr^{-1}.

Influence of TEA-enriched diet on fluid secretion rate and TEA flux

Fluid secretion rate and TEA flux of Malpighian tubules isolated from adult flies raised on diet containing 100 mmol l^{-1} TEA were both reduced by 40% relative to corresponding values for control tubules isolated from flies raised on TEA-free diet (Figs. 7A & 7C). There was no significant difference in secreted fluid TEA concentration between groups (Fig. 7B).

Discussion

The results demonstrate that larval *Drosophila melanogaster* are tolerant of high levels of the organic cation TEA in the diet and in the haemolymph, and that mortality increases when larvae are simultaneously exposed to TEA and either cimetidine or quinidine. The data also show that larvae reduce $[TEA]_{haem}$ rapidly, and that this reduction is dependent upon metabolic activity and is inhibitable by simultaneous exposure to cimetidine.

Larval mortality

Our results indicate that more than 75% of 3rd instar larvae survive on a diet containing 100 mmol l^{-1} TEA. *Drosophila* is somewhat less tolerant of diets containing high concentrations of salicylate and related organic anions. Survivorship of male, adult, *Drosophila* is reduced $\approx$ 42% by 28 mmol l^{-1} sodium salicylate (Massie, Williams and Iodice, 1985). For comparison, *Drosophila* appears to be relatively sensitive to low concentrations of other xenobiotics. Lethal levels of the fungal toxin α-amanitin for first

and second instar larvae are ≈ 200 to 400 μg l^{-1} of diet (Phillips, Willms and Pitt, 1982). Addition of as little as 80 ppm cadmium to the diet results in a decrease in adult emergence of nearly 75% (Callaghan and Denny, 2002).

The addition of the organic cations quinidine or cimetidine to diet enriched with 100 mmol l^{-1} TEA resulted in a synergistic, rather than additive, increase in larval mortality. The combined response was significantly greater than the sum of the mortalities associated with TEA and either cimetidine or quinidine alone. This has possible implications for the future development of insecticides. Compounds that exhibit low toxicity on their own may be highly toxic to a pest species when applied together. As discussed below, the synergism may result in part if one organic compound (e.g. quinidine) acts as a competitive inhibitor for the transport of another (e.g. TEA).

Haemolymph TEA concentration in larvae maintained on a TEA-enriched diet with or without cimetidine

$[TEA]_{haem}$ was ≈ 3% of the concentration of TEA in the diet. The concentrations of TEA in the haemolymph were much higher than those measured for the half-maximal rate of transport (K_t) of TEA by the Malpighian tubules or posterior midgut (0.18 – 0.22 mmol l^{-1}; Rheault and O'Donnell, 2004). At concentrations of ≈ 1 mmol l^{-1} or higher, the systems for the active removal of TEA from the haemolymph are nearly saturated (Rheault and O'Donnell, 2004). This would suggest that for larvae maintained on diets containing TEA at concentrations greater than ≈ 50 mmol l^{-1} the concentration of TEA in

the haemolymph is set primarily by the concentration in the gut lumen and the permeability of the gut wall to TEA.

The rate of $[TEA]_{haem}$ decline during the first 4 hours after transfer from diet containing 300 mmol l^{-1} TEA to a TEA-free diet was 2.6-fold greater than the rate of decline after transfer from diet containing 100 mmol l^{-1} TEA. The rate of decline was reduced by 58% when larvae were maintained on diet which contained 10 mmol l^{-1} cimetidine and 100 mmol l^{-1} TEA as compared to the rate of decline when the diet contained 100 mmol l^{-1} TEA alone (Fig. 4). Cimetidine effectively inhibits TEA transport by the Malpighian tubules (Rheault and O'Donnell, 2004) and is a well-known competitive inhibitor of several types of organic cation transporters in other epithelia (Lee and Kim, 2004). We suggest that cimetidine slows the rate of decline of $[TEA]_{haem}$ because it reduces the rate of TEA secretion by transporters in the Malpighian tubules and posterior midgut.

It is important to point out that larval mortality levels were relatively low even when millimolar levels of TEA were present in the haemolymph. Micromolar concentrations of TEA reduce motor end-plate current amplitudes in 3rd instar *Drosophila* larvae (Delgado *et al.*, 1992) and 5 mmol l^{-1} TEA broadens action potentials in cultured *Drosophila* neurons (Zhao and Wu, 1997). The delayed-rectifier potassium current in neurons of the *Drosophila* mutant rutabaga is reduced 46% by 10 mmol l^{-1} TEA (Alshuaib and Mathew, 2002). Organic cation transporters in the blood-brain barrier may protect the central nervous system of *Drosophila* from high levels of TEA in the haemolymph. An effective blood-brain barrier may be particularly important given that

haemolymph levels of TEA rise well above the concentrations associated with saturation of active organic cation transporters in the excretory system.

Locomotor activity was decreased in larvae maintained on diet enriched with both TEA and either cimetidine or quinidine suggesting that the combined effects of two organic cations affected the nervous system. Cimetidine increased haemolymph levels of TEA by 32% when both TEA and cimetidine were present in the diet at concentrations of 100 mmol l^{-1} relative to the concentration of TEA measured in the haemolymph when larvae were maintained on diet containing 100 mmol l^{-1} TEA alone. The decrease in locomotor activity may be the result of prolonged exposure to high levels of TEA because cimetidine inhibits TEA secretion by tissues such as the Malpighian tubules or the blood-brain barrier as discussed above. Conversely it may be the result of the effects high concentrations of cimetidine have upon the animal. Quinidine is also a known K^+ channel blocker (Nam *et al.*, 2004), so decreased locomotor activity in larvae maintained on diet containing both quinidine and TEA may be due to the combined effects of these compounds upon excretion of organic cations as well as actions upon K^+ channels.

