EFFECTS OF SALT STRESS ON EPITHELIAL ION TRANSPORT IN *DROSOPHILA*

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BY

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ABSTRACT:

Fruit flies (*Drosophila melanogaster*) are known for their ability to survive well on diets containing high concentrations (0.4 mol l^{-1} or more) of NaCl and KCl. *Drosophila* has been used in recent years as a model species for identification of genes involved in salt tolerance in insects and other animals. This thesis examined the extent of haemolymph volume regulation and ionoregulation in larval or adult *Drosophila* reared on control diet or on salt-rich diets that contained an additional 0.4 mol l^{-1} NaCl or KCl. K^+ concentrations in the haemolymph of adults reared on KCl-rich (0.4 mol l^{-1}) diet did not differ from the values for insects reared on the control diet, whereas Na^+ concentrations in the haemolymph of adults reared for 1d – 7d on NaCl-rich (0.4 mol l^{-1}) diet increased ~50 % relative to values for insects reared on the control diet. K^+ concentrations in the haemolymph of larvae reared on the KCl-rich diet increased transiently then returned to the control value within 48 hours whereas Na^+ concentrations in the haemolymph of larvae reared on the NaCl-rich diet increased ~50 % relative to values for insects reared on the control diet.

This thesis also examined the role of the Malpighian tubules and gut in haemolymph ionoregulation during salt stress. It was hypothesized that increases in secretion of excess ions (Na^+ or K^+) by the Malpighian tubules and hindgut as well as decreased absorption across the midgut would contribute to haemolymph ionoregulation. Changes in fluid and ion secretion by Malpighian tubules isolated from the larvae were measured using the Ramsay assay and ion-selective microelectrodes. The results showed that haemolymph ionoregulation in larvae reared on salt-rich diets involved both alterations in the basal secretion rates of Na^+ or K^+ by the Malpighian tubules as well as stimulatory effects produced by diuretic factors present in the haemolymph. Stimulation of tubule fluid and ion secretion may involve increases in intracellular Ca^{2+} in response to salt stress. Na^+ and K^+ fluxes across the isolated gut of larval *Drosophila melanogaster* reared on control or salt-rich diets were measured using the scanning ion-selective microelectrode technique (SIET). In larvae reared on KCl-rich diet, K^+ absorption across the anterior portion of the middle midgut was reduced relative to the same gut segment of larvae reared on the control diet and there was there was also an increase in the magnitude and extent of K^+ secretion across the posterior half of the middle midgut. In larvae reared on the NaCl-rich diet, there was a small reduction in Na^+ absorption by the middle midgut relative to larvae reared

on the control diet and Na^+ was secreted rather than absorbed across the hindgut. Overall, the results of the thesis suggest that haemolymph ionoregulation during salt stress involves a reconfiguration of the ion transport mechanisms of the gut and Malpighian tubules so that there are reductions in K^+ and Na^+ absorption and increases in K^+ and Na^+ secretion. These results indicate considerable phenotypic plasticity with respect to K^+ and Na^+ transport by the gut epithelia of larval *Drosophila*.

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Many thanks to my colleagues and friends Sarah Chahine and Ryan Belowitz for making the lab a convivial place to work. Their love and support shortened the long hours in the lab. I will never forget the great times we shared.

My deepest gratitude goes to my family for their love and support throughout my life; this dissertation is simply impossible without them. My parents have always worked hard to support the family and spare no effort to provide the best possible environment for us to study and follow our dreams. I love you mom and dad.

I am thankful to God for blessing me with a beautiful life, beautiful family, beautiful friends and helping me through tough times.

Lastly I offer my regards and blessings to all of those who supported me in any respect during the completion of the thesis.

Three passions have governed my life

Three passions have governed my life:
The longings for love, the search for knowledge,
And unbearable pity for the suffering of [humankind].

Love brings ecstasy and relieves loneliness.
...In the union of love I have seen
In a mystic miniature the prefiguring vision
Of the heavens that saints and poets have imagined.

With equal passion I have sought knowledge.
I have wished to understand the hearts of [people].
I have wished to know why the stars shine.

Love and knowledge led upwards to the heavens,
But always pity brought me back to earth;
Cries of pain reverberated in my heart
Of children in famine, of victims tortured
And of old people left helpless.
I long to alleviate the evil, but I cannot,
And I too suffer.

This has been my life; I found it worth living

[Bertrand, Russel]

THESIS ORGANIZATION AND FORMAT

This thesis is presented as a "sandwich thesis" with a general introduction presented in chapter1 followed by two research papers presented as chapter 2 and chapter 3, which are in the format of manuscripts to be submitted for publication in peer-reviewed journals. A general discussion is presented in chapter 4.

Chapter 1: General introduction and project objectives

Chapter 2: Alterations of fluid and ion transport by Malpighian tubules of *Drosophila melanogaster* in response to salt stress.

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Chapter 1

General introduction and thesis objectives

Introduction to Drosophila melanogaster.

The fruit fly, *Drosophila melanogaster* (Drosophilidae) is a relatively small, soft-bodied insect 3 – 4 mm long and weighing ~ 1 mg (Borror et al., 1989). Fruit flies are holometabolous insects with 4 life stages: egg, larva, pupa and adult.

Drosophila lives on various live yeast that grow in the presence of bacteria, moulds and other microorganisms on decaying organic matter, particularly fruit. Whereas an adult female may consume its own mass in food per day, the larva ingests 3 to 5 times its mass per day up to the time of pupation (Sang, 1978). *Drosophila* are easily raised in culture in the laboratory and their nutritional requirements have been investigated in both larvae and adults. The elements potassium, phosphate, magnesium and sodium are essential whereas calcium supplemental to that in the normal yeast diet does not appear to be essential to growth. Folic acid, choline, cholesterol and fructose or other sugars have been determined to be essential for the growth of healthy larvae. In recent years, *Drosophila* have become an important model system for studying the physiology and regulation of appetite and the links between diet and longevity (e.g. Ja et al., 2007; Grandison et al., 2009). In addition, the Malpighian (renal) tubules of *Drosophila* are now used for what is termed integrative physiology. Molecular genetic modification of specific cells or tissues can be used in conjunction with biochemical, pharmacological and physiological techniques for analysis of epithelial function (Dow et al., 1998).

Malpighian tubules and gut

The Malpighian tubules (MT) and hindgut of insects together comprise the functional kidney, and are the main organs for excretion, osmo- and iono-regulation. MTs are single layered blind-ended tubes and their number varies from two to several hundred in different

species. Electron micrographs show that the basolateral membrane is bounded by a basement lamina and that the apical membrane has a brush border with numerous microvilli (e.g. Wessing and Eichelberg, 1978). In contrast to hydrostatic filtration by the vertebrate kidney, fluid is filtered into the MT by active solute transport (Na^+, K^+, Cl^-) and passive influx of water. Ions and osmotically obliged water secreted into the tubule lumen then enter the gut at the junction of the midgut and hindgut. The composition of the tubular fluid can be subsequently modified by the proximal (lower) MT or the anterior hindgut and especially the rectum. Waste materials and toxins are excreted across the tubule wall either by passive filtration or through the actions of specific transport systems for molecules such as uric acid, organic anions and alkaloids such as nicotine and morphine.

Fluid and ion secretion by Malpighian tubules of Drosophila.

Drosophila has two pairs of MTs which lie free in the haemocoele. Both MT pairs join at a common ureter, which joins the alimentary canal at the midgut-hindgut junction (Figure 1; Wessing and Eichelberg, 1978). The anterior MTs are found in the anterior portion of the body cavity, whereas the posterior MTs are located in the lower part of the body cavity and are 25% longer than the anterior MTs (Wessing and Eichelberg, 1978). Morphologically the MTs can be divided into initial, transitional, main and lower segments (Figure 2). The initial segment of the anterior MT does not secrete fluid at measurable rates (Dow et al., 1994) but is a major site of Ca^{2+} transport (Dube et al., 2000 a,b). Water transport is a passive osmotic process that is directly coupled to active transport of Na^+, K^+ and Cl^- across the main segment (O'Donnell and Maddrell, 1983; Dow et al., 1998). Secreted fluid by the main segment is K^+ rich and is nearly iso-osmotic to the haemolymph. The lower segment of the *Drosophila* MT does not secret fluid but is responsible for the absorption of approximately 30% of the KCl and

water secreted by the main segment (O'Donnell and Maddrell, 1995). In addition, the lower tubule secretes Ca^{2+} and acidifies the urine.

Drosophila MTs are ideal for studies of epithelial ion transport because they transport at high rates and are easily removed undamaged from the adult fly or larva. Typical dimensions of a MT are 35 μm outer diameter, 17 μm luminal diameter and 2 mm in length (Dow et al., 1994). Secretion rates of MTs *in vitro* can be elevated approximately 10 fold by the addition of one or more diuretic factors. Given that a MT can secrete fluid at rates up to 6 nl/min, calculations of total cell volume based on these dimensions indicate that each cell may secrete its own volume of fluid in less than 10 seconds (Dow et al., 1994).

Mechanisms and control of fluid secretion and ion transport by MTs of Drosophila melanogaster

Malpighian tubules in *Drosophila* consist of two types of cells, principal (type I) and secondary or "stellate" (type II) cells (Sozen et al., 1997). Typically there are 22 stellate cells and 82 principal cells within one MT (Dow et al., 1998). The principal cell has at least two subpopulations, which express different genes and presumably differ in function. Type II cells are distributed throughout the initial, transitional and main segments of posterior MTs and within the main segment of anterior MTs. This cell type appears either bar or star shaped (Sozen et al., 1997).

Most of the work on this insect with respect to MT ion transport has focused on the transport of monovalent ions, Na^+, K^+ and Cl^- (O'Donnell and Maddrell, 1995; O'Donnell et al., 1996, 1998). The main segment is responsible for fluid secretion driven by active ion transport. The current model proposes an apical plasma membrane vacuolar (V-type) H^+-ATPase which is insensitive to ouabain but is inhibited by bafilomycin A, (Bertram et al.,

1991). The H^+-ATPase creates a proton gradient across the apical membrane of the principal cells; this gradient then drives movement of K^+ and Na^+ from cell to lumen, through amiloride-sensitive Na^+/H^+ or K^+/H^+ antiporters (Figure 3; O'Donnell et al., 1996; Dow and Davies, 2003) to achieve a net secretion of K^+ and Na^+ from cell to lumen. The entry of ions across the basolateral membrane involves both a bumetanide sensitive $Na^+/K^+/2Cl^-$ cotransporter and the basolateral Na^+/K^+-ATPase (Ianowski and O'Donnell, 2004). Much of the Na^+ that enters through the cotransporter is thus recycled back to the haemolymph by the Na^+/K^+ ATPase. Surprisingly, tubules secrete well for sustained periods (> 1 h) in K^+ free saline, and since bumetanide does not alter the rate of fluid secretion of MTs in K^+-free saline (Linton and O'Donnell, 1999), it is unlikely that Na^+ can substitute for K^+ in the Na^+:K^+:2Cl cotransporter, as has been found for tubules of *Rhodnius* (Ianowski et al., 2004). Some possible routes for Na^+ entry in K^+-free saline could be via coupled transport with organic solutes such as glucose or amino acids.

The vacuolar-type H^+-ATPase also establishes a favourable transepithelial electrical gradient for Cl^- to move from the cell to lumen through Cl^- channels in the stellate cells (O'Donnell et al., 1998) (Fig. 3). Water transport through aquaporins in stellate cells is a passive osmotic consequence of active and passive ion transport (Dow and Davies, 2003).

Classic work by Berridge (1968) showed that fluid secretion rate of isolated Malpighian tubules of the blowfly *Calliphora* increases in response to increases in bathing saline potassium concentration. The concentration of K^+ in the urine (100-140 mmol l^{-1}) is always much higher than its concentration in the bathing medium, especially at low concentrations of the latter (i.e. 8-60 mmol l^{-1}). Isolated tubules of *Drosophila* secrete fluids containing 120 mmol l^{-1} K^+ and 30 mmol l^{-1} Na^+ in saline containing 20 mmol l^{-1} K^+ (O'Donnell and Maddrell, 1995). The latter

value is typical of K^+ levels in the haemolymph of flies reared on a standard *Drosophila* diet.

Fluid and ion secretion by *Drosophila* MTs are affected by the actions of several neuropeptide hormones which allow for separate control of cation and anion transport (O'Donnell et al., 1996). The cardioacceleratory peptide CAP2B a member of the family of cardioacceleratory peptides (CAPs) first isolated from *Manduca sexta* (Davies el al., 1995), stimulates fluid secretion rate through elevation of intracellular cGMP levels. CAP2B binds to a putative receptor and acts via intracellular calcium to stimulate nitric oxide synthase. The nitric oxide then acts on guanylate cyclase to raise cGMP levels which then stimulates protein kinase G, which in turn stimulates the apical membrane V-ATPase. This stimulation increases the lumen positive potential (O'Donnell et al., 1996; Dow et al., 1998) implying activation of the apical H^+-ATPase in principal cells (Davies et al., 1995; Kean et al., 2002). In addition, a member of the corticotrophin releasing factor (CRF) family of neuropeptides acts through cAMP to increase fluid secretion (Cabrero et al., 2002). As for cGMP, the action of cAMP appears to be stimulation of the apical H^+-ATPase (O'Donnell et al., 1996).

Both the peptide leucokinin (LK) and thapsigargin stimulate fluid secretion and reduce the lumen-positive TEP (O'Donnell et al., 1996). Leukokinin receptors have been found in the stellate cells (Radford et al., 2002), and a role for stellate cell chloride channels has been proposed (O'Donnell et al., 1998; Yu et al., 2002). The *Drosophila* leucokinin is now referred to as Drosokinin and its effects are believed to be mediated through increases in intracellular calcium levels that bring about an increase in transcellular chloride conductance through channels in the stellate cell (O'Donnell et al., 1998; Rosay et al., 1997). Thapsigargin can be used experimentally to increase the levels of intracellular Ca^{2+} because it blocks the Ca^{2+} ATPase responsible for movement of Ca^{2+} from the cytosol into the endoplasmic reticulum.

Anatomy and physiology of the Drosophila *gut*

The anatomy of the gut is shown in Figure 1 (based on Shanbhag and Tripathi, 2009). The adult midgut consists of a cylindrical tube composed of a single layer of brush bordered, tall, columnar parenchymal cells. Short basal cells, which are believed to be regenerative, are interspersed among the columnar cells. An inner circular layer and an outer longitudinal layer of muscle fibers surround the parenchymal cells and tracheal cells (Gartner, 1985).

The parenchymal cells sit upon a basal lamina. The basal cell membrane is extensively infolded whereas the lateral cell membranes are relatively straight. There are numerous junctional complexes between adjacent cells. The midgut lumen is divided into two compartments by the presence of a non-cellular peritrophic membrane. There are numerous regular microvilli on the apical cell membrane but these do not contact the peritrophic membrane (Gartner, 1985).

Recent studies by the laboratory of Tripathi (Shanbhag and Tripathi, 2005, 2009) have shown that the pH of the luminal contents of the anterior midgut and the posterior part of the anterior midgut is between neutral and mildly alkaline (pH>7 and <8), the middle segment is highly acidic (pH<3.0) and the middle and posterior midgut is highly alkaline (pH>10). Studies to date have examined the transport of acid and base across the gut (Shanbhag and Tripathi, 2005, 2009) but relatively little is known of the patterns and mechanisms of K^+ and Na^+ transport across the *Drosophila* gut.

The larval gut transports acidic and basic equivalents at higher rates than the adult, consistent with higher feeding rates. The larval midgut has at least three segments: an anterior neutral zone, a short and narrow acid-secreting middle segment and a long and wider posterior

segment. Studies of the latter segment show that it secretes base (probably bicarbonate) into the lumen. The posterior midgut has a lumen-negative transepithelial potential (35–45 mV) and a high transepithelial electrical resistance (800–1400 ohms cm^2). This high resistance is correlated with a great reduction in lateral intercellular volume. The primary transport system driving base secretion into the lumen appears to be a bafilomycin-A1-sensitive, electrogenic H^+ V-ATPase located on the basal membrane, which extrudes acid into the haemolymph, as inferred from the extracellular pH gradients detected adjacent to the basal membrane. Longitudinal gradients of pH dye-indicators in the lumen of the adult midgut show that it is also segmented into anterior, middle and posterior regions. The anterior segment appears to be absorptive whereas the middle midgut secretes acid (pH<4.0) through a process dependent on a carbonic anhydrase-catalysed H^+ pool. Absorptive cells alternate with secretory cells in the middle segment. In the absorptive cells, the apical surface is folded so that its surface area is increased by ~9-fold relative to a flat surface, whereas the basal surface is amplified >90-fold by infolding. By contrast, the apical surface of the secretory cells is amplified by >90-fold and the basal surface by ~10-fold. Cells of the posterior segment have an extensively dilated basal extracellular labyrinth, with a volume larger than that of anterior segment cells, indicating more fluid absorption in the posterior segment (Shanbhag and Tripathi, 2005, 2009).

Surviving salt stress: functional roles of the gut and Malpighian tubules

In both the freshwater mosquito *Aedes aegypti* and the saltwater species *Ochlerotatus taeniorhynchus*, Malpighian tubule fluid secretion and ion transport are influenced by changes in the salinity of the larval rearing medium (Donini et al., 2006). For unstimulated tubules of both species, the K^+ concentration of secreted fluid is significantly lower when larvae are reared in 30% seawater, relative to tubules from freshwater-reared larvae. The Na^+ concentration of

secreted fluid from unstimulated tubules of *O. taeniorhynchus* reared in 30% or 100% seawater is higher relative to tubules from freshwater-reared larvae. These results indicate that changes in salinity of the larval rearing medium lead to sustained changes in ion transport mechanisms in unstimulated tubules. In addition, changes in the rate of ion transport by the tubules may result in either conservation of Na^+ under freshwater (Na^+-deprived) conditions or enhanced elimination of Na^+ in saline (Na^+-rich) conditions. Changes in rearing medium salinity also affect the nature and extent of stimulation of fluid and ion secretion by secretagogues. In response to cGMP, for example, tubules from larvae reared in high salinity secrete more Na^+ at the expense of K^+, relative to tubules from larvae reared in low salinity.

Wild-type *Drosophila* are very tolerant of high levels of Na^+ or K^+ in the diet (Huang et al., 2002), suggesting that they can provide a useful model species to examine the tissues involved in the regulation of haemolymph Na^+ and K^+ levels in response to dietary challenges. Roles for specific membrane transporters have been highlighted in recent studies examining survival of *Drosophila* during salt stress. The two proteins encoded by the inebriated (*ine*) gene, which are members of the Na^+/Cl^--dependent neurotransmitter/osmolyte transporter family, appear to be responsible for osmolyte transport within the Malpighian tubule and hindgut. Mutants lacking both *ine* gene isoforms are hypersensitive to osmotic stress, which can be assayed by maintaining flies on media containing NaCl, KCl, or sorbitol. Mutant flies showing such hypersensitivity can be completely rescued by high-level ectopic expression of the either of the two isoforms of *ine* (*ine*-P1 or *ine*-P2). Huang et al. (2002) concluded that *ine* mutations confer osmotic stress sensitivity by preventing osmolyte accumulation within the Malpighian tubule and hindgut. Dietary salt stress induces changes in *Drosophila* gene expression involving immune/stress responses, carbohydrate metabolism, and ion transport pathways (Stergiopoulos et

al., 2008). A tubule-specific gene called *salty dog* is expressed on the basolateral plasma membrane of the tubule and is strongly up regulated in response to dietary salt. Based on protein structure inferred from its gene sequence, *salty dog* is likely to be a Na^+/solute cotransporter, but the cotransporter substrate is unknown. Given that *salty dog* is localized to the basolateral membrane of the Malpighian tubule, its upregulation in response to dietary salt will tend to aid sodium clearance from the haemolymph. The authors speculate that flies may sense increasing Na^+ levels during dietary salt stress and increase Na^+ excretion by the tubule by upregulating basolateral Na^+ uptake. Surprisingly, flies in which *salty dog* expression in the principal cells is knocked down using an RNAi technique actually survive better on salt-rich diets. It is suggested that overexpression of *salty dog* during dietary salt stress is a miscalculated response of the organism, analogous to the detrimental cytokine storm elicited in healthy humans exposed to influenza.

Thus, although transport by the tubules and gut is likely to be involved in the survival of salt-stressed flies, the extent of haemolymph ionic homeostasis and changes in rates of transport by the tubules and gut of larval or adult *Drosophila* have not been examined directly. Such studies are the subject of this thesis. Epithelial function during salt stress has been assessed through the use of two electrophysiological techniques.

Analysis of epithelial transport using the Ramsay assay

The output of the Malpighian (renal) tubules in insects has long been studied using the Ramsay assay, in which isolated Malpighian tubules are placed in droplets of saline solution under liquid paraffin. The diameter of the secreted droplets collected at known intervals is measured, allowing calculation of the droplet volume and hence rate of secretion. The technique

can be used in conjunction with ion-selective microelectrodes (ISMEs) to measure the rate of secretion of particular ions. For *Drosophila*, an isolated pair of Malpighian tubules is placed in a droplet of bathing saline under paraffin oil. One tubule remains in the saline, and the other is pulled out and wrapped around a stainless steel pin embedded in the Sylgard-line base of a Petri dish. Secreted fluid droplets which form at the ureter, positioned just outside the bathing saline droplet, are collected on glass rods and placed on the bottom of the dish adjacent to calibration droplets containing known concentrations of Na^+ or K^+ in *Drosophila* saline. For each droplet, the potential difference between the ion-selective microelectrode and the reference microelectrode is measured by a high impedance ($>10^{15}$ Ω) operational amplifier and recorded on a PC-based data acquisition system. Ion concentration in the secreted droplets is calculated from the voltage difference between the secreted droplet and the calibration droplets. Transepithelial flux (pmol min^{-1}) for each tubule can then be calculated as the product of fluid secretion rate (nl min^{-1}) and ion concentration (mmol l^{-1}) determined by the ISME. Multiple droplets can be collected from 20 or more tubules set up in the Ramsay assay (Figure 4)

Analysis of epithelial transport using the scanning ion electrode technique (SIET)

This thesis also makes use of the scanning ion electrode technique which permits non-invasive analysis of epithelial ion transport by isolated tissues. For small insects such as *Drosophila*, transport can be measured along the entire length of the gut and attached Malpighian tubules. SIET exploits ionic concentration gradients created in the unstirred layer by ion transport across cell membranes or epithelial layers (Figure 5). The isolated tissue and the ion-selective microelectrode (ISME) are observed through a microscope equipped with a CCD camera. The microelectrode is positioned by an orthogonal array (*X, Y, Z*) of computer-controlled stepper motors and is moved between two points at each measurement site. The first point is

close to the cell surface (within 5 to 10 μm) and the second point is 30–100 μm farther away, at right-angles to the surface. The voltage difference between the two limits of the microelectrode excursion (ΔV) is used to calculate a corresponding concentration difference (ΔC) using the electrode calibration curve. The concentration difference is then converted to net flux (mol cm^{-2} s^{-1}) using Fick's Law: $J = D\Delta C/\Delta X$, where D is the diffusion coefficient of the ion of interest and ΔX is the excursion distance. SIET is particularly useful for spatial and temporal analysis of ion transport, especially for small insects such as fruit flies and mosquitoes that are not amenable to the Ussing chamber studies used for gut segments of larger species such as locusts and *Manduca sexta*.

Organization and goals of the thesis

Given the evidence for growth and survival of *Drosophila* on salt-rich diets, the central questions of this thesis, therefore are:

1) Do adult and larval Drosophila regulate the levels of Na^+ and K^+ in the haemolymph in response to changes in dietary Na^+ and K^+ levels?

2) What mechanisms contribute to the regulation of haemolymph Na^+ and K^+ levels?

The maintenance of a particular level of K^+ or Na^+ in the haemolymph reflects a balance between the rate of absorption of these ions from the gut and the rate of excretion by other regions of the gut or the Malpighian tubules. Question (2) can be thus be addressed by studies examining a series of questions relating to absorption and excretion:

a) *Does the Na^+/K^+ ratio of the fluid secreted by the Malpighian tubules change when larvae are reared on high or low Na^+ or K^+ levels in the diet?*

b) *What are the sites of uptake of dietary Na^+ and K^+ into the haemolymph (e.g. esophagus, gastric caeca, anterior midgut, posterior midgut)?*

c) *Do the rates of uptake across the midgut change in flies reared on diets containing elevated levels of K^+ or Na^+?*

d) *Is there secretion or absorption of K^+ and Na^+ across the hindgut (ileum/rectum) in flies reared on control or Na^+/K^+-rich diets?*

Chapter 2 of this thesis makes use of the Ramsay assay for collection of fluid secreted by isolated Malpighian tubules. The levels of Na^+ and K^+ in the secreted fluid droplets are determined using ion-selective microelectrodes. The rate of secretion of each ion can then be calculated as the product of the rate of fluid secretion (determined from measurements of fluid droplet diameter) and the concentration of each ion in the secreted fluid. Secretion rates of Na^+ and K^+ can then be compared in tubules from adults and larvae reared on diets containing the control or elevated levels of Na^+ or K^+. Since addition of high concentrations of Na^+ or K^+ will elevate the osmolality of the food, ion secretion will also be examined in tubules from flies reared on diets in which saline osmolality is elevated by inclusion of a non-electrolyte (sucrose).

Chapter 3 exploits the scanning ion electrode technique (SIET) to examine rates of Na^+ and K^+ transport by different segments of guts isolated from flies reared on salt-rich or control diets.

Chapter 4 integrates the results of chapters 2 and 3 and provides an overview of the changes in epithelial ion transport in response to dietary salt stress. In addition, avenues for future research are discussed.

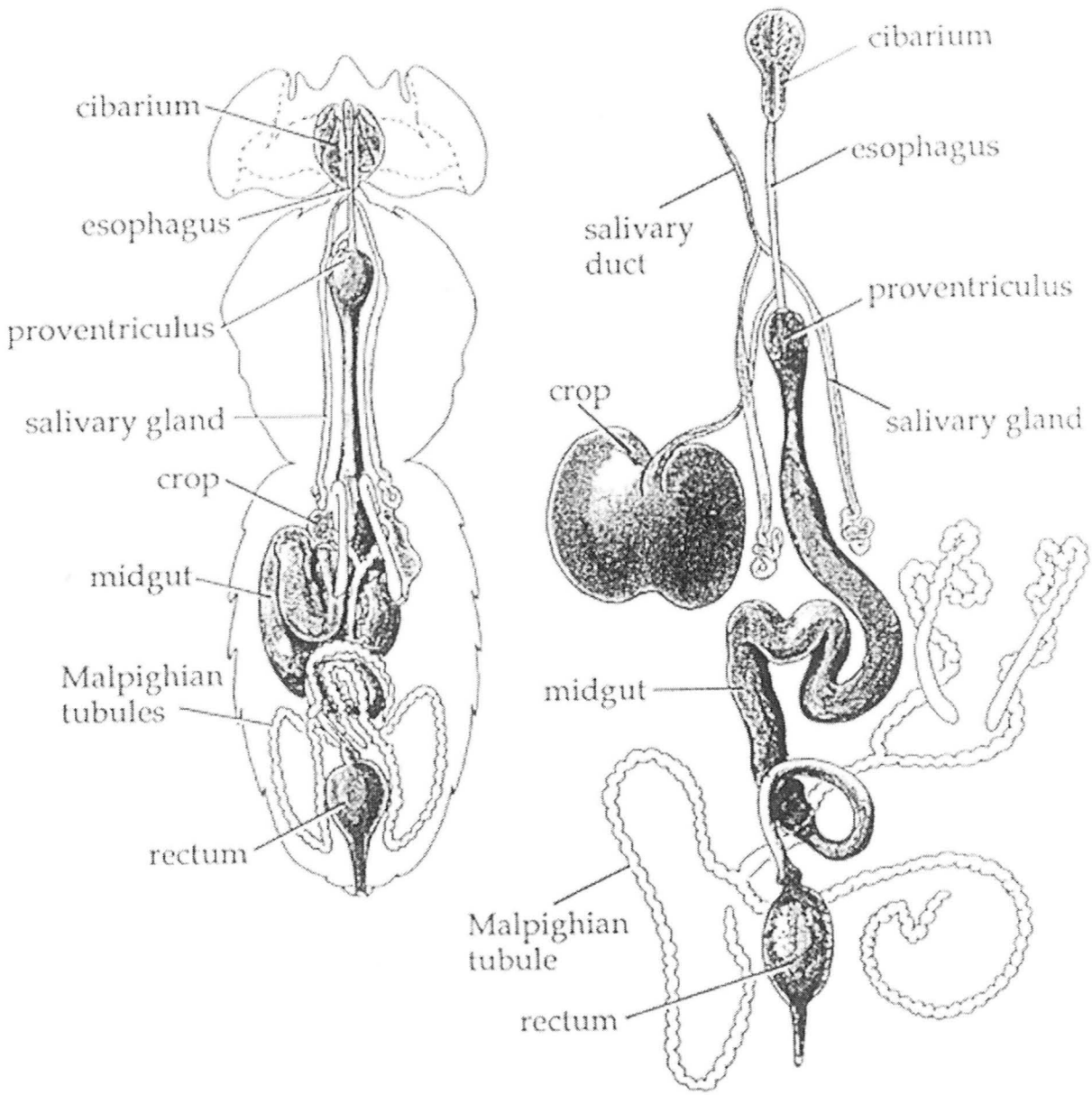


Figure 1:- Schematic representation of insect gut

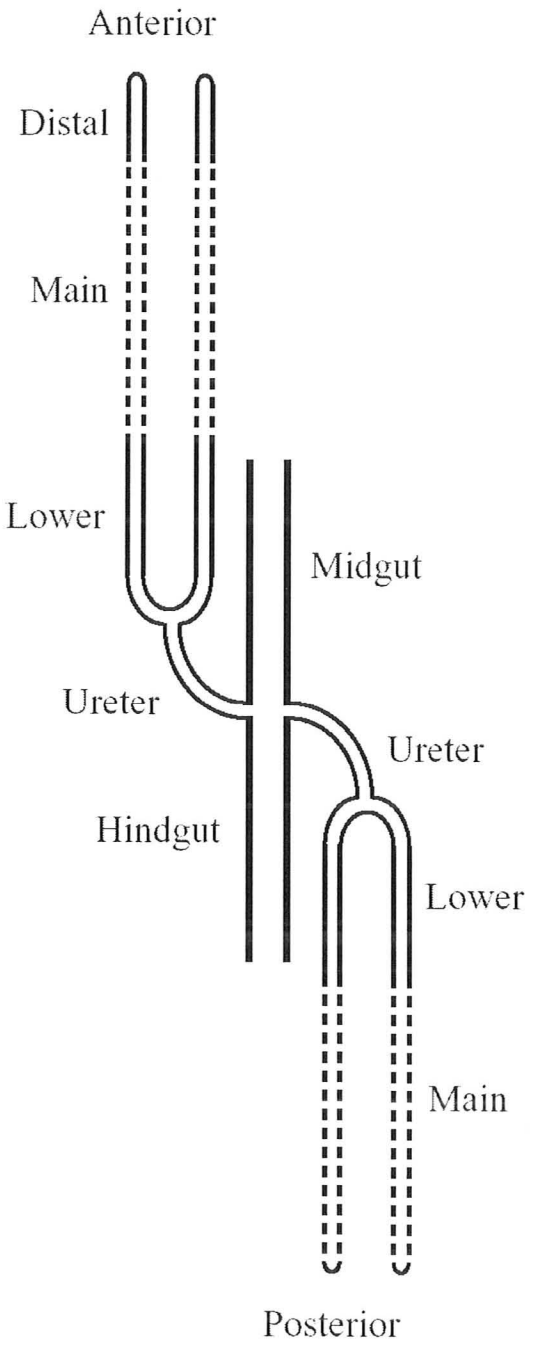


Figure 2:- Schematic representation of Malpighian tubules

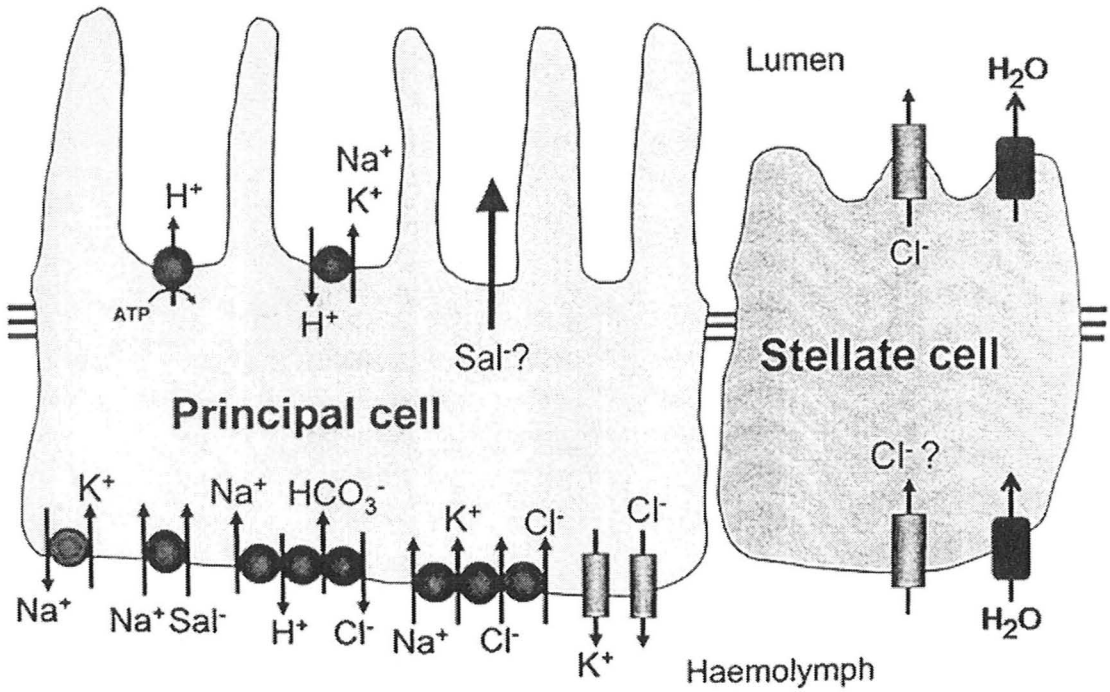
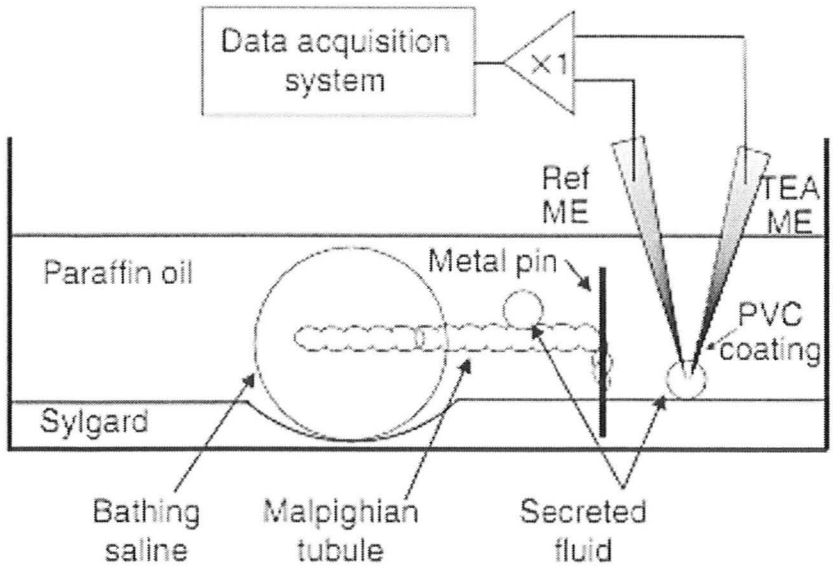


Figure 3:- Schematic diagram of ion transporters in the cells of the Malpighian tubules of Drosophila melanogaster