Effects of temperature on the rate of decline of $[TEA]_{haem}$

Slowing the metabolic rate by chilling completely blocked the decline of $[TEA]_{haem}$ after transfer to a TEA-free diet. Typical Q_{10} values range from 2.4 for the beetle, *Phoracatha semipunctata,* (Rogowitz and Chappell, 2000), 1.86 for the grasshopper, *Melanoplus sanguinipes* (Rourke, 2000) and 1.96 for the fruit fly, *Drosophila simulans* (Lanciani *et al.*, 1990). The animals do not feed when chilled, so

TEA-rich diet will remain in the gut lumen after transfer to TEA-free diet. Active transport of TEA by transporters in the gut and Malpighian tubules may be reduced several fold when chilled, whereas passive diffusion of TEA from gut lumen into the haemolymph will be reduced only a few percent. The net effect will be that haemolymph levels of TEA remain high when the larvae are chilled.

Injection of TEA into the haemocoel

After injection of TEA had raised the concentration of TEA in the haemolymph to 10.9 mmol l^{-1} the concentration of TEA in the haemolymph declined at a rate of 3.40 mmol l^{-1} hr^{-1}. This rate can be compared with estimates of the rate of decline based upon TEA transport rates of isolated Malpighian tubules. Maximal TEA flux across a single isolated tubule is 1.52 pmol min^{-1} $tubule^{-1}$ (Rheault and O'Donnell, 2004). Assuming that haemolymph volume of a 3rd instar larvae is ≈ 2 µl (Carton *et al.*, 2002), then 4 tubules transporting at the maximal rate will reduce haemolymph TEA concentration at a rate of 0.182 mmol l^{-1} hr^{-1}. This rate is ≈ 19 times less than the observed rate of decline. The rate of decline that can be accounted for by the posterior midgut can also be estimated based upon the maximal flux rate (3.7 pmol cm^{-2} min^{-1}; Rheault and O'Donnell, 2004) and the surface area of the posterior midgut (0.0248 cm^2). The latter mean value was based on morphometric measurements of the guts of 10 larvae. Based on these values, the midgut transporting at the maximal rate will reduce haemolymph TEA concentration at a rate of 0.163 mmol l^{-1} hr^{-1}, more than 20-fold lower than the observed rate. The combined actions of the posterior midgut and MTs would reduce haemolymph TEA

concentration at a rate of 0.208 mmol l^{-1} hr^{-1}, which is 16-fold lower than the observed rate.

These calculations show that haemolymph TEA concentrations decline much more rapidly than can be attributed to active, saturable transport across the MTs and posterior midgut. It is worth noting that our results do not reveal whether TEA has been excreted, sequestered within tissues or metabolized to other compounds. However, we suggest that passive TEA transport into the gut, when its contents contain lower concentrations of TEA than the haemolymph, may contribute to the rapid decline in haemolymph TEA concentrations. When animals are injected with TEA, the gut contents are TEA-free, and there will be a large gradient favouring passive diffusion of TEA from haemolymph to gut. This movement may be relatively rapid because of the large surface area of the gut. Similarly, when animals are transferred from TEA-enriched diets to TEA-free diet, continued feeding and progression of material through the gut will lead to replacement of TEA-rich contents by TEA-free contents. In this case as well, haemolymph TEA concentrations will at some point exceed those in the gut lumen and passive movement of TEA across the gut wall will contribute to a drop in haemolymph TEA concentrations. This line of reasoning, although admittedly speculative, suggests that the gut may play an important role in eliminating toxins from the haemolymph, providing that the insect can feed upon toxin-free foods after ingestion or absorption of high levels of toxins

It is important to point out that the rate of decline of haemolymph TEA can be accounted for by the actions of the MTs and posterior midgut when haemolymph TEA

levels are lower. For example, Fig. 4A showed that the concentration of TEA in the haemolymph declined at a rate of 0.163 mmol l^{-1} hr^{-1} between 4 and 8 hours after larvae were transferred from a diet containing 100 mmol l^{-1} TEA to TEA-free diet. This value is similar to the rate of 0.208 mmol l^{-1} hr^{-1} that can be explained on the basis of active transport by the MTs and posterior midgut together.

TEA secretion by Malpighian tubules of flies raised on a TEA-enriched diet

Malpighian tubules isolated from adult flies raised on a 100 mmol l^{-1} TEA-enriched diet secreted fluid at significantly lower rates when compared to tubules isolated from flies raised on TEA-free diet (Fig. 7). Current models of ion transport by the main segment of the Malpighian tubules of *Drosophila* propose that active transepithelial ion transport drives the flow of osmotically obliged water (Maddrell and O'Donnell, 1992; Ianowski and O'Donnell, 2004). The basis for the reduction in fluid secretion rate may reflect non-lethal, but nonetheless deleterious, effects of sustained exposure to high levels of TEA in the diet and the haemolymph.

The finding that TEA secretion was not enhanced by ingestion of TEA-enriched diet is in contrast to the effects of ingestion of a protein-rich diet on organic anion transport by MTs of the blood-feeding insect *Rhodnius prolixus*. PAH transport by isolated tubules is greatly increased 1-2 days after the animal has consumed a protein-rich meal. Similarly, uric acid transport by isolated tubules of *Rhodnius* is increased 1-2 days after consuming a blood meal (O'Donnell, Maddrell and Gardiner, 1983). It is important to point out that we examined TEA transport by unstimulated MTs. It is conceivable that

exposure to TEA in the diet may augment the release of diuretic factors which have been shown to increase TEA transport by isolated MTs (Bijelic and O'Donnell, 2004).

It is important to note that TEA transport was measured in tubules isolated from adult *Drosophila* after sustained exposure of the larvae to high levels of TEA in the diet. It is possible that TEA transport by isolated tubules is increased by exposure of the larvae to TEA-enriched diet, but that the upregulation is not apparent after pupation. Measuring TEA secretion rates across isolated larval Malpighian tubules was attempted however stable rates of fluid secretion were not achieved.