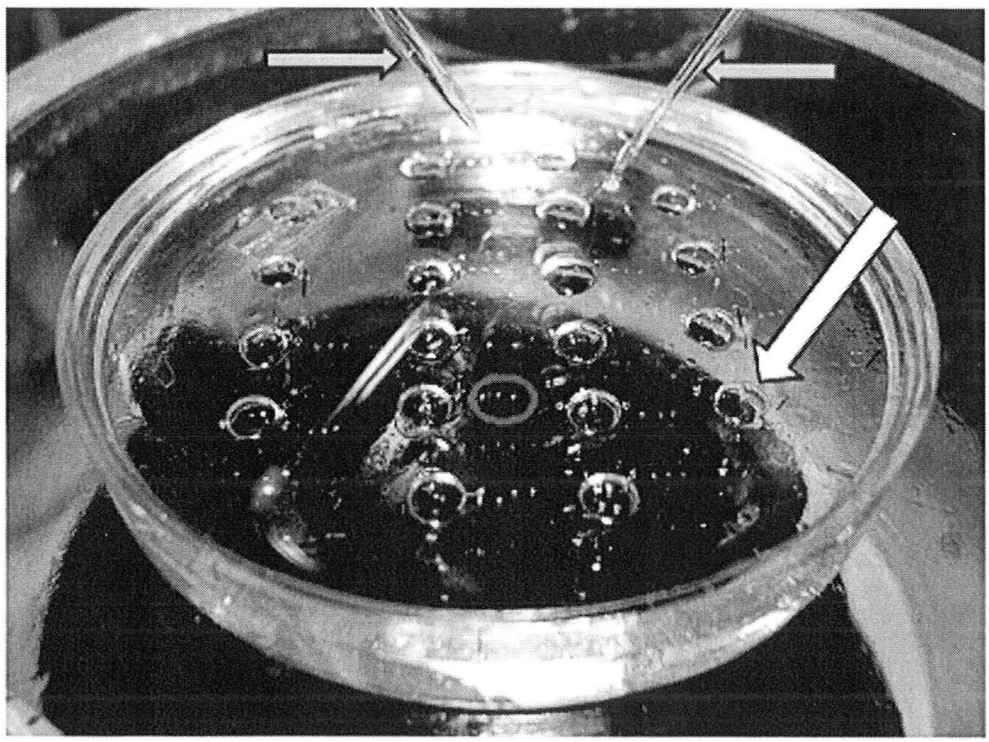


Figure 4:- The Ramsay assay

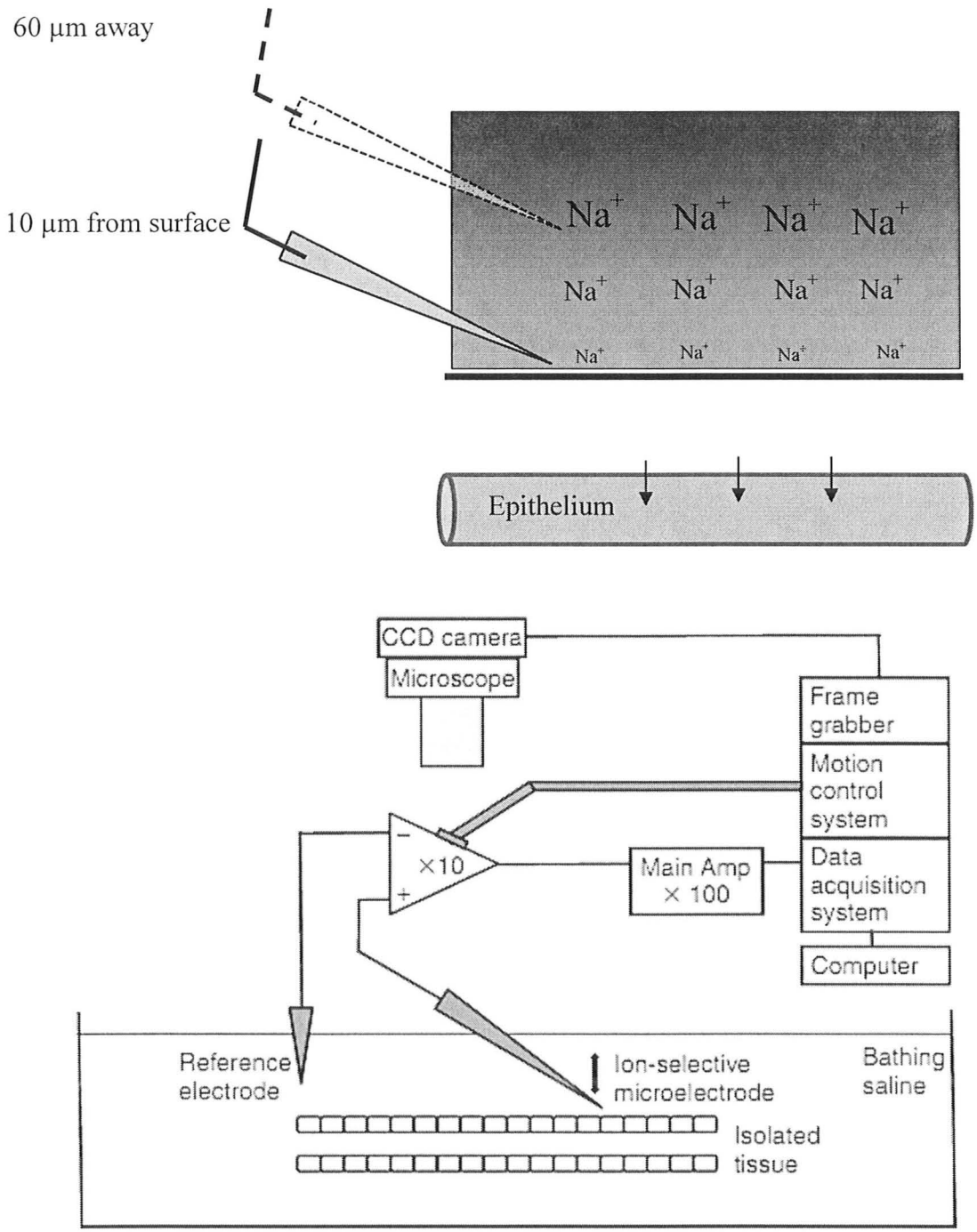


Figure 5:- Measurement of Na^+ fluxes using scanning ion-selective electrode technique

Chapter 2

Alterations of fluid and ion transport by Malpighian tubules of *Drosophila melanogaster* in response to salt stress.

W. Naikkhwah and M. J. O'Donnell

Abstract

The effects of NaCl-rich and KCl-rich diets on haemolymph ionoregulation and Malpighian tubule (MT) fluid and ion secretion were examined in larval and adult *Drosophila melanogaster*. K^+ concentrations in the haemolymph of adults reared on KCl-rich (0.4 mol l^{-1}) diet did not differ from the values for insects reared on the control diet. In the haemolymph of larvae reared on the KCl-rich diet, K^+ concentrations increased from 23 mmol l^{-1} to 75 mmol l^{-1} after 6 hours, then returned to the control value within 48 hours. Na^+ concentrations in the haemolymph of adults or larvae reared for 1d – 7d on NaCl-rich (0.4 mol l^{-1}) diet increased ~50 % relative to values for insects reared on the control diet. Secretion of fluid, Na^+ and K^+ by MTs isolated from larvae reared on the NaCl-rich diet for >6 h and bathed in control saline containing 20 mmol l^{-1} K^+ did not differ from the values for tubules of larvae reared on the control diet. Secretion of fluid and K^+ by MTs isolated from larvae reared on the KCl-rich diet for >6 h and bathed in saline containing 60 mmol l^{-1} K^+ increased >50% relative to the values for tubules of larvae reared on the control diet. Secretion of fluid, Na^+ and K^+ increased when tubules were bathed in haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diets. Secretion was further increased by addition of exogenous cAMP but not by addition of thapsigargin to the haemolymph. The results show haemolymph ionoregulation in larvae reared on salt-rich diets involves both alterations in the basal secretion rates of Na^+ or K^+ as well as the stimulatory effects of diuretic factors present in the haemolymph. The results are consistent with a stimulation of tubule fluid and ion secretion through increases in intracellular Ca^{2+} in response to salt stress.

Introduction

Insects are known to survive a wide variety of desiccating or hypersaline environments that pose challenges to maintenance of homeostasis. Larvae of mosquitoes, chironomids and the alkali fly *Ephydra hians* are known to live in osmotically stressful hypersaline conditions (Neuman, 1976; Nayar, 1969; Scudder, 1969; Maddrell and Phillips, 1974). Both the gut, particularly the hindgut, and the Malpighian tubules are known to contribute to haemolymph pH and ionoregulation. In mosquito larvae (*Aedes campestris*) inhabiting hypersaline lakes, the rectum secretes hyperosmotic fluid containing elevated levels of Na^+, K^+, Mg^{2+}, Cl^- and HCO_3^-, whereas the Malpighian tubules are the major site of SO_4^{2-} excretion (Phillips and Bradley, 1977). Larvae of salt-tolerant mosquitoes *Ochlerotatus taeniorhyncus* show evidence of phenotypic plasticity in response to salt stress. The Malpighian tubules secrete more Na^+ at the expense of K^+ when the larvae are reared in 30% or 100% sea water (Donini et al., 2006).

Recent research has highlighted the usefulness of *Drosophila* for studies of salt stress. Fifty percent of adult flies reared on diets contain 0.85 M NaCl survive 4 days (Stergiopoulos et al., 2008), and >75% of flies survive exposure to 0.4 M NaCl or KCl (Huang et al., 2002). Although this species is not normally exposed to such high levels of salt stress, its advantages as a genetic model make it an attractive species to study, and ion transport by both the Malpighian tubules and the gut can be studied using electrophysiological methods. In this study, we have examined the effects of salt stress on haemolymph ion regulation, and on the rates of fluid and ion secretion by isolated Malpighian tubules bathed in saline or in haemolymph collected from larvae reared on control or experimental diets. In addition, we have examined the effects of secretagogues on secretion of fluid, Na^+ and K^+ by isolated tubules.

Materials and Methods

Insects and diet preparation

The Oregon R strain of *D. melanogaster* were raised on standard artificial diet and maintained at 21° – 23°C in laboratory culture. The control diet was prepared as described by Roberts and Stander (1998). Solution A consisted of 800 ml tap water, 100 g sucrose, 18 g agar, 8 g KNa tartrate, 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgCl_2$, and 0.5 g $CaCl_2$. Solution B consisted of 200 ml tap water and 50 g dry active yeast. The two solutions were autoclaved, combined and stirred. After cooling to 55°C, 10 ml of an acid mix (11 parts tap water, 10 parts propionic acid, and 1 part 85% o-phosphoric acid) and 7.45 ml of 10% phydroxybenzoic acid methyl ester (Tegosept) dissolved in ethanol were added to the mixture. Experimental diets were prepared by addition of 0.4 mol l^{-1} NaCl, 0.4 mol l^{-1} KCl or 0.8 mol l^{-1} sucrose to the standard diet. For experiments involving the larvae we used third instar larvae which had been transferred as third instars for 6, 12, 24 or 48 hours to the experimental diet. Larvae which were chronically exposed to salt rich diet had been raised from eggs on the diet and were therefore exposed to the diet for 7 days or more by the time they reached the 3rd instar.

Measurement of haemolymph volume

Haemolymph volume was estimated by a blotting technique (Folk et al., 2001). This involved weighing the larvae, tearing the cuticle with fine forceps, blotting haemolymph with tissue paper and reweighing the larvae. Haemolymph volume was then calculated by subtracting the final from the initial weight and assuming that haemolymph density was 1 mg/µl.

Haemolymph collection

Adults were placed ventral surface upwards in a dish containing paraffin oil. One leg was cut distal to the femur with fine scissors and pressure was applied to the thorax with forceps. Exuded haemolymph droplets were collected and pooled using a fine glass rod. Haemolymph was collected from 3rd instar larvae under paraffin oil by tearing the cuticle with forceps and lifting the larva through the air-oil interface, thus leaving behind a droplet (~ 0.5 µl) of haemolymph. Haemolymph was collected and pooled from ~ 30 larvae that had fed chronically on control diets or diets enriched with 0.4 mol l^{-1} NaCl, 0.4 mol l^{-1} KCl or 0.8 mol l^{-1} sucrose. Care was taken to collect only clear samples of haemolymph, free of any haemocytes.

Malpighian tubule dissection and Ramsay assays:

Tubules were dissected under standard *Drosophila* saline solution containing (mmol l^{-1}) 117.5 NaCl, 20 KCl, 2 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 15 Hepes, 20 glucose and 10 glutamine and adjusted to pH 7.0 with NaOH. A saline containing 60 mmol l^{-1} K^+ was made by equimolar substitution of KCl for NaCl. Dissections were performed using forceps as described by Dow et al., (1994), and one pair of Malpighian tubules joined by a common ureter were then removed and transferred using fine glass probes to the droplets of bathing saline (20 µl) or haemolymph (5 µl) under paraffin oil in the Ramsay assay dish. Haemolymph was stirred with a glass rod prior to use to remove clots.

The tubule pairs were arranged so that one end of the tubule was bathed in the saline well and the other end was wrapped around a 0.15 mm diameter minuten pin positioned approximately 2-3 mm away from the droplet. The ureter was positioned so that the main (secretory) segment of one tubule was bathed in saline or haemolymph. Droplets of secreted fluid were collected from the ureter after 60 min using a fine glass probe and placed under

paraffin oil on the Sylgard-lined bottom of the dish. The diameter (d) of the droplet was measured with an ocular micrometer under at 80x magnification, and droplet volume was calculated as $(\pi d^3)/6$. Secretion rates (nl/min) were calculated by dividing droplet volume by the time over which it formed.

Measurement of K^+ and Na^+ concentration in haemolymph and secreted fluid droplets

Ion-selective microelectrodes were used to measure the concentration of Na^+ and K^+ in samples of haemolymph or secreted fluid under paraffin oil. Micropipettes were pulled from 1.5 mm o.d. unfilamented borosilicate glass capillary tubing using a vertical micropipette puller (Narishige, Tokyo, Japan), and dried on a hot plate at 200°C for 10 min before silanization. The latter process makes the glass surface hydrophobic and facilitates retention of the hydrophobic ionophore cocktails. A drop of dimethyldichlorosilane (~1 µl) was pipetted onto the inside of a 150 mm diameter Pyrex Petri dish, which was then inverted over the micropipettes which had been placed on the hot plate. Micropipettes were removed after a minimum of 20 min exposure to the silane vapour, and could be stored over silica gel for up to 2 weeks before filling. This degree of silanization is sufficient to retain the ionophore cocktail but avoids capillary rise of paraffin oil into the pipette tip. Appropriately silanized microelectrodes for use under paraffin oil are characterized by a flat meniscus at the interface between the cocktail and the backfill solution.

K^+-selective microelectrodes were first backfilled with 150 mmol l^{-1} KCl using a plastic 1 ml syringe pulled out over a low flame to a fine tip (Thomas, 1978) and then tip-filled with a column (~ 500 µm) of K^+ ionophore I, cocktail B (Fluka, Buchs, Switzerland). Na^+-selective microelectrodes were first backfilled with 150 mmol l^{-1} NaCl and then tip filled with a Na^+ ionophore cocktail which consisted of 10% Na^+ ionophore X, 89.75% nitrophenyl octyl ether and

0.25% sodium tetraphenylborate (Messerli et al., 2008). Reference microelectrodes were pulled from 1.5 mm o.d. filamented glass tubing and were filled with 150 mmol l^{-1} KCl. Electrodes were connected through chlorided silver wires to an electrometer of high input impedance (>10^{13} Ω) and signals were recorded using a computer-based data acquisition and analysis system (PowerLab, ADInstruments, Bella Vista NSW, Australia) running Chart software.

Na^+ and K^+ concentrations in drops of haemolymph or secreted fluid were measured under paraffin oil by positioning ion-selective and reference electrodes in the drop and measuring the potential change relative to that in drops of calibration solutions. Na^+-selective microelectrodes were calibrated in NaCl-KCl mixtures in which the sum of both cation concentrations was 150 mmol l^{-1}. Ion concentrations in the samples were calculated from the equation:

$$[\text{Ion}]_{\text{sample}} = [\text{Ion}]_c 10^{(\Delta V/S)}$$

Where $[\text{Ion}]_{\text{sample}}$ is the ion concentration of the droplet of haemolymph or tubule secretion, $[\text{Ion}]_c$ is the concentration of a calibration drop, ΔV is the change in potential (mV) between the sample and the calibration drop, and S is the slope (mV) for a tenfold change in ion concentration. All experiments were done at room temperature, 23°C.

Although ion-selective electrodes measure ion activity and not concentration, data can be expressed in terms of concentrations if it is assumed that the ion activity coefficient is the same in calibration and experimental solutions. Expression of data in terms of concentrations simplifies comparisons with studies in which ion concentrations are measured by techniques such as atomic absorption spectroscopy.

Sodium or potassium flux (ρmol min^{-1} tubule) was calculated as the product of fluid secretion rate (nl min^{-1}) and secreted fluid K^+ or Na^+ concentration (mmol l^{-1}).

Effects of secretagogues

All compounds were obtained from Sigma and added to bathing saline at concentrations of 100 μmol l^{-1} (cAMP) or 10 μmol l^{-1} (thapsigargin). Secreted droplets were collected from each unstimulated tubule after 40 minutes, the secretagogue was then added and a second droplet was collected after a further 40 min.

Graphing and statistics

Data were plotted using GraphPad Instat (GraphPad Software, San Diego, California). Values are expressed as means ± S.E.M. for the indicated number of tubules (*N*). Two-sample *F*-tests were used to compare the variances of the data for the control and experimental groups. Depending on the outcome of each *F*-test, differences between experimental and control groups were compared using unpaired Student's *t*-tests assuming either equal or unequal variances. The responses of the same group of tubules before and after an experimental treatment were compared using a paired *t*-test. Differences were considered significant if $P<0.05$. Data in which concentration of ions varied or saline ionic composition varied were analyzed by one-way ANOVA with Tukey's post-hoc multiple comparison ($p<0.05$).

Results

Estimates of haemolymph volume in 3rd instar larvae

The average mass of a 3rd instar larva reared on the control diet was 1.86 ± 0.06 mg and haemolymph volume was 0.57 ± 0.02 µl, equivalent to 31% of larval mass (Figure 1A,B). There was a significant decrease of 26% and 30% in the weight of larvae maintained on the KCl or NaCl-rich diets, respectively. Haemolymph volume of larvae reared on the KCl-rich diet was maintained but haemolymph volume of larvae reared on the NaCl-rich diet decreased to 52% of the control value (20% of larval mass).

Effects of salt-rich diets on haemolymph concentrations of Na^+ and K^+.

The haemolymph of adult flies maintained on the control diet contained 26 mmol l^{-1} K^+ (Figure 2A). There was no significant increase in K^+ concentration in the haemolymph of adult flies maintained for as long as 7 days on the KCl-rich diet. By contrast, there was a transient increase in K^+ concentration in the haemolymph of larvae reared on KCl-rich diet (Figure 2B). Haemolymph K^+ concentration increased more than 3 fold to 75 mmol l^{-1} after 6 hours and remained at approximately twice the control level for the first 24 hours. For larvae maintained 48 hours or longer on the KCl-rich diet, the haemolymph K^+ was restored to the control level.

Na^+ concentration in haemolymph of adults increased by 32% - 57% after 12 hours or longer on the NaCl-rich diet (Fig. 2C). The Na^+ concentration in the haemolymph of the larvae on the NaCl-rich diet increased by more than 70% above the control level after 12 hours, then declined to a value ~ 56% above the control level (Fig. 2D).

The increases in K^+ and Na^+ concentrations in haemolymph of larvae reared on the KCl-rich and NaCl-rich diets, respectively, were not due simply to the increased osmotic pressure of

the diet. K^+ and Na^+ concentrations in the haemolymph of larvae maintained on diet containing 0.8 mol l^{-1} sucrose were somewhat reduced, relative to larvae reared on the control diet (Fig. 2E, F)

Effects of salt-rich diets on secretion of Na^+, K^+ and fluid by larval Malpighian tubules isolated in control saline.

We used the Ramsay assay and ion-selective microelectrodes to assess the rates of fluid and ion (Na^+, K^+) transport of larval tubules maintained chronically on the NaCl-rich or KCl-rich diets.

For larvae reared on the NaCl-rich diet, there were no significant changes, relative to tubules isolated from larvae reared on control diet, in fluid secretion rate (Fig. 3A) or secreted fluid concentrations of Na^+ or K^+ (Fig. 3B). With the exception of a reduction in K^+ flux after 6 hours, there were no significant changes in the flux of Na^+ or K^+ (Fig. 3C).

An unexpected finding was an increase in the flux of Na^+ by tubules isolated from larvae reared on the KCl-rich diet for 48 hours or longer. Fluid secretion rates of tubules increased after 24 hours or more on the KCl-rich diet, by up to 56% in the chronic exposure group (Fig. 4A). There was an increase in secreted fluid Na^+ concentration and a corresponding decrease in K^+ concentration (Fig. 4B). As a consequence, K^+ flux was maintained but Na^+ flux increased by 178 % at 48 hours and by 278% in the larvae that were chronically exposed to the KCl-rich diet (Fig. 4C).

Effects of the KCl-rich diet on secretion of Na^+, K^+ and fluid by larval Malpighian tubules isolated in saline containing 60 mmol l^{-1} K^+

The effects of the KCl-rich diet on secretion by larval tubules was further explored by isolating the tubules in saline containing high levels of K^+. The rational for these experiments was that the potassium level of the haemolymph of larvae reared on the KCl- rich diet is elevated for the first 24 hours (Figure 2) and that the tubules may secrete higher levels of K^+ relative to controls when the saline contains higher levels of K^+. The results (Figure 5) show that fluid secretion rate in 60 mmol l^{-1} K^+ saline before the transfer to the KCl-rich diet (0 hours) did not differ from that of tubules isolated in control (20 mmol l^{-1} K^+) saline (Figure 5A). There was an increase in secreted fluid K^+ concentration (Fig. 5B) and K^+ flux (Fig. 5C) and a corresponding decrease in Na^+ concentration and Na^+ flux in 60 mmol l^{-1} K^+ saline before the transfer to the KCl-rich diet (0 hours) relative to tubules bathed in control saline. Importantly, there was an increase in fluid secretion rate in 60 mmol l^{-1} K^+ saline for tubules isolated from larvae maintained on the KCl-rich diet for 6 hours or longer. As a consequence, K^+ flux and Na^+ flux of tubules isolated from larvae maintained on the KCl-rich diet for 6 hours or longer were much greater than for tubules isolated from larvae before transfer to the KCl-rich diet and bathed in either control saline or 60 mmol 1^{-1} K^+ saline (Fig. 5C). K^+ flux in 60 mmol 1^{-1} K^+ saline increased by 47% - 56% in tubules isolated from larvae maintained for 6 hours or more on the KCl-rich diet, relative to tubules from larvae before transfer. These results indicate that there are sustained changes in the ion transport mechanisms of the tubules as a consequence of rearing the larvae on the KCl-rich diet.

Effects of salt-rich diets on secretion of Na^+, K^+ and fluid by Malpighian tubules of larvae isolated in haemolymph.

We also wished to determine whether the secretion of Na^+, K^+ and fluid by the tubules of larvae on the different diets was altered by factors circulating within the haemolymph. Tubules were therefore isolated in haemolymph that had been collected from larvae reared on control diet or from larvae that had been exposed to the KCl-rich diet or the NaCl-rich diet for 7 days or more (chronic exposure).

Fluid secretion rates of tubules isolated from larvae reared on control diet and bathed in haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diets increased, relative to the rate when the tubules were bathed in haemolymph collected from larvae reared on the control diet (Fig. 6A). There was no change in secreted fluid K^+ concentration for tubules from larvae reared on the control diet and bathed in haemolymph collected from larvae reared on the NaCl -rich or KCl-rich diet. However, there was a small increase in the Na^+ concentration of fluid secreted by tubules bathed in haemolymph collected from larvae reared on the NaCl-rich diet (Fig. 6B). The changes in fluid secretion rate resulted in increases in K^+ secretion of 95 % and 111 %, relative to tubules bathed in haemolymph collected from larvae reared on the control diet. The changes in fluid secretion rate and secreted fluid Na^+ concentration resulted in increases in Na^+ secretion of 175% and 50%, relative to the controls, for tubules bathed in haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diet, respectively (Fig. 6C).

Fluid secretion rate of tubules isolated from larvae reared on the NaCl-rich diet and bathed in haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diets increased, relative to the rate in haemolymph collected from larvae reared on the control diet (Fig. 7A). There were no changes in the K^+ or Na^+ concentration of fluid secreted by tubules bathed in

haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diets (Fig. 7B). The changes in fluid secretion rate resulted in increases in Na^+ secretion of 200% for tubules bathed in haemolymph collected from larvae reared on the NaCl-rich relative to tubules bathed in haemolymph collected from larvae reared on the control diet. The changes in fluid secretion rate of tubules isolated from larvae reared on the NaCl-rich diet also result in increases in K^+ secretion of 110% and 58% for tubules bathed in haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diets, respectively, relative to tubules bathed in haemolymph collected from larvae reared on control diet (Fig. 7C).

Fluid secretion rate of tubules isolated from larvae reared on the KCl-rich diet increased when bathed in haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diets, relative to the rate in haemolymph collected from larvae reared on the control diet (Fig. 8A). There were also increases in the Na^+ concentration of fluid secreted by tubules bathed in haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diet (Fig. 8B). The changes in fluid secretion rate and secreted fluid Na^+ concentrations resulted in increases in Na^+ secretion of 167% and 150 % and increases in K^+ secretion of 39% and 72% by tubules bathed in haemolymph collected from larvae reared on the NaCl-rich and KCl-rich diets, respectively, relative to tubules bathed in haemolymph collected from larvae reared on the control diet (Fig. 8C).

Effects of secretagogues on fluid and ion transport by tubules isolated from larvae reared on control or salt-rich diets.

The results of experiments using haemolymph collected from larvae reared on control or salt-rich diets as the bathing medium for isolated tubules suggested that diuretic factors were

present in the haemolymph of larvae reared on salt-rich diets. The effects of known diuretic factors in *Drosophila* tubules are mediated through the second messengers cAMP and cGMP, and Ca^{2+} (Dow and Davies, 2003). Cyclic AMP and GMP both lead to increased ion transport and fluid secretion through stimulation of the vacuolar-type H^+-ATPase in the apical membrane of the principal cells in the tubule. The primary effect of increases in cytosolic Ca^{2+} is to increase Cl^- permeability mediated by Cl^- channels in the stellate cells (O'Donnell et al., 1998). Increases in cytosolic Ca^{2+} levels can be induced through addition of thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase. We hypothesized that if the V-ATPase or stellate cell Cl^- permeability had been activated by diuretic factors present in the haemolymph, then subsequent addition of cAMP or thapsigargin, respectively, would have no additional stimulatory effect.

The fluxes of both K^+ and Na^+ increased in response to addition of cAMP to tubules isolated from larvae reared on the KCl-rich diet and bathed in haemolymph collected from larvae reared on any of the three diets (Figure 9A). These increases in flux were due to increases in fluid secretion rate rather than increases in secreted fluid concentrations of Na^+ and/or K^+ (data not shown). K^+ flux increased and Na^+ flux was unchanged when tubules isolated from larvae reared on the NaCl-rich diet were bathed in haemolymph collected from larvae reared on any of the three diets (Figure 9B). These data indicate that the tubules retain responsiveness to cAMP when bathed in haemolymph isolated from larvae reared on the NaCl-rich or KCl-rich diets, and that such haemolymph does not contain diuretic factors which are mediated through increases in intracellular cAMP.

The flux of K^+ increased in response to addition of thapsigargin to tubules isolated from larvae reared on the KCl-rich or NaCl-rich diets and bathed in haemolymph collected from

larvae reared on the control diet (Figure 10A, 10B). The increase in flux was due to an increase in fluid secretion rate rather than increases in secreted fluid concentrations of K^+ (data not shown). K^+ flux also increased and Na^+ flux was unchanged in response to addition of thapsigargin to tubules isolated from larvae reared on the KCl-rich diet and bathed in haemolymph collected from larvae reared on the NaCl-rich diet (Figure 10A). However, there were no increases in the fluxes of either K^+ or Na^+ in response to addition of thapsigargin to tubules of larvae reared on NaCl-rich diets and bathed in haemolymph collected from larvae reared on the KCl-rich or NaCl-rich diets (Figure 10B). These data indicate that the tubules retain responsiveness to thapsigargin when bathed in haemolymph isolated from larvae reared on control diets, but that there was no increase in K^+ or Na^+ flux when tubules from larvae reared on either the KCl-rich or NaCl-rich diets were bathed in haemolymph collected from larvae reared on KCl-rich diet. The results suggest that a factor acting through increases in intracellular Ca^{2+} was present in the haemolymph of larvae reared on the KCl-rich diet.

Discussion

The results provide new insights into the extent and patterns of haemolymph ionoregulation during dietary salt stress, the phenotypic plasticity of ion transport by the Malpighian tubules, and the role of haemolymph-borne factors in controlling ion transport.

Haemolymph ionoregulation in response to dietary salt loading

These results indicate that adult and larval *Drosophila* show considerable capacity for regulation of haemolymph Na^+ and K^+ levels even when the flies are fed diets containing high levels (0.4 mol l^{-1} above that in the control diet) of Na^+ or K^+. When adults were reared on the KCl-rich diet the potassium concentration in the haemolymph was maintained at the control level throughout the study period (up to 7 days). In the larvae, haemolymph K^+ increased above the control level during the first 24 hours, then returned to the control level by 48 hours. The increase in haemolymph K^+ concentration to 75 mmol l^{-1} at 6 hours was of interest because this level would presumably tend to depolarize nerves and muscles. In particular, previous studies have shown that exposure of neurohaemal areas to solutions of elevated K concentration (above 40 mmol l^{-1}) causes a maximal release of diuretic hormone in the hemipteran *Rhodnius prolixus* and the dipteran *Glossina austeni* (Maddrell and Gee, 1974). In *Glossina*, addition of K-rich (40 mmol l^{-1}) solution which had been circulated over the abdominal tissues under liquid paraffin resulted in transient diuresis of isolated tubules in the Ramsay assay. The blood brain barrier (Treherne and Schofield, 1981) may provide protection for the central nervous system during transient periods of elevated haemolymph K^+.

Although there was a sustained increase in Na^+ concentration in the haemolymph of both adults and larvae on the Na-rich diet, the results are indicative, nonetheless, of a homeostatic

response. In spite of a 4-fold increase in dietary Na^+ concentration (from 145 mmol l^{-1} in the control diet to 545 mmol l^{-1} in the diet containing an additional 0.4 mol l^{-1} NaCl), haemolymph Na^+ concentration increased after 48 hours by only 80% in the larvae and 53% in the adults.

Changes in ion transport by Malpighian tubules of salt-stressed larvae

There is no change in the rates of secretion of fluid, Na^+ or K^+ in Malpighian tubules isolated from larvae reared on the NaCl-rich diet. Although there is a sustained increase in haemolymph Na^+ concentration in these larvae, the maximum concentration is still well below that in the diet, suggesting that other epithelia contribute to haemolymph Na^+ regulation, perhaps through reduced absorption across the midgut or enhanced excretion across the hindgut.

By contrast, there are several dramatic changes in transport by the Malpighian tubules of larvae reared on the KCl-rich diet. Firstly, there is a sustained increase in fluid secretion rate after larvae are reared on the KCl-rich diet for 24 hours or longer. Secondly, there is a paradoxical decrease in secreted fluid K^+ concentration and a corresponding increase in Na^+ concentration when the tubules are bathed in saline containing 20 mmol l^{-1} K^+. The homeostatic benefits of these changes are seen when the tubules are bathed in saline containing 60 mmol l^{-1} K^+, mimicking the increase in haemolymph K^+ concentration during the first 24 hours on the KCl-rich diet. The dramatic increase in fluid secretion rate of tubules isolated after 6 hours or more on the KCl-rich diet is correlated with an increase in secretion of both Na^+ (256%) and K^+ (56%), relative to tubules isolated prior to transfer to the KCl-rich diet and bathed in 60 mmol l^{-1} saline. These changes indicate that there is a sustained increase in secretion of fluid, Na^+ and K^+ in tubules from larvae reared on the KCl-rich diets. Since diuretic factors present in the haemolymph are washed off during the 40 minutes required for dissection and in the large

volume (20 µl) of the bathing saline droplets, these changes appear to indicate a sustained increase in the capacity of the tubules to eliminate K^+. It is important to note that the increase in fluid secretion rate of KCl-exposed tubules in response to saline containing 60 mmol l^{-1} K^+ is due to an increase in basal secretion rate, indicating phenotypic plasticity in the tubules isolated from larvae reared on the KCl-rich diet. Thus *in vivo* both high levels of salt in the haemolymph and exposure of tubules to the salt may be required to increase K^+ and Na^+ secretion rate.

The role of diuretic/clearance factors in the response to salt loading.

Diuresis has been well characterized as a response to fluid loading in blood feeding insects such as *Rhodnius* (Orchard & Paluzzi, 2009) and mosquitoes (Beyenbach, 2003). There are also situations where increases in fluid secretion can be induced *in vitro* in tubules of desert insects such as Tenebrionid beetles by application of extracts of the corpora cardiaca. However, simultaneous injection of the extracts and the dye amaranth into the haemocole shows that the most of the dye transported by the Malpighian tubules of desert beetles moves anteriorly into the midgut, indicating fluid recycling by this route. The most likely function for a diuretic factor in this species, then, is clearance of metabolic wastes from the haemolymph (Nicholson, 1991). As such, the factors which stimulate fluid secretion by the Malpighian tubules *in vitro* may function as 'clearance hormones' *in vivo* (Nicholson, 1991). In this context, the increase in fluid secretion rate by Malpighian tubules isolated from *Drosophila* larvae in response to haemolymph collected from larvae on KCl-rich diets may function primarily to enhance elimination of K^+ in response to dietary salt loading. Water balance may be maintained either by enhanced absorption from the semi-liquid diet in the midgut or through absorption in the hindgut. For larvae reared on the NaCl-rich diet, there is enhanced secretion of Na^+ and also K^+ when bathed in haemolymph

collected from larvae reared on the NaCl-rich diet. In this case, K^+ balance may be maintained either through enhanced absorption by the midgut or absorption of K^+ by the hindgut.

There was no evidence for the presence of a natriuretic factor in larval haemolymph as a means of enhancing secretion of Na^+ by the Malpighian tubules. The mosquito natriuretic peptide (MNP) is a member of the CRF-related family of peptides and its actions are mediated through the effects of cAMP (Beyenbach, 2003). MNP selectively increases tubule secretion of NaCl and water, leading to the elimination of the NaCl load resulting from ingestion of the blood meal by the adult female mosquito. Tubules isolated from *Drosophila* larvae reared on the NaCl-rich or KCl-rich diets retained responsiveness to cAMP when bathed in haemolymph collected from larvae reared on the control, NaCl-rich or KCl-rich diets. This finding suggests that the actions of the diuretic factor present in the haemolymph of larvae reared on the NaCl-rich or KCl-rich diets is not mediated by cAMP.

By contrast, our experiments provide evidence that is consistent with an elevation of intracellular Ca^{2+} as part of the pathway leading to enhanced secretion of K^+ and fluid by tubules bathed in haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diets. Addition of thapsigargin, which elevates intracellular Ca^{2+} through SERCA inhibition, did not increase secretion of Na^+ or K^+ by tubules from larvae reared on the NaCl-rich diets and bathed in haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diets. Thapsigargin did increase K^+ secretion by tubules of larvae reared on the KCl-rich diets when the tubules were bathed in haemolymph collected from larvae reared on the NaCl-rich diets, but not when bathed in haemolymph collected from larvae reared on the KCl-rich diets. Taken together, the results suggest that the diuretic factors responsible for the increase in fluid secretion and K^+ secretion in tubules isolated from larvae reared on the NaCl-rich or KCl-rich diets may act through increases

in intracellular Ca^{2+}. The effects of both the kinins (O'Donnell et al., 1998; Terhzaz et al., 1999) and tyramine (Blumenthal, 2003) are mediated through increases in intracellular Ca^{2+} which lead to an increase transepithelial chloride permeability. It would therefore be of interest to determine if kinins or tyramine are present in the haemolymph of *Drosophila* larvae reared on the NaCl-rich or KCl-rich diets.

Estimates of haemolymph clearance rates for Na^+ and K^+

The rate of transport of K^+ by the Malpighian tubules is ~ 500 pmol min^{-1} (125 pmol min^{-1} $tubule^{-1}$ x 4 tubules). The haemolymph K^+ content is approximately 18 nmol (25 mmol l^{-1} x 0.71 µl). If the tubules in larvae reared on KCl-rich diets transport K^+ *in vivo* at rates similar to those of the isolated tubules used in this study, then the entire haemolymph content of K^+ could be transported by the 4 tubules in ~ 36 min. The rate of Na^+ transport by the 4 tubules of larvae reared on NaCl-rich diets is ~ 40 pmol min-1 (10 pmol min^{-1} $tubule^{-1}$ x 4 tubules) and the haemolymph Na^+ content is ~ 53 nmol (75 mmol l^{-1} x 0.71 µl). For Na^+ then, the clearance time is ~1300 minutes. These calculations suggest that whereas the Malpighian tubules may play an important role in regulating haemolymph K^+ concentration, other epithelia are likely to be involved in minimizing the increase in haemolymph Na^+ when larvae are reared on NaCl-rich diets. Subsequent studies using the scanning ion-selective electrode technique will examine how absorption and excretion of Na^+ and K^+ by the gut are influenced by the diet on which the larvae are reared.

Figure captions:

Figure 1. Larval weight and haemolymph volume. (A) Weight of larvae reared on the control, KCl-rich (0.4 mol l^{-1}) or NaCl-rich (0.4 mol l^{-1}) diets. (B) Haemolymph volume in the larvae reared on control, KCl-rich or NaCl-rich diets. All data in this and subsequent figures are shown as means ± S.E.M; The asterisks denote significant differences ($P < 0.05$) relative to control values.; *N= 14*

Figure 2. K^+ or Na^+ concentrations in haemolymph of larvae or adults reared on the control diet or after acute (6, 12, 24, 48 h) or chronic (7 d) exposure to KCl-rich (0.4 mol l^{-1}), NaCl-rich (0.4 mol l^{-1}) or sucrose-rich (0.8 mol l^{-1}) diet. (A) K^+ concentration in haemolymph of adults on the KCl-rich diet. (B) K^+ concentration in haemolymph of larvae on the KCl-rich diet. (C) Na^+ concentration in haemolymph of adults on the NaCl-rich diet. (D) Na^+ concentration in haemolymph of larvae on the NaCl-rich diet. (E) K^+ concentration in haemolymph of larvae on the sucrose-rich diet. (F) Na^+ concentration in haemolymph of larvae on the sucrose-rich diet. The asterisks denote significant differences ($P < 0.05$) relative to control values.; *N= 19-22*

Figure 3. The effects of acute (6, 12, 24, 48) or chronic exposure of larvae to the NaCl-rich diet on: (A) Malpighian tubule fluid secretion rate (B) the concentration of K^+ and Na^+ in the secreted fluid and (C) transepithelial flux of K^+ and Na^+ for tubules bathed in control saline. In the chronic exposure, eggs were laid on the NaCl-rich diet and 3rd instar larvae were dissected ~ 7 days later. The asterisks denote significant differences ($P < 0.05$) relative to control values.; *N=10*

Figure 4. The effects of acute or chronic exposure of the larvae to the KCl-rich diet on: (A) Malpighian tubule fluid secretion rate (B) the concentration of K^+ and Na^+ in the secreted fluid and (C) transepithelial flux of K^+ and Na^+ for tubules bathed in control saline. The asterisks denote significant differences ($P < 0.05$) relative to control values.; *N=10*

Figure 5. The effects of acute or chronic exposure of the larvae to the KCl-rich diet on: (A) Malpighian tubule fluid secretion rate (B) the concentration of K^+ and Na^+ in the secreted fluid and (C) transepithelial flux of K^+ and Na^+ for tubules bathed in control saline or 60 mmol l^{-1} K^+ saline. The asterisks denote significant differences ($P < 0.05$) relative to tubules isolated before transfer of the larvae from control to KCl-rich diet (0 hours); *N=10*

Figure 6. The effects of haemolymph source on tubules isolated from larvae reared on the control diet. Tubules were bathed in haemolymph collected from 3rd instar larvae reared on the control diet or after chronic exposure to the NaCl-rich (chronic Na) or KCl-rich (chronic K) diet. (A) Malpighian tubule fluid secretion rate (B) concentration of K^+ or Na^+ in the secreted fluid , (C) transepithelial flux of K^+ or Na^+. The asterisks denote significant differences ($P < 0.05$) relative to control values; *N=15.*