This study demonstrated the tolerance of *Drosophila* larvae to high concentrations of TEA in the diet and in the haemolymph. Our results suggest that the nervous system must be well protected against high levels of organic cations by transporters within the blood-brain barrier. Although the mechanisms for organic cation transport by the Malpighian tubules and midgut are not sufficient to account for the rate of decline observed *in vivo,* it is suggested that dilution of the gut contents and subsequent passive diffusion of TEA from the haemolymph augments the active transport performed by the excretory system. Clearly, *Drosophila* has evolved multiple mechanisms to defend against high levels of organic cations in the haemolymph. Further research needs to be performed, however, to identify how large a role diffusion of TEA, from the haemolymph into the gut, plays in determining the overall rate of TEA decline.

Figure 1. Mortality rate of larvae maintained on TEA-enriched diet. N = 30 for each data point. Inset: Probit analysis of data including 95% confidence intervals.

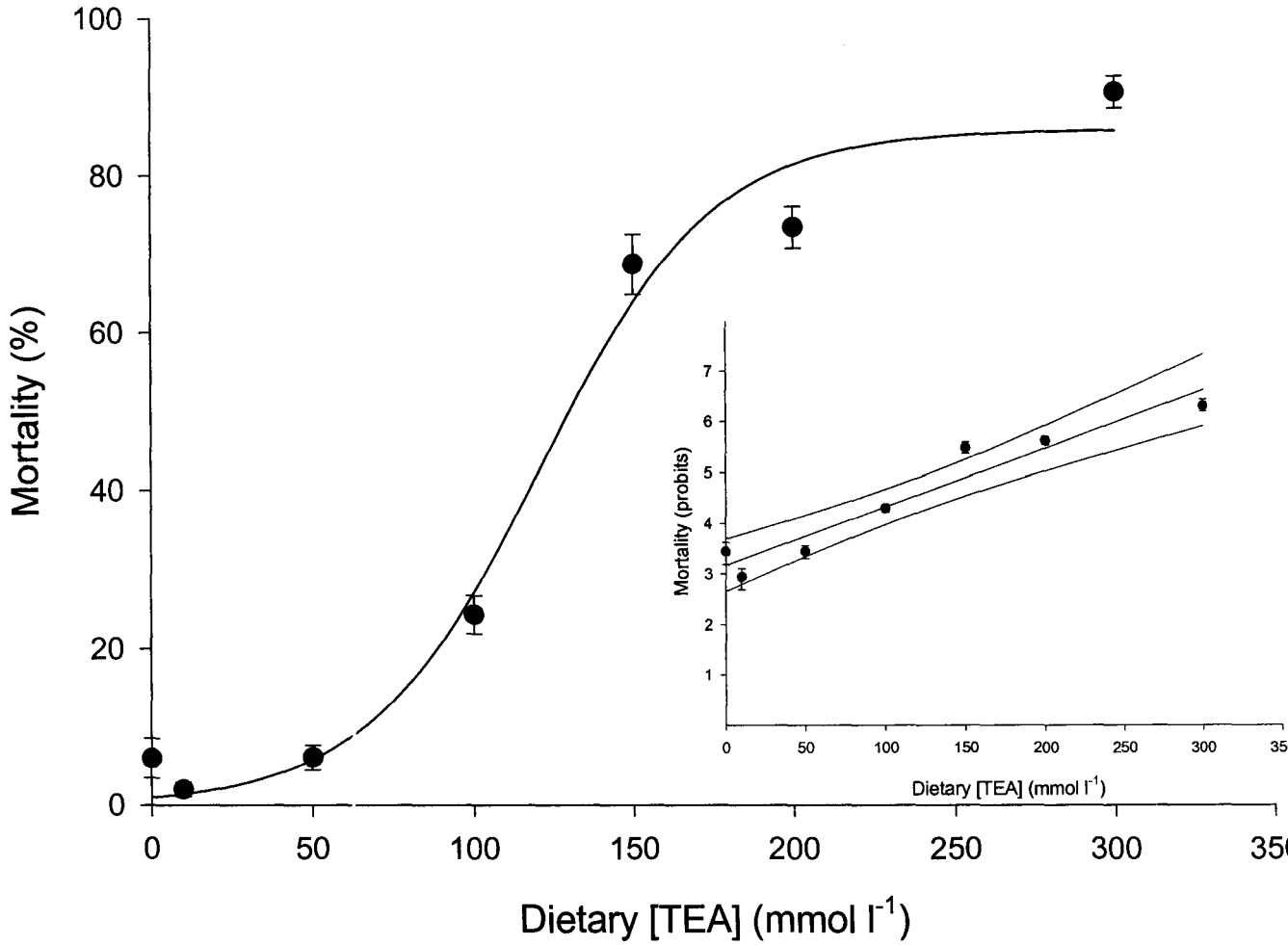

Mortality (%)
100
80
60
40
20
0
0
50
100
150
200
250
300
350
Dietary [TEA] (mmol l-1)
Mortality (probits)
7
6
5
4
3
2
1
0
50
100
150
200
250
300
350
Dietary [TEA] (mmol l-1)

Figure 2. Mortality of larvae maintained on diet containing both TEA and another organic cation. Quinidine (quin) or cimetidine (cimet) was added to diet containing 100 mmol l^{-1} TEA. Mortality levels significantly different from that produced by diet containing 100 mmol l^{-1} TEA alone are indicated by an asterisk (z-statistic). All concentrations are mmol l^{-1}. Corresponding N values are provided on each bar.

Mortality (%)
90
80
70
60
50
40
30
20
10
0
*
90
322
153
150
203
201
189
185
0 TEA
100 TEA
10 Quin
100 Cimet
100 TEA & 1 Quin
100 TEA & 10 Quin
100 TEA & 10 Cimet
100 TEA & 100 Cimet

Figure 3. $[TEA]_{haem}$ of 3rd instar larvae maintained on TEA-enriched or TEA plus cimetidine-enriched diets for 24 hours. N = 30 for each data point.

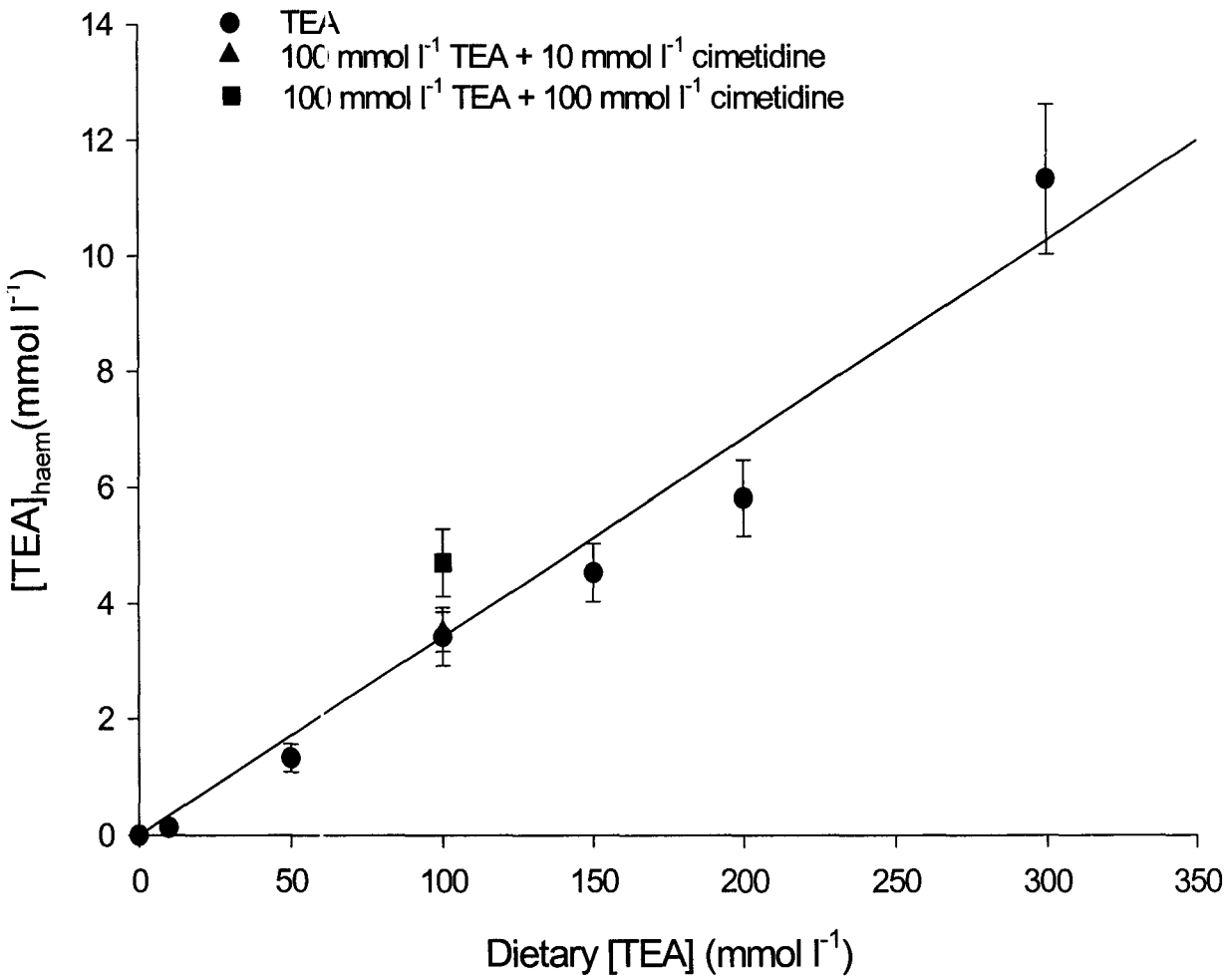
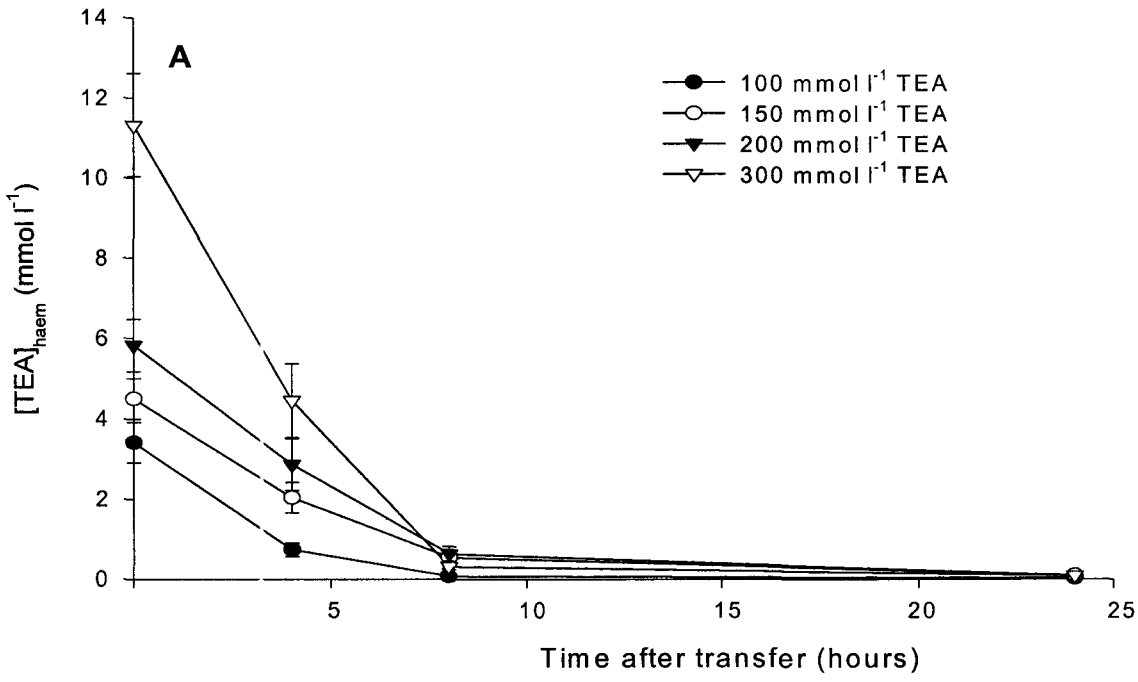

TEA
100 mmol l-1 TEA + 10 mmol l-1 cimetidine
100 mmol l-1 TEA + 100 mmol l-1 cimetidine
[TEA]haem(mmol l-1)
Dietary [TEA] (mmol l-1)
0
2
4
6
8
10
12
14
0
50
100
150
200
250
300
350

Figure 4. Changes in haemolymph TEA concentration after transfer of larvae from (A) diet containing TEA alone or (B) diet containing both TEA and cimetidine to a TEA-free diet. N = 30 larvae per data point.