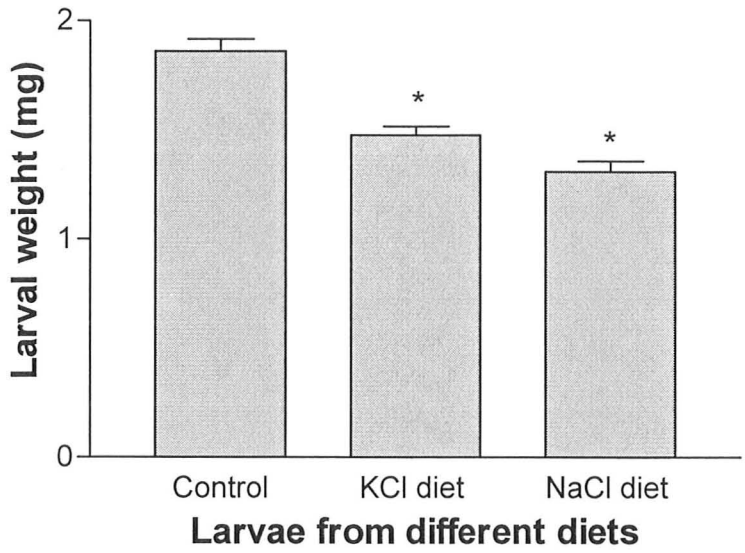
Figure 7. The effects of haemolymph source on tubules isolated from larvae reared on the NaCl-rich diet. Tubules were bathed in haemolymph collected from 3rd instar larvae reared on the control diet or after chronic exposure to the NaCl-rich (chronic Na) or KCl-rich (chronic K) diet. (A) Malpighian tubule fluid secretion rate (B) concentration of K^+ or Na^+ in the secreted fluid , (C) transepithelial flux of K^+ or Na^+. The asterisks denote significant differences ($P < 0.05$) relative to control values; *N=14*

Figure 8. The effects of haemolymph source on tubules isolated from larvae reared on the KCl-rich diet. Tubules were bathed in haemolymph collected from 3rd instar larvae reared on the control diet or after chronic exposure to the NaCl-rich (chronic Na) or KCl-rich (chronic K) diet. (A) Malpighian tubule fluid secretion rate (B) concentration of K^+ or Na^+ in the secreted fluid , (C) transepithelial flux of K^+ or Na^+. The asterisks denote significant differences ($P < 0.05$) relative to control values; *N=15*

Figure 9. The effects of cAMP and haemolymph source on transepithelial flux of K^+ and Na^+ in tubules isolated from larvae reared on (A) the KCl-rich diet or (B) the NaCl-rich diet and bathed in haemolymph collected from larvae reared on the control, KCl-rich (chronic K) or NaCl-rich (chronic Na) diets. Fluxes were determined before (basal) or after stimulation with 0.1 mmol l^{-1} cAMP. The asterisks denote significant differences ($P < 0.05$) relative to control values.; *N=6*

Figure 10. The effects of thapsigargin and haemolymph source on transepithelial flux of K^+ and Na^+ in tubules isolated from larvae reared on (A) the KCl-rich diet or (B) the NaCl-rich diet and bathed in haemolymph collected from larvae reared on the control, KCl-rich (chronic K) or NaCl-rich (chronic Na) diets. Fluxes were determined before (basal) or after stimulation with 10 μmol l^{-1} thapsigargin. The asterisks denote significant differences ($P < 0.05$) relative to control values.; *N=6*

A)


2
1
0
Larval weight (mg)
*
*
Control
KCl diet
NaCl diet
Larvae from different diets

B)

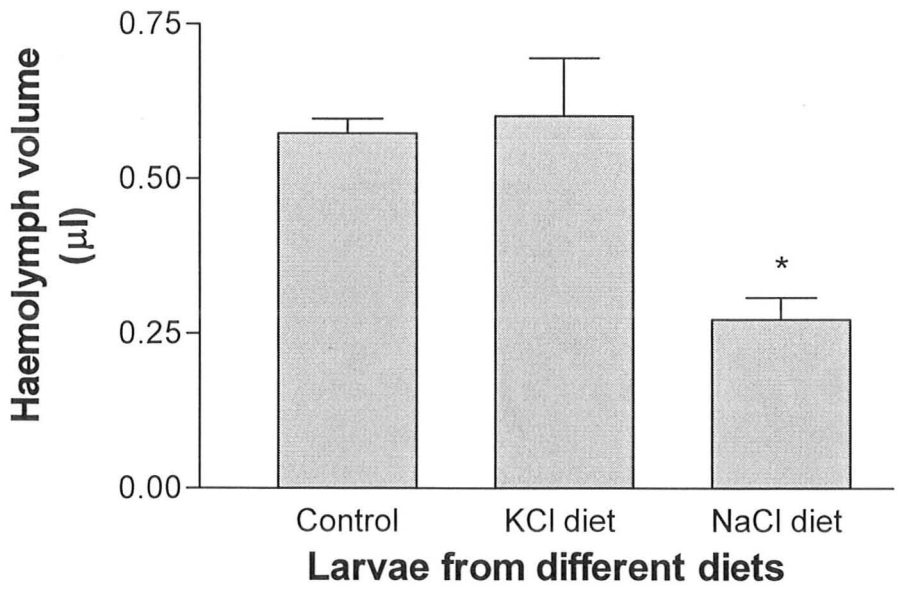
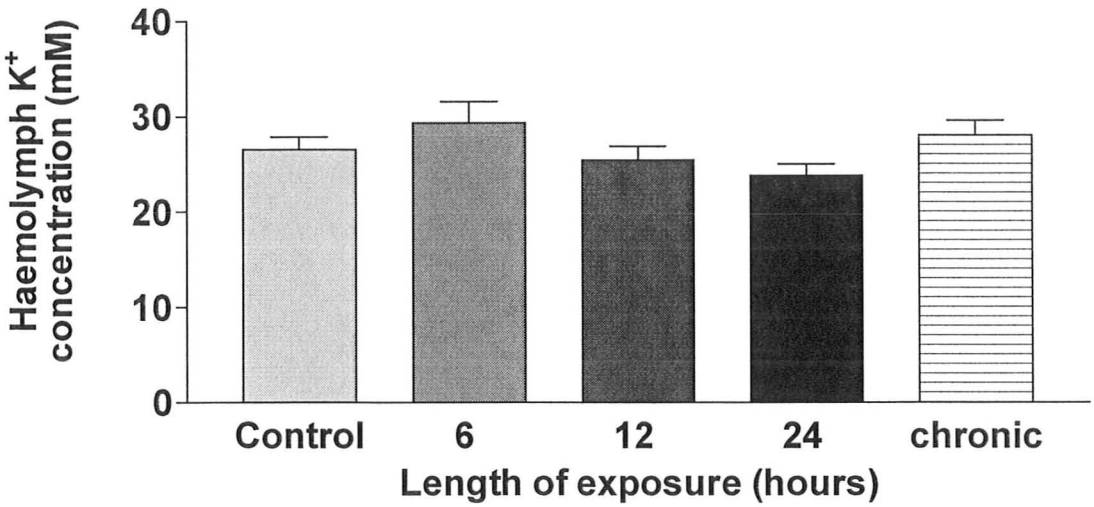
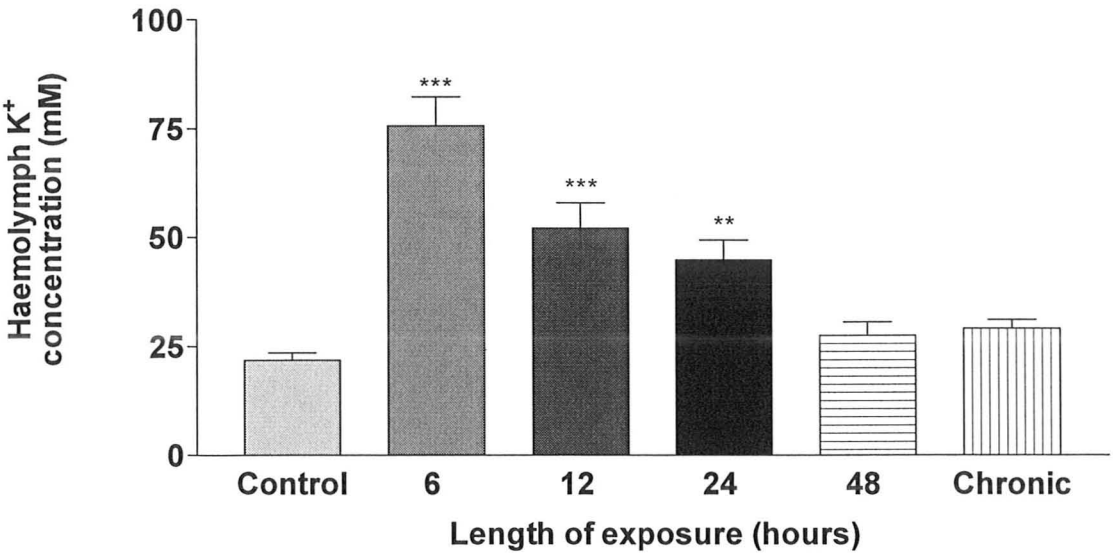

0.75
0.50
0.25
0.00
Haemolymph volume (µl)
*
Control
KCl diet
NaCl diet
Larvae from different diets

Figure 1

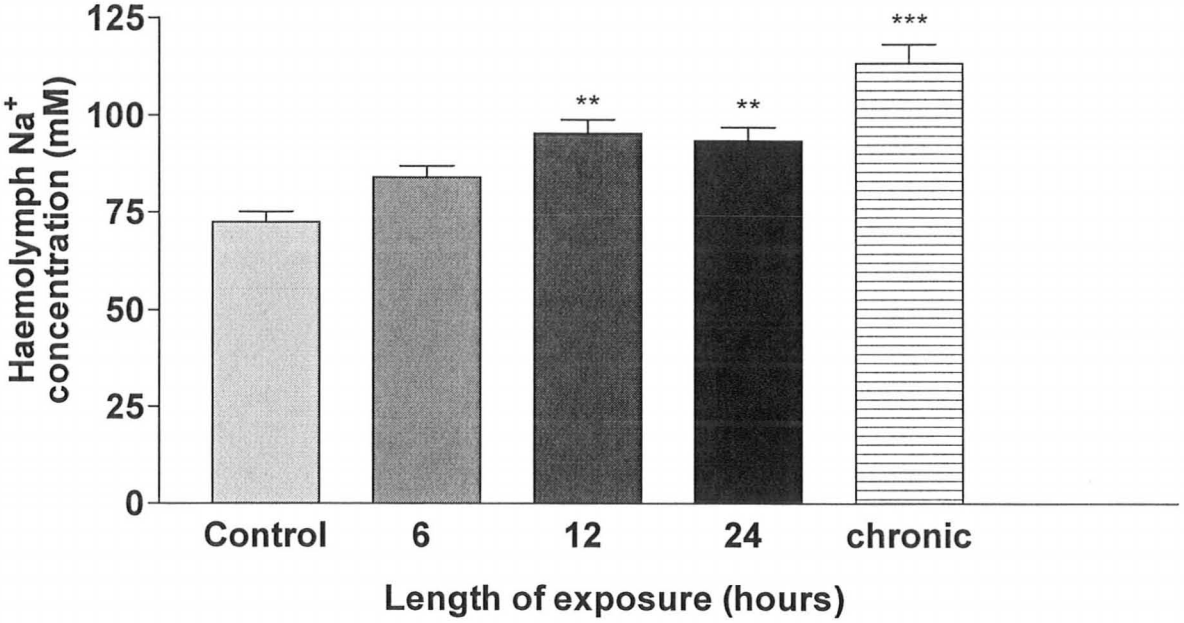
A)


Haemolymph K+ concentration (mM)
40
30
20
10
0
Control
6
12
24
chronic
Length of exposure (hours)

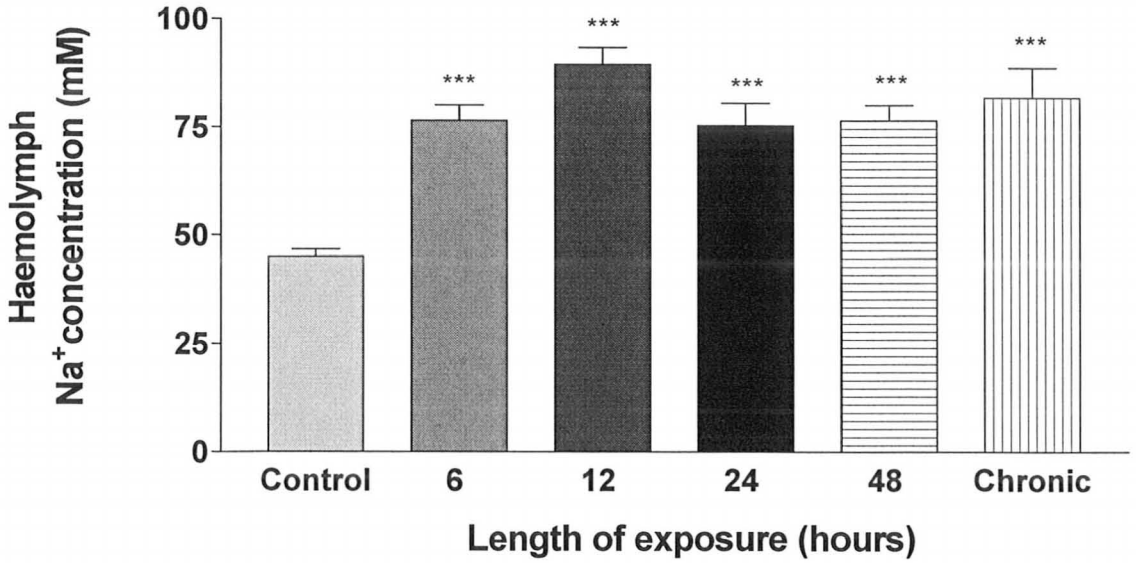
B)


Haemolymph K+ concentration (mM)
100
75
50
25
0
***
***
**
Control
6
12
24
48
Chronic
Length of exposure (hours)

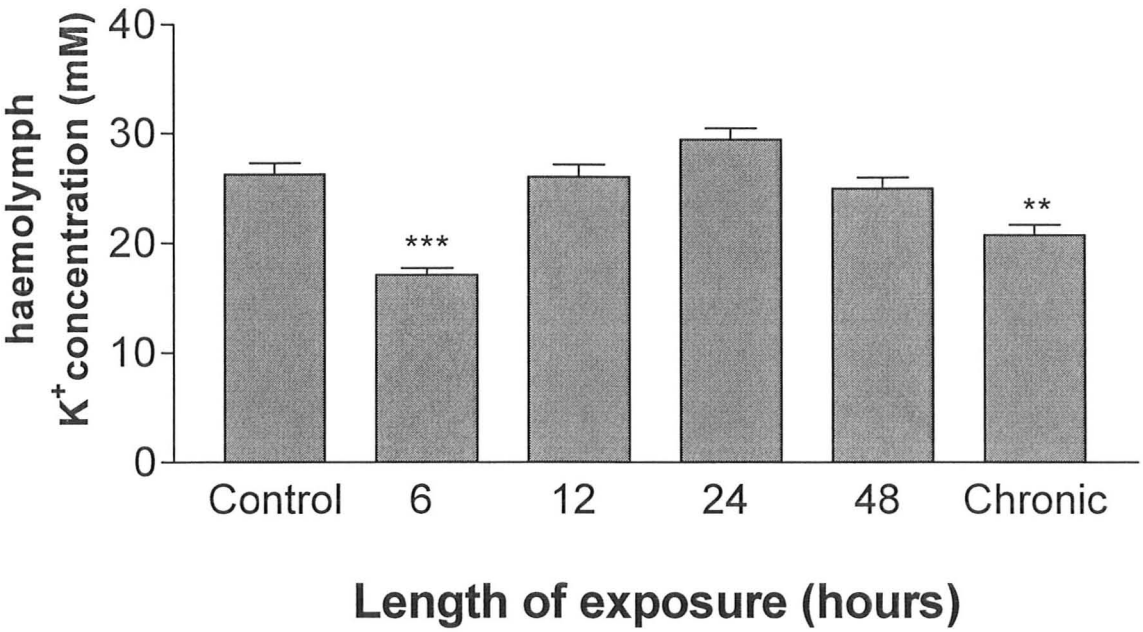
C)


125
100
75
50
25
0
Haemolymph Na+ concentration (mM)
**
**
***
Control
6
12
24
chronic
Length of exposure (hours)

D)


100
75
50
25
0
Haemolymph Na+concentration (mM)
***
***
***
***
***
Control
6
12
24
48
Chronic
Length of exposure (hours)

E)


haemolymph
K+ concentration (mM)
40
30
20
10
0
***
**
Control
6
12
24
48
Chronic
Length of exposure (hours)

F)

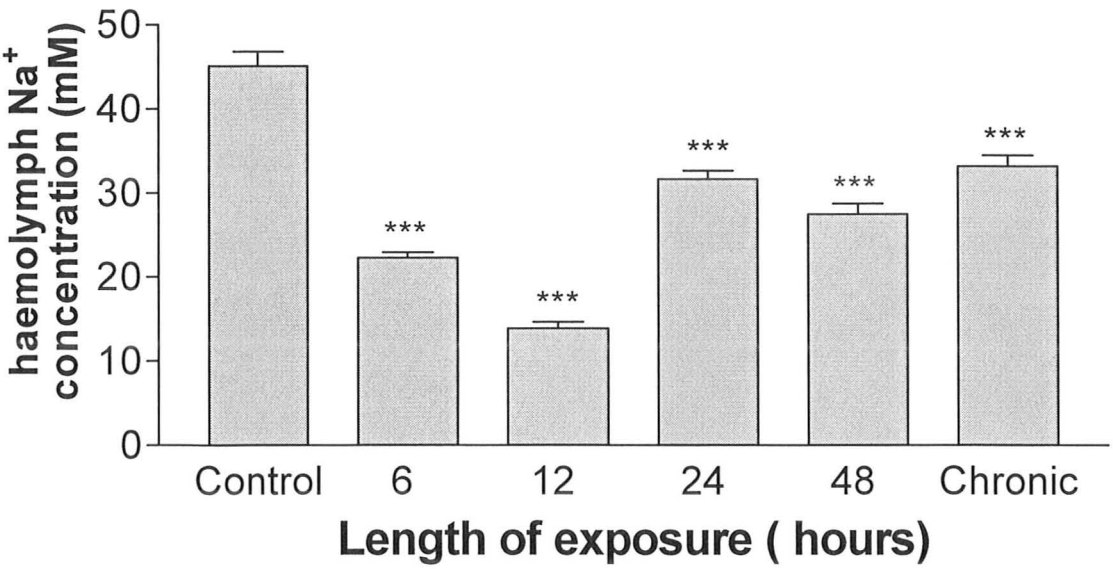
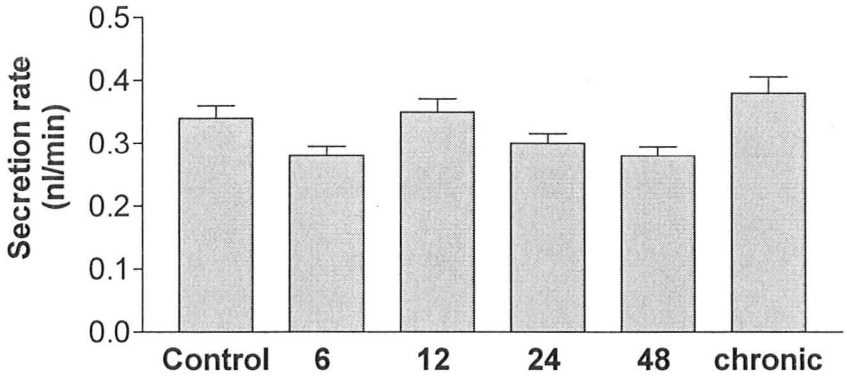
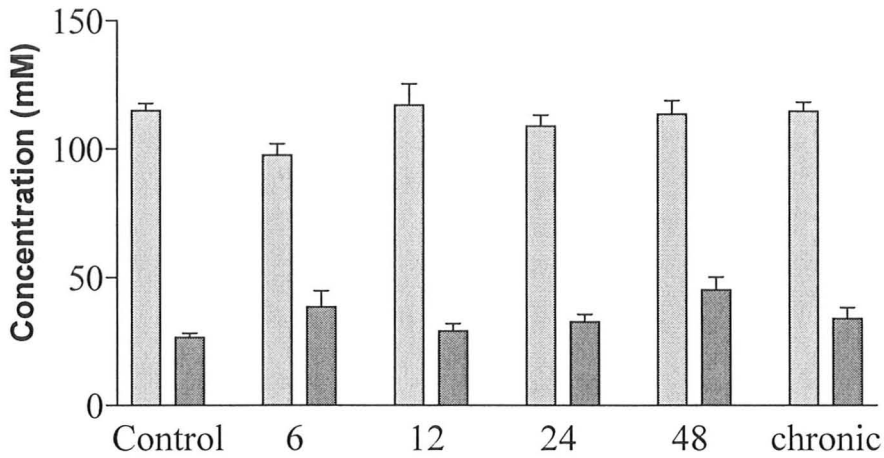

haemolymph Na+
concentration (mM)
50
40
30
20
10
0
***
***
***
***
***
Control
6
12
24
48
Chronic
Length of exposure (hours)