A
100 mmol l-1 TEA
150 mmol l-1 TEA
200 mmol l-1 TEA
300 mmol l-1 TEA
[TEA]haem (mmol l-1)
Time after transfer (hours)

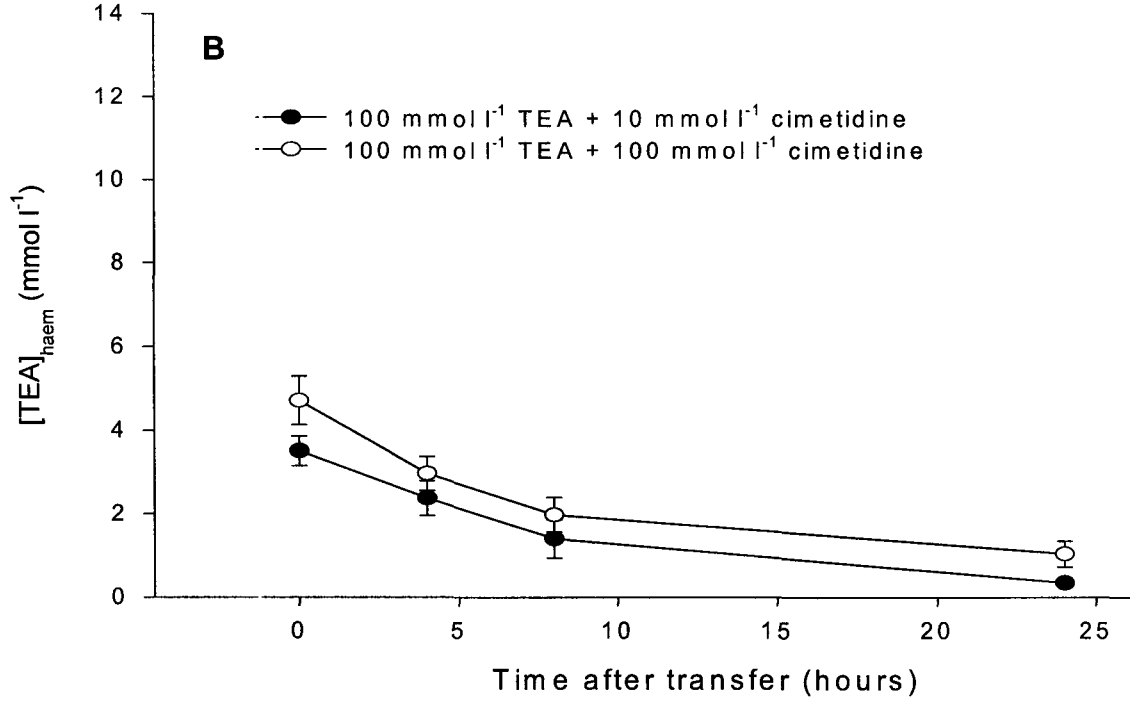

B
100 mmol l-1 TEA + 10 mmol l-1 cimetidine
100 mmol l-1 TEA + 100 mmol l-1 cimetidine
[TEA]haem (mmol l-1)
Time after transfer (hours)

Figure 5. Effect of temperature on haemolymph TEA concentration. Haemolymph TEA concentrations were measured immediately after larvae were maintained on diet containing 150 mmol l^{-1} for 4 hours (initial) and after larvae were transferred to TEA-free diet and maintained either at room temperature or chilled for 4 hours. Significant differences from the initial TEA concentration are indicated by an asterisk (*t*-test, $P < 0.05$). N values are indicated above each bar.

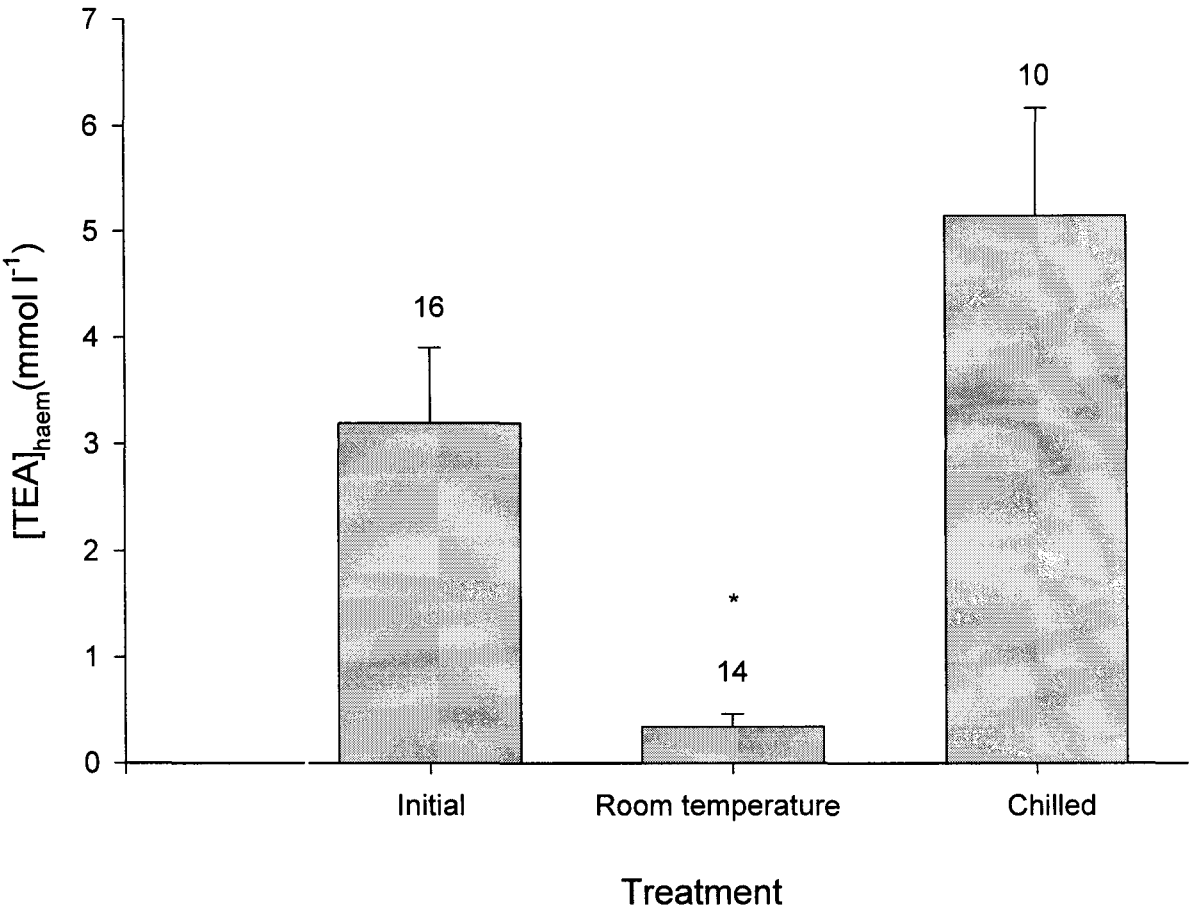

7
6
5
4
3
2
1
0
[TEA]haem(mmol l-1)
16
*
14
10
Initial
Room temperature
Chilled
Treatment

Figure 6. Rate of decline of $[TEA]_{haem}$ after injection of 100 mmol l^{-1} TEA into the haemocoel of 3rd instar larvae. N = 12 larvae.

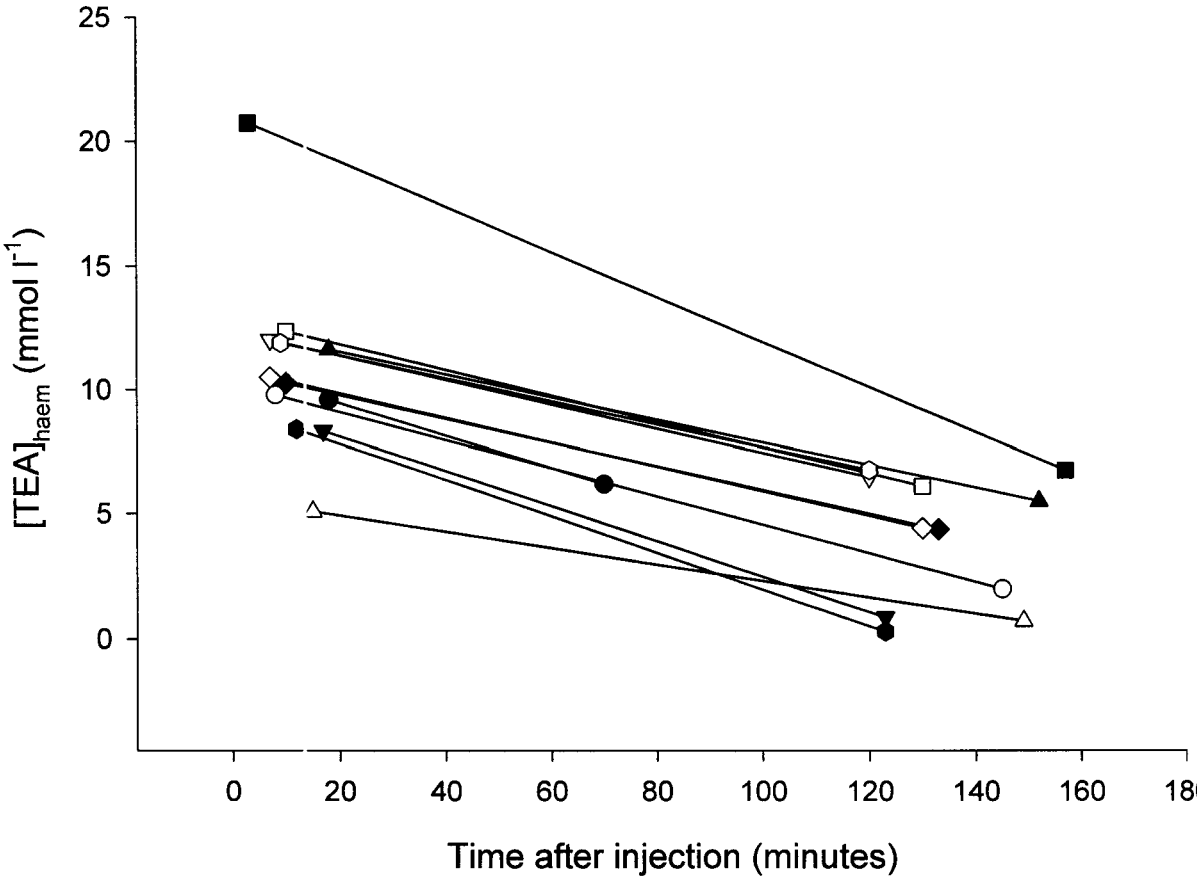
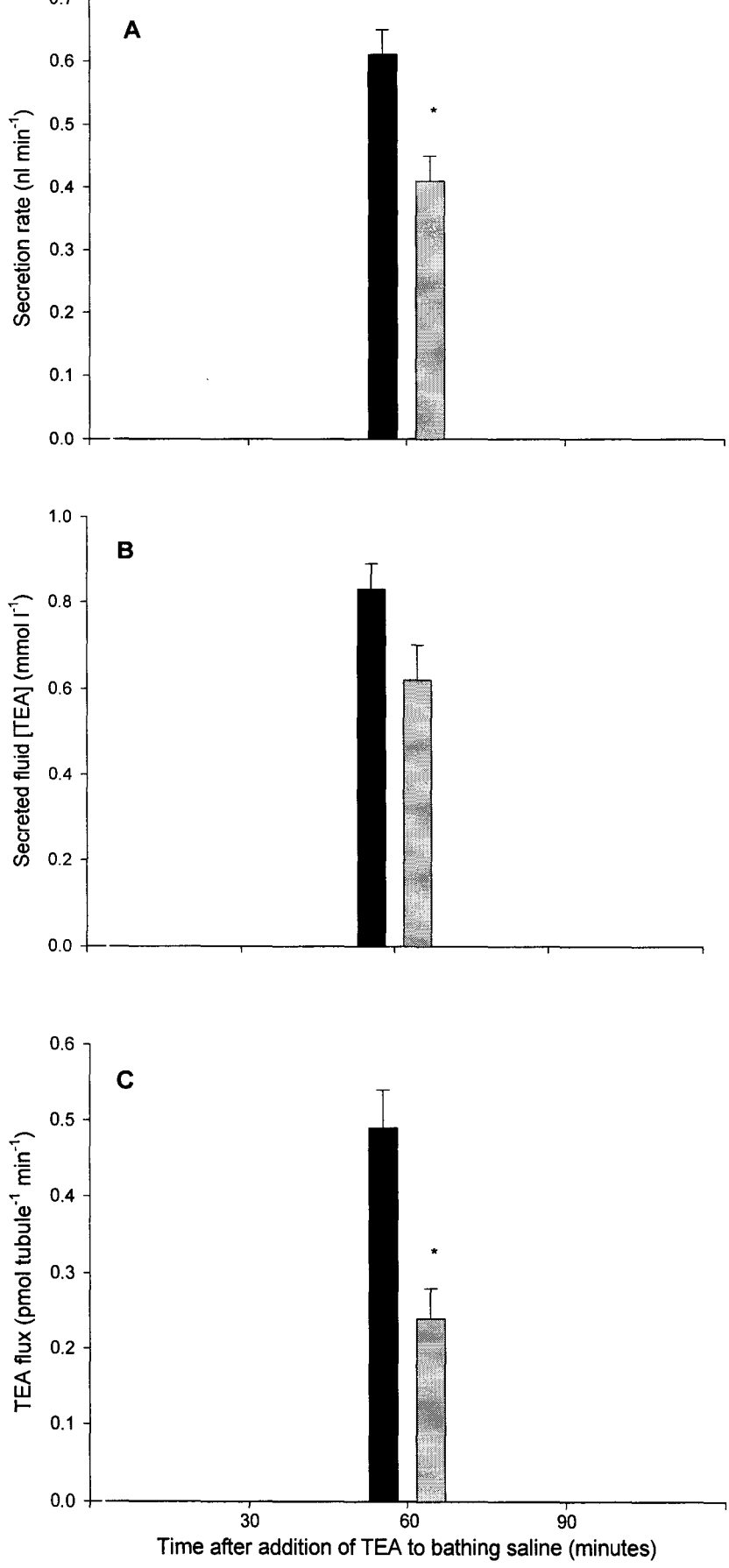