Figure 2

A)


Secretion rate (nl/min)
0.5
0.4
0.3
0.2
0.1
0.0
Control
6
12
24
48
chronic

B)


Concentration (mM)
150
100
50
0
Control
6
12
24
48
chronic

C)

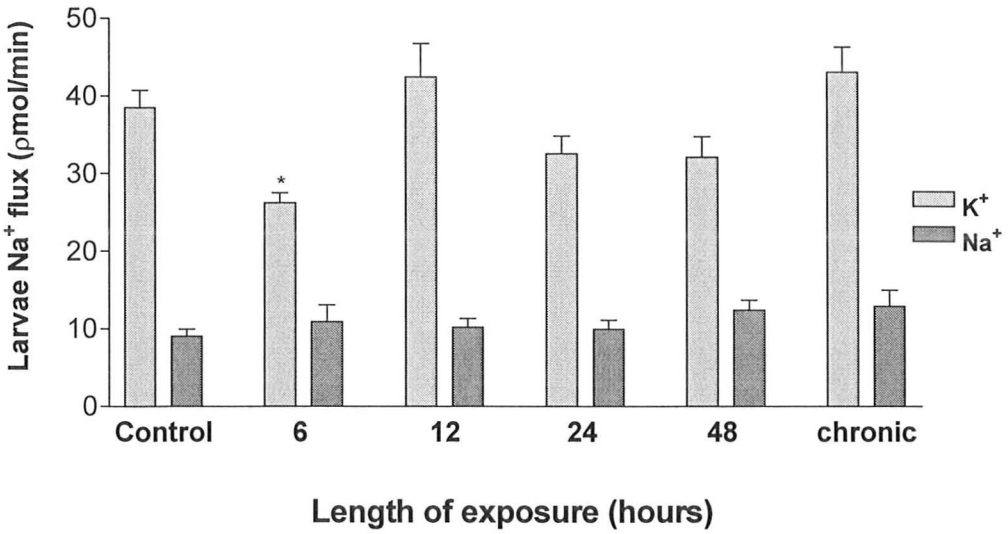
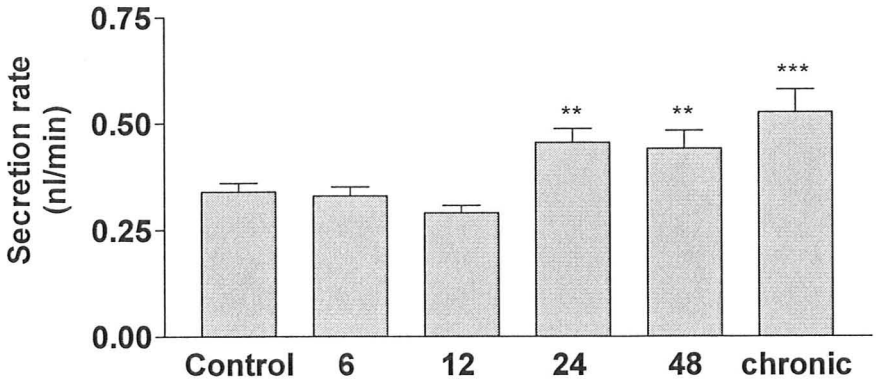
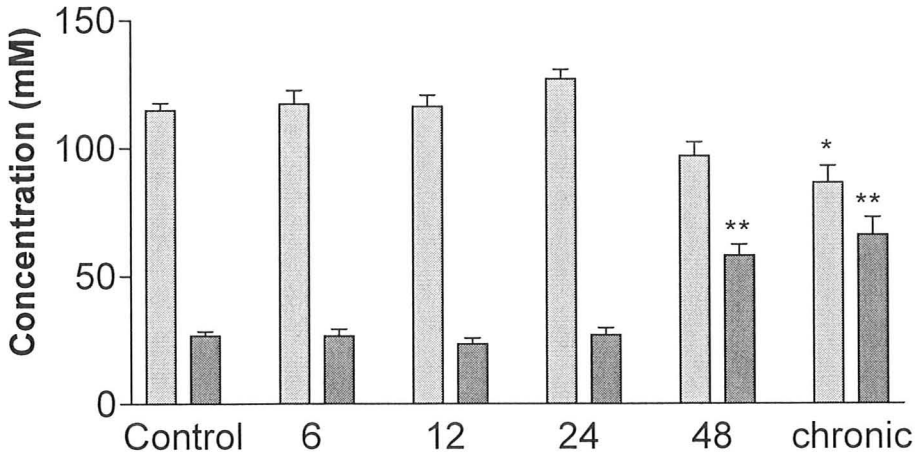

Larvae Na+ flux (ρmol/min)
50
40
30
20
10
0
*
Control
6
12
24
48
chronic
K+
Na+
Length of exposure (hours)

Figure 3

A)


Secretion rate (nl/min)
0.75
0.50
0.25
0.00
**
**
***
Control
6
12
24
48
chronic

B)


Concentration (mM)
150
100
50
0
**
*
**
Control
6
12
24
48
chronic

C)

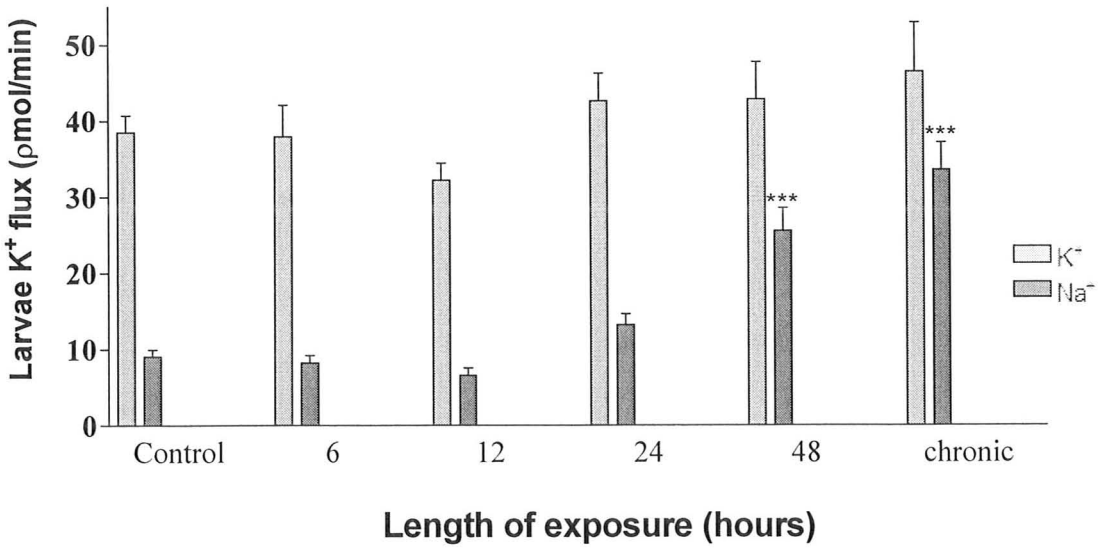
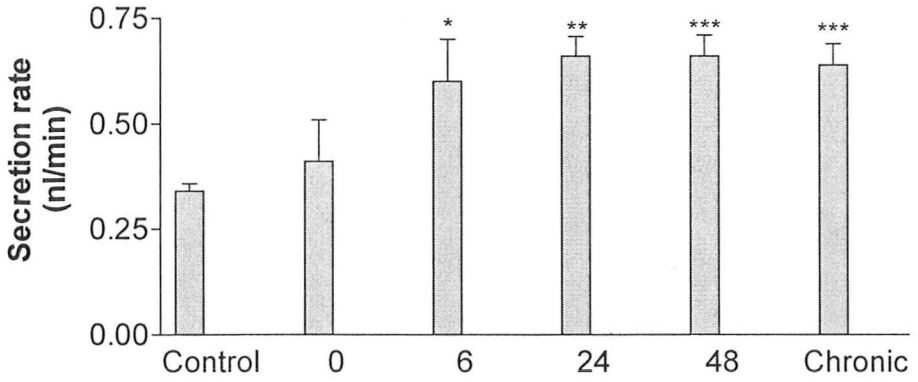
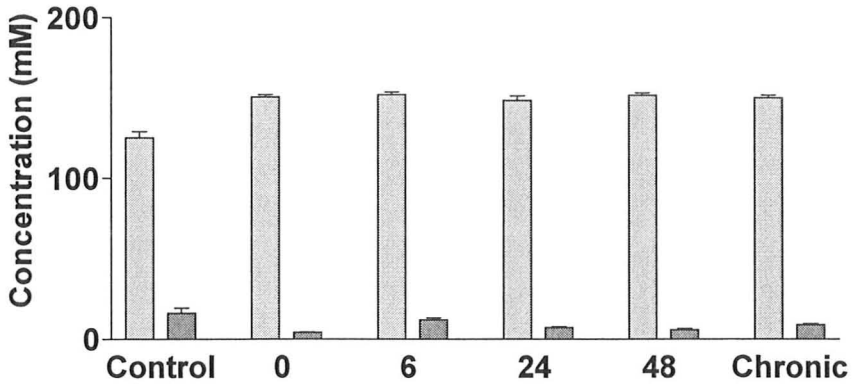

Larvae K+ flux (ρmol/min)
50
40
30
20
10
0
***
***
K+
Na+
Control
6
12
24
48
chronic
Length of exposure (hours)

Figure 4

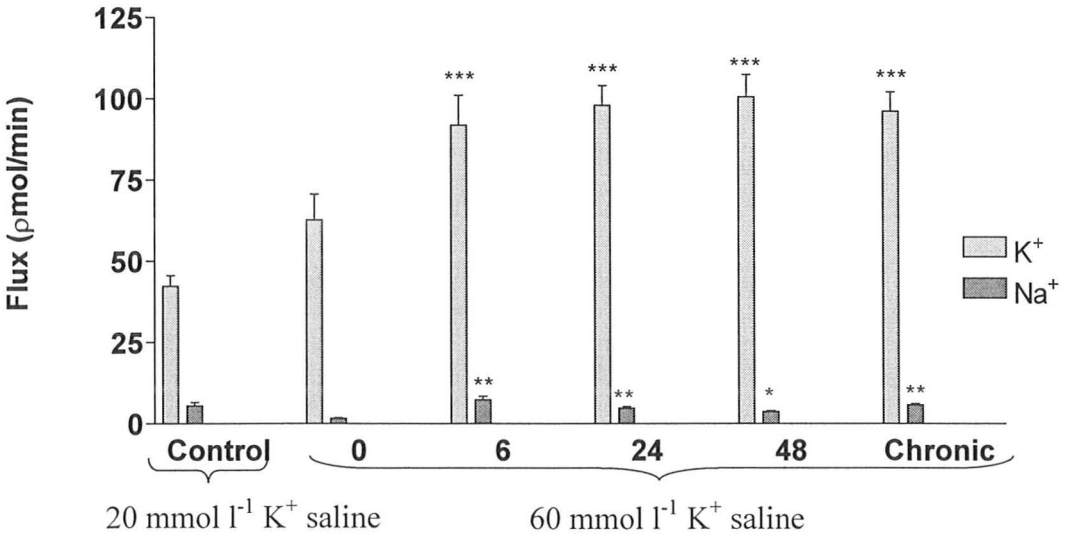
A)



B)



C)



Length of exposure

Figure 5

Tubules isolated from larvae reared on the control diet

A)

B)

C)

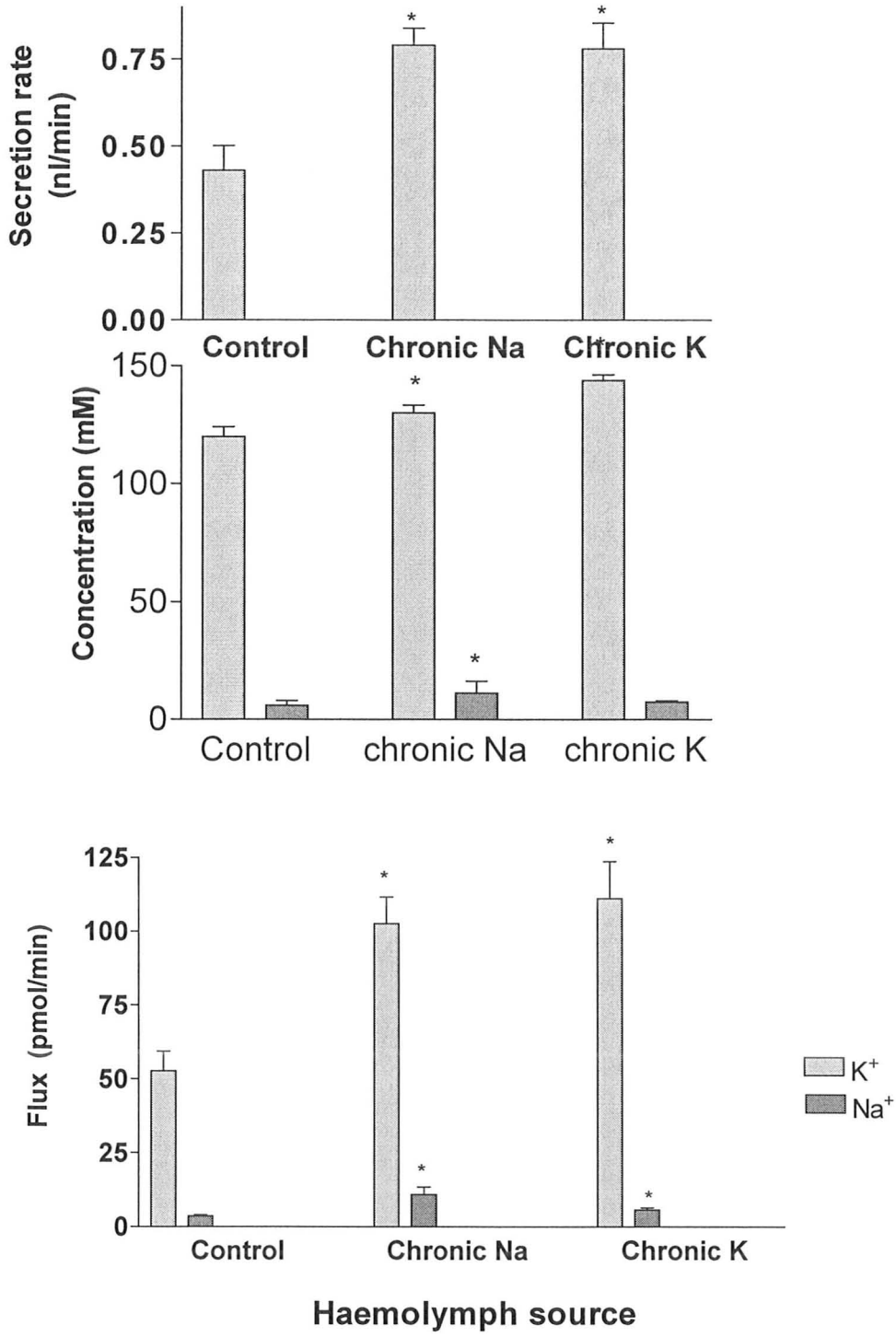


Figure 6

Tubules isolated from larvae reared on the NaCl-rich diet

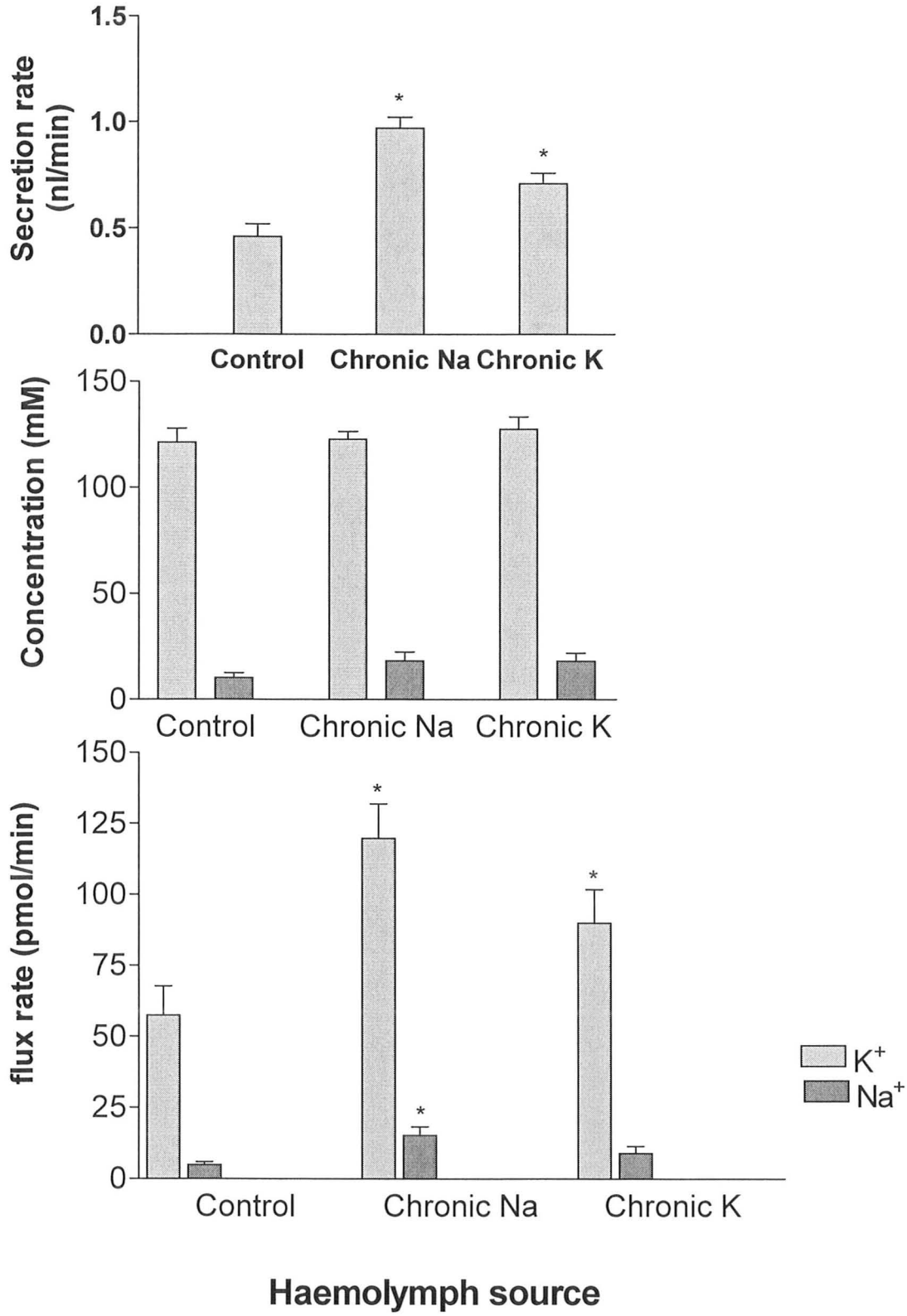
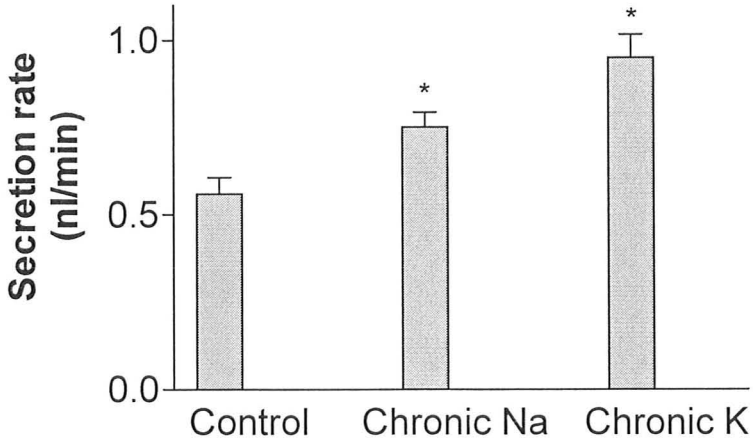


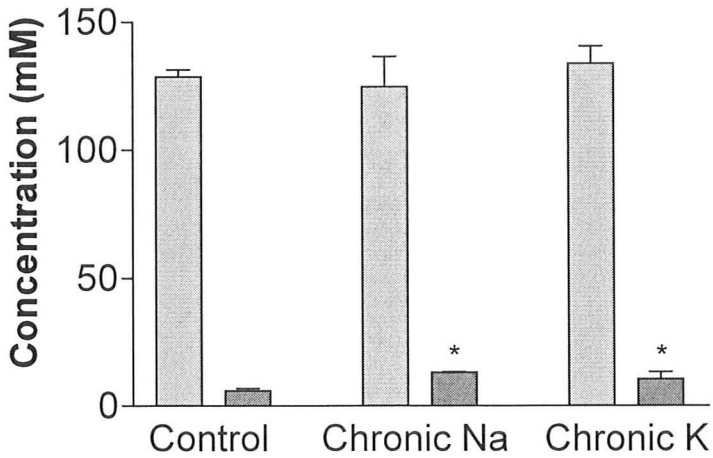
Figure 7

Tubules isolated from larvae reared on the KCl-rich diet

A)



B)



C)

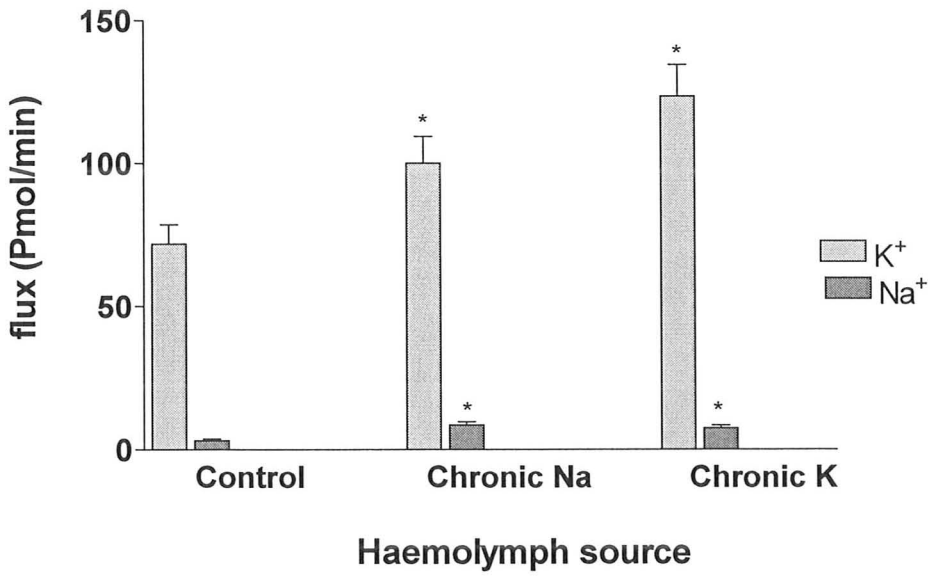
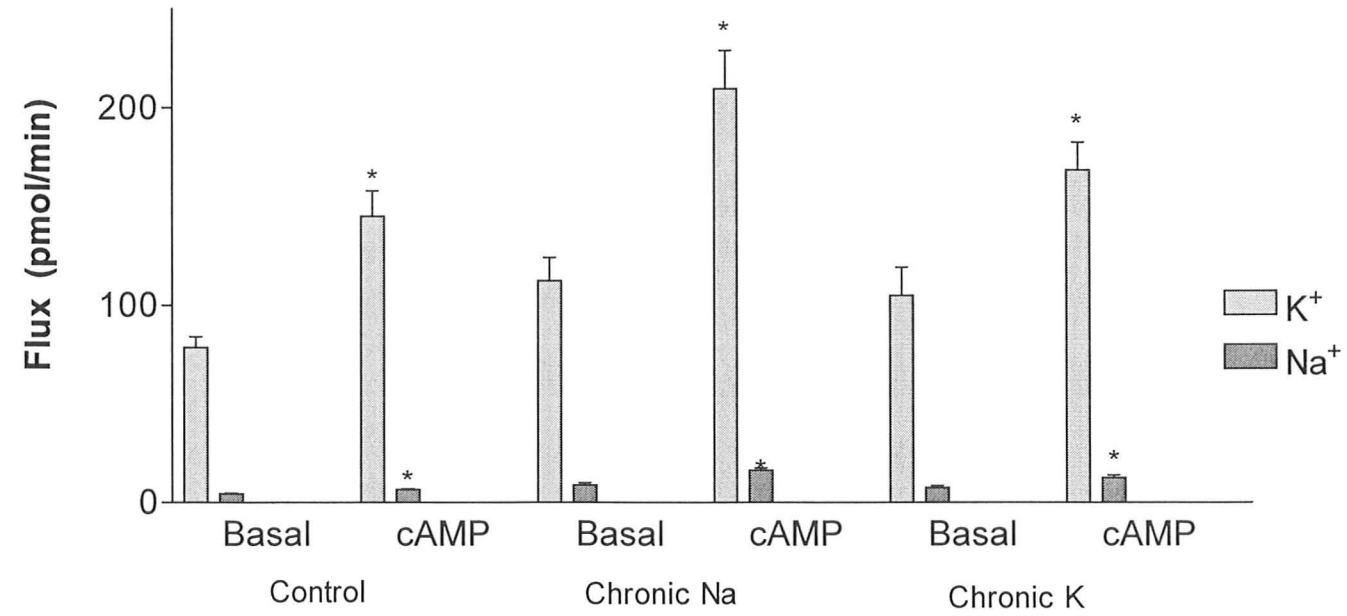


Figure 8

A)


Flux (pmol/min)
200
100
0
Basal
cAMP
Basal
cAMP
Basal
cAMP
Control
Chronic Na
Chronic K
K+
Na+

B)

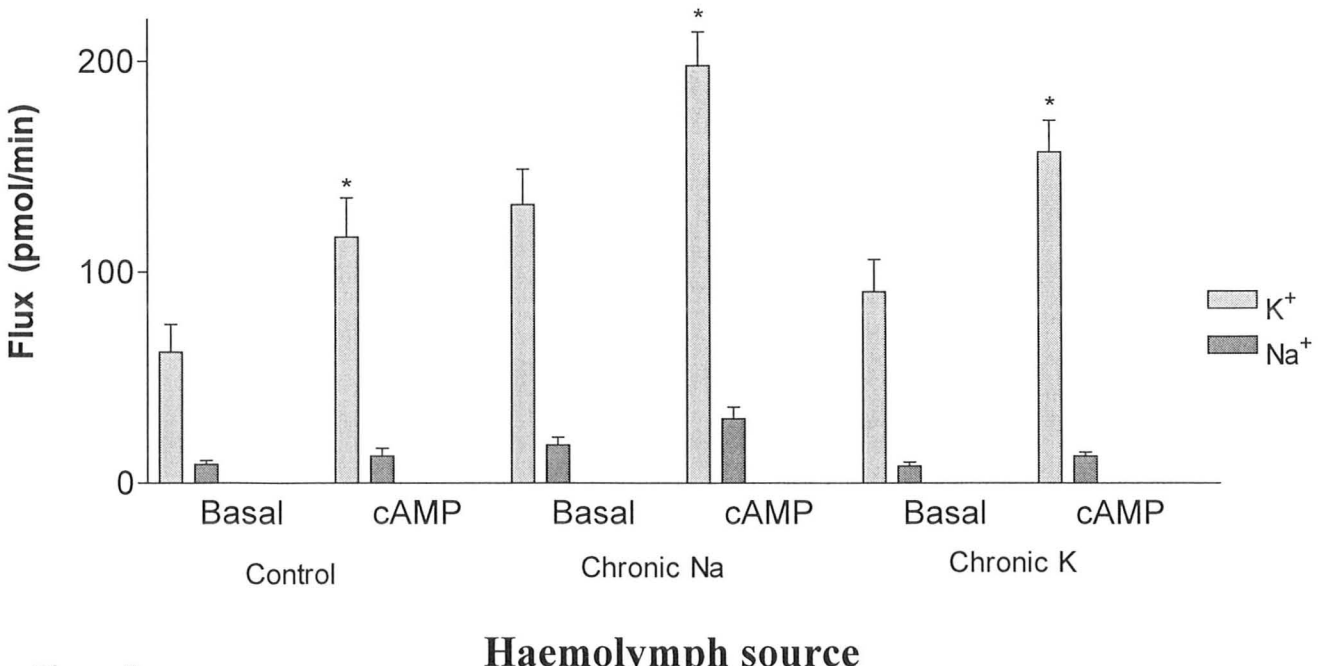
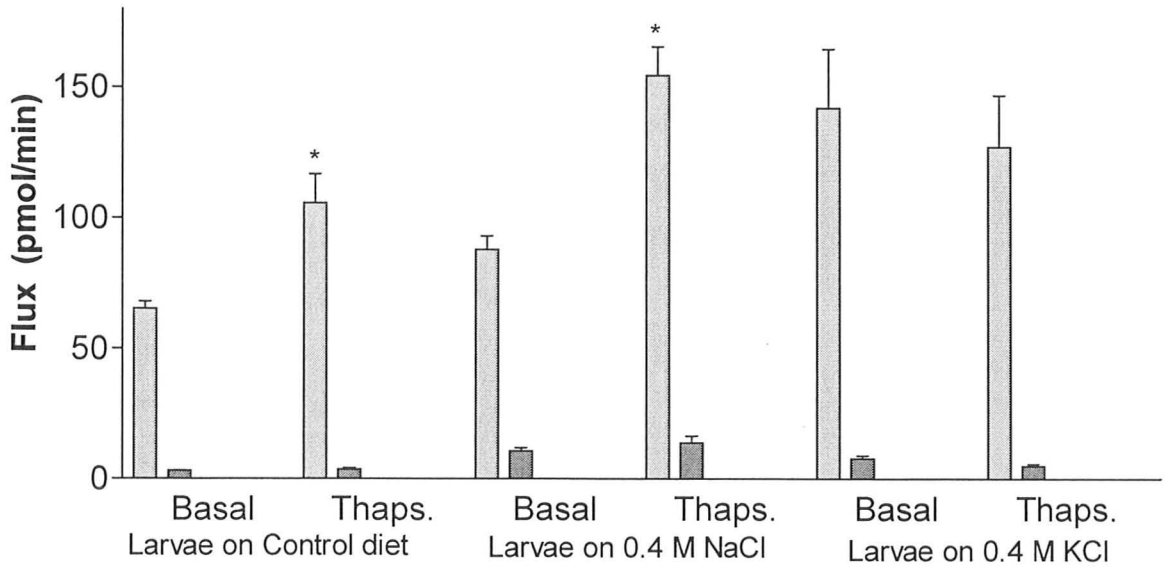

Flux (pmol/min)
200
100
0
Basal
cAMP
Basal
cAMP
Basal
cAMP
Control
Chronic Na
Chronic K
K+
Na+
Haemolymph source

Figure 9

A)


Flux (pmol/min)
150
100
50
0
*
*
Basal
Thaps.
Basal
Thaps.
Basal
Thaps.
Larvae on Control diet
Larvae on 0.4 M NaCl
Larvae on 0.4 M KCl

B)

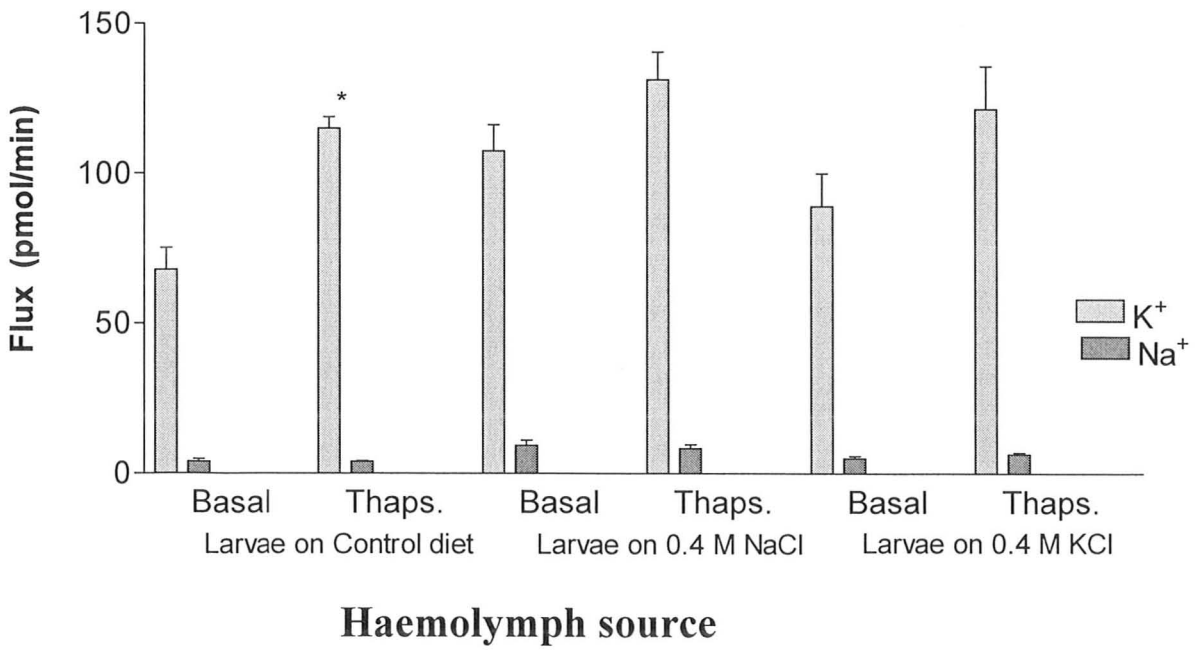

Flux (pmol/min)
150
100
50
0
*
K+
Na+
Basal
Thaps.
Basal
Thaps.
Basal
Thaps.
Larvae on Control diet
Larvae on 0.4 M NaCl
Larvae on 0.4 M KCl
Haemolymph source

Figure 10

Chapter 3

Effects of salt-rich diets on ion transport by the gut of *Drosophila melanogaster* larvae.

W. Naikkhwah and M. J. O'Donnell

Abstract

This study assessed whether alterations in Na^+ and K^+ transport by the gut of larval *Drosophila melanogaster* reared on salt-rich diets contribute to haemolymph ionoregulation. Na^+ and K^+ fluxes across the isolated gut of 3rd instar larvae reared on control or salt-rich diets were measured using the scanning ion-selective microelectrode technique (SIET). K^+ absorption across the anterior portion of the middle midgut of larvae reared on diet in which the concentration of KCl was increased 0.4 mol l^{-1} above that in the control diet was reduced 8-fold, relative to the same gut segment of larvae reared on the control diet. There was also an increase in the magnitude and extent of K^+ secretion across the posterior half of the middle midgut. Na^+ was absorbed across the hindgut of larvae reared on the control diet, but secreted across the hindgut of larvae reared on diet in which the concentration of NaCl was increased 0.4 mol l^{-1} above that in the control diet. There was also a small reduction in the extent of Na^+ absorption across the middle midgut of larvae reared on the NaCl-rich diet. The results indicate considerable phenotypic plasticity with respect to K^+ and Na^+ transport by the gut epithelia of larval *Drosophila*. SIET measurements of K^+ and Na^+ fluxes along the length of the gut suggest that the ion transport mechanisms of the gut are reconfigured during salt stress so that there are reductions in K^+ and Na^+ absorption and increases in K^+ and Na^+ secretion. Together with previously described changes in salt secretion by the Malpighian tubules, these changes contribute to haemolymph ionregulation.

Introduction

In many insects, the gut and/or the Malpighian (renal) tubules play important homeostatic roles in response to salt stress associated with dehydration or dietary loading. Larvae of mosquitoes inhabiting hypersaline lakes, for example, excrete excess salt through the rectum (Bradley and Phillips, 1977). In addition, the tubules of salt-tolerant species may increase the transport of Na^+ at the expense of K^+ when reared in salt-rich waters (Donini et al., 2006). In tenebrionid beetles living in deserts or dry stored products, the distal ends of the Malpighian tubules are applied to the surface of the rectum and enveloped in a layer of perinephric cells which limit water flow from the haemolymph to the tubule lumen. Production of elevated salt concentrations within the tubule lumen creates elevated osmotic pressures responsible for absorption of water from the rectal lumen (O'Donnell and Machin, 1991). *Drosophila* are also capable of precise haemolymph osmoregulation in response to addition of 300 mmol l^{-1} sucrose or NaCl to the diet (Pierce et al., 1999). Molecular genetic studies of the fruit fly *Drosophila* implicate both the Malpighian tubules and the gut in salt tolerance. Two proteins encoded encoded by the inebriated (*ine*) gene have been implicated in osmolyte transport within the Malpighian tubule and hindgut. Mutants lacking both *ine* gene isoforms are hypersensitive to osmotic stress (Huang et al., 2002). Most genes which are upregulated or downregulated by salt stress are highly enriched in the Malpighian tubules and/or the hindgut, suggesting that these tissues are likely to play major roles in the response to salt stress (Stergiopoulos et al., 2008). Expression of the GLUT4/8-like sugar transporter gene CG6484 is elevated 2700-fold in the midgut, relative to whole flies, whereas expression of the putative sodium/halide symporter gene salty dog (CG 2196) is 7000-fold higher in the Malpighian tubules than in whole flies. The

Drosophila gene *NFAT* may also play an important role in the response to salt stress, perhaps through NFAT regulation of target genes, as yet unknown, that are involved in the accumulation of organic osmolytes (Keyser et al., 2007).