25
20
15
10
5
0
[TEA]haem (mmol l-1)
0
20
40
60
80
100
120
140
160
180
Time after injection (minutes)

Figure 7. (A) Fluid secretion rates, (B) secreted fluid TEA concentrations and (C) transepithelial TEA flux by the main segment of Malpighian tubules isolated from adult flies raised on diet containing 100 mmol l^{-1} TEA (black bars) or TEA-free diet (grey bars). Significant differences are indicated by an asterisk (*t*-test, $P < 0.05$). N = 11.


A
Secretion rate (nl min-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
*
B
Secreted fluid [TEA] (mmol l-1)
0.0
0.2
0.4
0.6
0.8
1.0
C
TEA flux (pmol tubule-1 min-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
*
30
60
90
Time after addition of TEA to bathing saline (minutes)

Chapter 4

General Discussion and Conclusions

My results demonstrate that factors which stimulate fluid secretion and inorganic ion transport by the isolated Malpighian tubules of *Drosophila* also stimulate transepithelial TEA secretion. Questions still remain however with regards to the physiological significance of multiple diuretic factors in *Drosophila*. In particular, it is not known which stimuli promote the release of diuretic factors. It is conceivable that one of the diuretic factors may be released in response to ingestion of organic cations such as TEA and may therefore lead to clearance of the organic cation from the haemolymph through its effects on fluid secretion and organic cation transport.

In this context, a useful analogy can be made with the blood-feeding insect *Rhodnius prolixus*. After a blood meal, release of serotonin (5-HT) in response to distension of abdominal stretch receptors leads to numerous effects such as cuticular plasticization (Reynolds, 1974), stimulation of fluid secretion by the Malpighian tubules and reabsorption of KCl by the downstream segment of the tubule (Collier and O'Donnell, 1997). In addition, there is a CRF-related peptide whose effects on fluid secretion are additive to those of 5-HT but which do not have effects on the cuticle for example (Te Brugge and Orchard, 2002). Some of the diuretic factors in *Drosophila* may also have effects on tissues other than the Malpighian tubules and these factors may be released in response to other stimuli such as feeding, larval moulting or diuresis after emergence of the adult. Moreover, although all of the factors increased transepithelial

TEA flux, some may have additional effects on the transport of other molecules, such as organic anions. For example, cAMP, but not cGMP or leucokinin-I, stimulates transepithelial secretion of the organic anion salicylate (E. Ruiz-Sanchez, personal communication).

One of the striking outcomes of this thesis was the demonstration of the extraordinary tolerance of *Drosophila* larvae for the organic cation TEA. This was particularly surprising because TEA levels in the haemolymph become highly elevated after larvae have been maintained on diets containing 100 to 300 mmol l^{-1} TEA. An implicit result of Chapter 3 is that *Drosophila* larvae must be endowed with organic cation transporters in the blood-brain barrier, since the levels of TEA in the haemolymph were at or above those found to interfere with neuronal function. It is worth noting that Murray *et al.*, (1994) found the presence of P-glycoprotein (P-gp) in the blood-brain barrier of the tobacco hornworm, *Manduca sexta* and propose that this transporter is important during ingestion of high levels of nicotine. P-gps in the Malpighian tubules and blood-brain barrier both act to lower the concentration of nicotine in the haemolymph and protect sensitive neuronal tissues. Similar coordination of the organic cation transporters in the Malpighian tubules and blood-brain barrier could be important for larvae ingesting small organic cations such as TEA.

One putative candidate for a basolateral organic cation transporter in *Drosophila* is the protein encoded for by the gene ORCT. Although initially ascribed to the carnitine family of transporters, recent evidence suggests that the *Drosophila* transporter, although in the solute carrier (SLC22) family, is as similar to the organic anion transporters as it is

to the organic cation transporters of vertebrates. Attempts to identify organic cation transporters using immunohistochemical techniques such as those used for P-gp in *Manduca* may not be feasible. Antibodies which recognize vertebrate organic cation transporters may not recognize the transporters in *Drosophila*.

An intriguing finding of this thesis was the increased larval mortality due to the synergistic effect of combining TEA with either quinidine or cimetidine in the diet. This finding may open new avenues in the development of insecticides. Combining compounds which have relatively low toxicity on their own may produce mortality levels which are much higher when the compounds are combined. It would be of interest to find out if our findings extend to other pest species of insects. Work by other researchers has demonstrated TEA transport across the Malpighian tubules of insects such as cockroaches, crickets, locusts and milkweed bugs (M. Rheault, J. Plaumann and M. O'Donnell, personal communication).

My results also show that the Malpighian tubules and posterior midgut cannot account for the rapid rate of decline in haemolymph TEA levels after larvae previously maintained on a TEA-enriched diet were transferred to TEA-free diet or after injection of TEA into the haemocoel. Given that *Drosophila* feed primarily on the yeasts of rotting fruit, the rate of transport of organic cations may be different among insects whose primary diet is plant materials which are rich in alkaloids such as nicotine. The findings of Chapter 3 suggest that ingestion of TEA-free diet may enhance the rate of decline of TEA from the haemolymph. It is worth noting in this context that a recent study of New Guinean rainforest plants, and the insects that feed on them, yielded a new and much

lower estimate of the number of species on the planet. The estimate, which lowers the number of species from approximately 31 million to between four and six million, is based on the finding that insects specialize their feeding not on individual species of plants, but on genera and even families of plants (Novotny *et al.*, 2002). It would be of interest to know whether pest species will switch food preferences if haemolymph levels of particular toxins begin to rise. This might allow them to flush out ingested toxins simply by switching to a diet that contains lower concentrations of that particular toxin.

The possibility that the gut contents act as a sort of sump for high levels of TEA in the haemolymph could be tested in future studies. After injection of TEA into the haemocoel of 3rd instar larvae as I have described in Chapter 3, the gut can be isolated and placed into a capillary tube containing a known volume of deionized water. The volume of the gut contents can be determined by measuring the displacement of the water. TEA concentration can be determined in a sample of the water using a TEA-selective microelectrode.

Another dramatic finding of this thesis was that raising *Drosophila* on a diet containing 100 mmol l^{-1} TEA causes a non-lethal, yet deleterious effect on the rate of fluid secretion by isolated Malpighian tubules. The findings of Chapter 3 indicate that the rate of fluid secretion by unstimulated tubules isolated from adult flies raised on TEA-enriched diet is lower relative to tubules isolated from flies raised on TEA-free diet. It would be of interest to determine whether the decline in fluid secretion rate is due to the secretion of a particular ion e.g. Na^+. In this context, it would be useful to determine what effect factors which stimulate fluid secretion and transepithelial inorganic ion

transport have on Malpighian tubules isolated from flies raised on TEA-enriched diet. For example, TEA transport *in vivo* may be higher when flies are raised on a TEA-enriched diet if there is enhanced release of diuretic factors that augment TEA secretion by the Malpighian tubules. Also, given that the posterior midgut transports TEA it would be of interest to compare the transport of TEA across the midgut of flies maintained on a TEA-enriched diet relative to those maintained on a TEA-free diet.

Additionally, the flies may have been raised on diet that contained an excessive concentration of TEA which resulted in pathological effects. It may be more appropriate to raise larvae on diet containing a lower concentration of TEA which, although increases mortality relative to TEA-free diet, is not beyond the organism's excretion capability. In this context, future research should examine TEA transport and fluid secretion rate of isolated larval Malpighian tubules. I attempted this but was unable to obtain secretion rates that were stable however this technique was recently refined by the O'Donnell lab. It is possible that induction of TEA transport occurs but is not apparent once the animal begins pupation.

To conclude, future work in this field should focus on identifying the transporter(s), involved in the excretion of organic cations. In spite of the reservations expressed above, the best candidate for a TEA transporter in *Drosophila* is the protein encoded by the ORCT gene. It would be of interest to use reverse transcriptase polymerase chain reaction technology to express this gene in tissues such as the midgut and Malpighian tubules. It would also be of interest to examine organic cation transport by Malpighian tubules isolated from *Drosophila* species known to feed on diets rich in

these compounds. For example, cactophilic species from the southwest United States are known to ingest food rich in isoquinoline alkaloids which induce high levels of cytochrome P450 activity. It would be of interest to determine if these flies also have a higher capacity to excrete either toxic components of the diet or metabolites produced by P450 enzymes (Danielson *et al.*, 1997).

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