Our previous study (Naikkhwah and O'Donnell, 2010) examined changes in haemolymph Na^+ and K^+ levels as well as Malpighian tubule fluid and ion transport in salt-stressed flies. Haemolymph Na^+ concentration increases from 72 to 114 mmol l^{-1} in adult flies chronically exposed to a diet containing an additional 0.4 mol l^{-1} NaCl above the level in the control diet. In larvae, the corresponding increase in haemolymph Na^+ concentration is from 45 to 80 mmol l^{-1}. Regulation of haemolymph K^+ is even more precise. There is no significant change in K^+ concentration in the haemolymph collected from adults at 6 , 12 , 24 hours or 7 days after transfer from control diet to diet containing an additional 0.4 mol l^{-1} KCl. In the larvae, haemolymph K^+ concentration increases from 22 to 75 mmol l^{-1} 6 hours after the transfer to KCl-rich diet, but the control level is restored by 48 h. Although increases in Na^+ and K^+ secretion by tubules from larvae reared on salt-rich diets contribute to elimination of excess salt under some conditions, a role for the gut is also implied by the results (Naikkhwah and O'Donnell, 2010).

Epithelia of the gut may contribute to ionic homeostasis either through absorption of less Na^+ or K^+ from diets enriched in these ions, or through transport of ions from haemolymph into the gut lumen and subsequent elimination. In *Drosophila* selected over multiple generations for survival on diets containing high concentrations of urea, for example, the mechanism of adaptation appears to involve reduction of uptake rather than increases in excretion (Etienne et al., 2001). The foregut and cuticle are lined with chitin which may be relatively impermeable to urea and it is suggested that the midgut and hindgut are possible sites for urea uptake since they

are the sites of nutrient absorption and urine modification, respectively (Etienne et al., 2001). In this study we have used the scanning ion-selective electrode technique to measure transport of Na^+ and K^+ across the foregut, midgut and hindgut (anterior half of ileum) of flies reared on either a standard *Drosophila* diet on diets enriched in Na^+ or K^+.

Materials and Methods

Insects and diet preparation

The Oregon R strain of *D. melanogaster* were raised on standard artificial diet and maintained at 21° – 23°C in laboratory culture. The control diet was prepared as described by Roberts and Stander (1998). Solution A consisted of 800 ml tap water, 100 g sucrose, 18 g agar, 8 g KNa tartrate, 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgCl_2$, and 0.5 g $CaCl_2$. Solution B consisted of 200 ml tap water and 50 g dry active yeast. The two solutions were autoclaved, combined and stirred. After cooling to 55°C, 10 ml of an acid mix (11 parts tap water, 10 parts propionic acid, and 1 part 85% o-phosphoric acid) and 7.45 ml of 10% phydroxybenzoic acid methyl ester (Tegosept) dissolved in ethanol were added to the mixture. Experimental diets were prepared by addition of 0.4 mol l^{-1} NaCl, 0.4 mol l^{-1} KCl or 0.8 mol l^{-1} sucrose to the standard diet.

Ion selective Microelectrodes (ISMEs) and Scanning Ion Electrode Technique (SIET)

Electrodes were pulled on a P-97 Flaming-Brown pipette puller (Sutter Instruments Co., Novato, CA) from 1.5 mm borosilicate glass (World Precision Instruments Inc., Sarasota, FL). This gave an electrode with a short shank and a tip opening of approximately 3–5 µm. The electrodes were then silanized with N,N-dimethyltrimethylsilylamine at 200°C for 30 min, and were stored at room temperature in a dessicator. K^+-selective microelectrodes were made by first backfilling them with 150 mmol l^{-1} KCl using a plastic 1 ml syringe pulled out over a low flame

to a fine tip (Thomas, 1978) and then tip-filled with a column (~ 500 μm) of K^+ ionophore I, cocktail B (Fluka, Buchs, Switzerland). Na^+-selective microelectrodes were made by first backfilling them with 150 mmol l^{-1} NaCl and then tip filling with a Na^+ ionophore cocktail which consisted of 10% Na^+ ionophore X, 89.75% nitrophenyl octyl ether and 0.25% sodium tetraphenylborate (Messerli et al., 2008). The K^+ ionophore cocktail is $10^{3.9}$ times, $10^{4.9}$ times and $10^{4.9}$ times more selective for K^+ relative to Na^+, Ca^{2+}, and Mg^{2+}, respectively (Amman et al., 1987). The Na^+ ionophore cocktail is $10^{2.6}$ times, $10^{3.5}$ times and $10^{3.7}$ times more selective for K^+ relative to Na^+, Ca^{2+}, and Mg^{2+}, respectively (Messerli et al., 2008). Microelectrodes were calibrated in solutions bracketing the concentration range of interest for each ion. K^+-selective and Na^+-selective microelectrodes were calibrated in solutions of 150 mmol l^{-1} KCl and 15 mmol l^{-1} KCl/135 mmol l^{-1} NaCl, and 150mmol l^{-1} NaCl and 15mmol l^{-1} NaCl/135 mmol l^{-1} KCl respectively. Slopes (mV) for a tenfold change in ion concentration were [mean ± s.e.m. (*N*)] 52.1± 2 (6) for K^+-selective microelectrodes and 57 ± 2 (6) for Na^+-selective microelectrodes. Reference electrodes were constructed from 10 cm borosilicate glass capillaries that were bent from the end at 45° angle, to facilitate placement in the sample dish. Capillaries were filled with boiling *Drosophila* saline solution containing 3–5% agar and were stored at 4 °C in *Drosophila* saline solution.

SIET measurements were made with hardware from Applicable Electronics (Forestdale, MA) and Automated Scanning Electrode Technique (ASET) software (version 2.0) from Science Wares, (Falmouth, MA). The ISMEs were connected to the amplifier headstage, which was connected to three computer controlled stepper motors that moved the probe in the X, Y, and Z axes with submicron accuracy and repeatability. The microelectrode was moved perpendicular to the tissue surface between two positions separated by 50 μm at each measurement site. The inner

position was within 5 μm of the tissue surface. First, the microelectrode was moved at 200 μm s^{-1} to one extreme of the 50 μm excursion. The microelectrode then remained stationary during the 3.5 s wait period to allow ion gradients near the tissue to re-establish after the localized stirring during the movement period. No data were collected during the wait period. Lastly, the microelectrode voltage was recorded for 0.5 s during the sampling period. The microelectrode was then moved 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 4.25 s. Voltage measurement at both extremes of microelectrode excursion thus required a total of ~8.5 s. Preliminary experiments showed that a 'wait' time of 3 s was more than sufficient to record the full voltage gradient. Increasing the 'wait' time beyond 3.5 s did not lead to larger gradients when the microelectrode was positioned in a concentration gradient. Voltage measurements taken at the limits of the excursion were amplified 1000-fold and used to calculate a voltage difference over the excursion distance of the microelectrode. This differential signal was then converted into a K^+ or Na^+ activity difference using a standard microelectrode calibration curve that related voltage output to K^+ or Na^+ activity in *Drosophila* saline. Three replicate measurements were made at each site. Within each segment of the gut, measurements were made at sites separated by ~ 30 μm. Regions of the gut that were examined in detail are shown in Figure 1.

Calculation of ion fluxes

Voltage gradients obtained from the ASET software were converted into concentration gradients using the following equation:

$$\Delta C = C_B \times 10^{(\Delta V/S)} - C_B$$

where ΔC is the concentration gradient between the two points measured in $\mu mol\ l^{-1}\ cm^{-3}$; C_B is the background ion concentration, calculated as the average of the concentration at each point measured in $\mu mol\ l^{-1}$; ΔV is the voltage gradient obtained from ASET in μV; and *S* is the slope of the electrode. Although ion-selective microelectrodes measure ion activity and not concentration, data can be expressed in terms of concentrations if it is assumed that the ion activity coefficient is the same in calibration and experimental solutions. This is particularly true for the calibration solutions and salines used in the present study because they are of similar ionic strength. Expression of data in terms of concentrations simplifies comparisons with previous studies in which ion concentrations were used (e.g. Stobbart, 1965).

The concentration gradient was subsequently converted into flux using Fick's first law of diffusion in the following equation:

$$J_I = D_I(\Delta C)/\Delta x$$

where J_I is the net flux of the ion in $pmol\ cm^{-2}\ s^{-1}$; D_I is the diffusion coefficient of the ion ($1.55 \times 10^{-5}\ cm^2\ s^{-1}$ for Na^+, $1.92 \times 10^{-5}\ cm^2\ s^{-1}$ for K^+; Lide, 2002); ΔC is the concentration gradient in $pmol\ cm^{-3}$; and Δx is the distance between the two points measured in cm.

Net fluxes of K^+ and Na^+ across gut segments of larvae reared on control or salt-rich diets.

The net K^+ or Na^+ flux across each region of the gut was calculated by multiplying the flux measured by SIET (pmol cm^{-2} s^{-1}) by the surface area of the tissue (cm^2). In essence, this considers the gut to be a series of contiguous cylinders, and the area of each cylinder is calculated as the product of the circumference times the length of each cylinder. The circumference is calculated as π times the cylinder diameter, whereas where the length of each cylinder is equivalent to the distance between the sites of ion-selective microelectrode placement during the SIET scans.

Graphing and statistics

Data were plotted using GraphPad Instat (GraphPad Software, San Diego, California). Values are expressed as means ± S.E.M. for the indicated number of preparations (*N*). Two-sample *F*-tests were used to compare the variances of the data for the control and experimental groups. Depending on the outcome of each *F*-test, differences between experimental and control groups were compared using unpaired Student's *t*-tests assuming either equal or unequal variances. The responses of the same group of tubules before and after an experimental treatment were compared using a paired *t*-test. Differences were considered significant if $P<0.05$. Data in which concentration of ions varied or saline ionic composition varied were analyzed by one-way ANOVA with Tukey's post-hoc multiple comparison ($p<0.05$).

Results

Preliminary scans of along the entire length of isolated guts were performed in order to identify the primary areas of K^+ and Na^+ absorption and/or secretion. The areas with the largest fluxes of K^+ and Na^+ (N= 3 and N = 2, respectively) were (in order of anterior to posterior position) the gastric caeca, the acid zone of the anterior midgut, the anterior middle midgut, the ureters of the Malpighian tubules and the hindgut (Figure 1). Fluxes in other regions were much smaller. K^+ fluxes in the midregion and posterior region of the middle midgut, for example, were 11% and 1%, respectively, of the flux in the anterior middle midgut. Na^+ fluxes in the midregion and posterior region of the middle midgut were 33% and 10%, respectively, of the flux in the anterior middle midgut. K^+ and Na^+ fluxes across the posterior half of the ileum were 17% and 42%, respectively, of the fluxes across the anterior half of the ileum. An example of a K^+ flux measurements across the middle midgut is shown in figure 2 and figures 3 to 7 summarize the fluxes in the regions of the gut with the largest fluxes.

Caeca

K^+ was secreted from haemolymph to lumen across much of the length of the ceaca of guts isolated from larvae reared on the control diet, except for a small region of absorption within ~ 100 μm of the tip (Figure 3A). This pattern was maintained in caeca of guts isolated from larvae reared on the KCl-rich diet, although there was a reduction in magnitude of both the secretory and absorptive fluxes, relative to the controls (Fig. 3B). Na^+ was absorbed from haemolymph to the lumen across the entire length of the caeca of guts isolated from larvae reared on the control diet (Figure 3C) but there was a 2-fold to 3-fold reduction in the magnitude of the flux across caeca isolated from larvae reared on the NaCl-rich diet (Figure 3D).

Anterior midgut (acid-zone)

In control larvae, K^+ was absorbed at rates of ~ 300 pmol cm^{-2} s^{-1} across the first 200 μm of the anterior midgut, and at rates of ~ 80 pmol cm^{-2} s^{-1} across the remainder (Fig. 4A). By contrast, K^+ fluxes were negligible across the anterior midguts of larvae reared on the KCl-rich diet (Fig. 4B). Na^+ fluxes were similar across the anterior midguts of larvae reared on both the control and the NaCl-rich diets (Fig. 4C,D). Na^+ was absorbed at low rates (~100 pmol cm^{-2} s^{-1}) across the anterior 700 - 800 μm of the anterior midgut, then secreted over the remaining 200 μm.

Middle midgut

K^+ was strongly absorbed across the anterior region of the middle midgut of larvae reared on the control diet, with the peak in absorption exceeding 4000 pmol cm^{-2} s^{-1} at a point approximately 450 μm from the start of the middle midgut (Figure 5A). K^+ was secreted across the remaining 300 μm of the middle midgut of the controls. There was a dramatic reduction in K^+ absorption across the midguts of larvae reared on the KCl-rich diet as well as an increase in the magnitude of K^+ secretion and the extent of the middle midgut across which K^+ was secreted (Figure 5B).

In contrast to the pattern of K^+ transport, Na^+ was secreted across the anterior region of the middle midgut and absorbed across the more posterior regions of the middle midgut (Fig. 5C.D). Relative to the middle midguts of larvae reared on the control diets (Fig. 5C); Na^+ transport across both secretory and absorptive regions was reduced in middle midguts of larvae reared on the NaCl-rich diet (Fig. 5D).

Ureter

Our previous study examined Na^+ and K^+ transport across the Malpighian tubules using the Ramsay assay (Naikkhwah & O'Donnell, 2010). The application of SIET allows measurement of Na^+ and K^+ transport by the ureter which connects each pair of tubules to the gut. K^+ was absorbed from lumen to haemolymph across much of the length of the ureter of guts isolated from larvae reared on the control diet, except for a small region of secretion within ~ 100 μm of the Malpighian tubules (Figure 6A). The pattern and magnitude of K^+ transport were similar in ureters of guts isolated from larvae reared on the KCl-rich diet (Fig. 6B). Na^+ was absorbed at similar rates from haemolymph to lumen across the entire length of the ureters isolated from larvae reared on the control or NaCl-rich diets (Figure 6C, D).

Hindgut (anterior half of the ileum)

K^+ was secreted across the anterior half of the ileum of larvae reared on the control or KCl-rich diets (Fig. 7A,B). Although K^+ secretion increased at most sites along the anterior ileum of larvae reared on the KCl-rich diet, there was a high degree of variability due to a single preparation in which fluxes exceeded 5000 pmol cm^{-2} s^{-1} at several points.

Na^+ was absorbed at rates of 100 – 200 pmol cm^{-2} s^{-1} across the anterior ileums of larvae reared on the control diet (Fig. 7C) but was secreted across most of the length of anterior ileum of larvae reared on the NaCl-rich diet (Fig. 7D).

Net fluxes of K^+ and Na^+ across gut segments of larvae reared on control or salt-rich diets.

Figure 8 shows the net flux across each of the gut segments described above. In addition, Figure 8 presents the rates of secretion of Na^+ and K^+ for isolated Malpighian tubules bathed in control saline. The rate of K^+ secretion for tubules isolated from larvae reared on the KCl-rich diet and bathed in control saline is 40 pmol min^{-1} tubule, equivalent to 2.6 pmol s^{-1} for all 4 tubules (Naikkhwah and O'Donnell). The rate of Na^+ secretion for tubules isolated from larvae reared on the NaCl-rich diets and bathed in control saline is 10 pmol min^{-1} tubule, equivalent to 0.67 pmol s^{-1} for all 4 tubules (*ibid)*. The significance of the patterns and magnitudes of K^+ and Na^+ transport are discussed below.

Discussion

The results indicate a considerable degree of phenotypic plasticity with respect to K^+ and Na^+ transport by the gut epithelia of larval *Drosophila*. Our measurements of K^+ and Na^+ fluxes along the length of the gut suggest that the ion transport mechanisms of the gut are reconfigured during salt stress so that there are reductions in K^+ absorption and increases in K^+ and Na^+ secretion. These changes contribute to haemolymph ion homeostasis.

Alterations of epithelial K^+ transport in response to dietary KCl loading

The most striking changes in K^+ flux in response to the KCl-rich diet are the dramatic decreases in K^+ absorption across the acid zone of the anterior midgut and the middle midgut. There is an 8-fold reduction in K^+ absorption across the anterior portion of the middle midgut of larvae reared on the KCl-rich diet, relative to the middle midgut of larvae reared on the control diet (Figure 8). There is also an increase in the magnitude and extent of K^+ secretion across the

posterior half of the middle midgut. As a result, K^+ is secreted across the middle midgut as a whole. Although there is a reduction in K^+ secretion across the caeca of larvae reared on KCl-rich diet, the small surface area of these structures limits the impact of this change on haemolymph K^+ regulation. As well, K^+ secretion across the hindgut is maintained or, in some larvae, dramatically increased.

In conjunction with our previous study (Naikkhwah and O'Donnell, 2010) the results presented here indicate that increases in dietary KCl evoke a coordinated set of homeostatic responses in multiple epithelia. Relative to controls, K^+ secretion by the Malpighian tubules is increased in larvae reared on the KCl-rich diet and these changes involve both an increase in the capacity to secrete K^+ when haemolymph K^+ levels are increased and diuretic factors which increase K^+-secretion more than 2-fold. At the same time, K^+ absorption by the anterior and middle midgut is reduced, so that there is less K^+ entering the haemolymph from the gut lumen. The latter change is noteworthy because of the large change in the chemical gradient tending to drive K^+ from the gut lumen into the haemolymph in larvae reared on the KCl-rich diet.

Alterations of epithelial Na^+ transport in response to dietary NaCl loading

Whereas the most dramatic alterations in K^+ transport in response to the KCl-rich diet are seen in the midgut, the most significant qualitative alteration of Na^+ transport involves the anterior half of the ileum. Whereas there is net absorption of Na^+ in the anterior ileums of larvae reared on the control diet, net secretion is seen in the anterior ileums of larvae reared on the NaCl-rich diet. Fluxes of Na^+ across the caeca and anterior midgut are similar in larvae reared on control or NaCl-rich diets, and there is a small reduction in the extent of Na^+ absorption across the middle midgut.

Our previous study (Naikkhwah & O'Donnell, 2010) showed that the Malpighian tubules are far more effective at eliminating K^+ than Na^+. K^+ is secreted by the tubules of larvae reared on the KCl-rich diet and bathed in saline at ~ 3 pmol s^{-1}. This rate is ~ 4 times the rate of Na^+ secretion by tubules of larvae reared on the NaCl-rich diet and bathed in saline (Figure 8). Secretion of Na^+ by the ileum thus represents a significant contribution towards limiting the rise in haemolymph Na^+ concentration in larvae reared on the NaCl-rich diet.

The possible contribution of the rectum to transport of K^+ or Na^+ was not determined because it was not possible to dissect the rectum free of the cuticle of the posterior region of the larva without damage. However, it is worth noting that the rectum is a much shorter segment in the larva relative to the adult. Moreover, the diameter of the larval rectum is much smaller than that of the ileum in the larvae (Fox and Spradling, 2009), whereas the adult rectum is of larger diameter than the ileum and contains 4 conspicuous cone-shaped rectal papillae that are associated with ion recycling and water absorption in other dipterans such as *Calliphora* (Gupta et al., 1980). These considerations suggest that the rectum is unlikely to play a dominant role in haemolymph ionoregulation in the larva.

Multiple epithelia contribute to ionoregulation

Figure 8 shows that the Na^+ fluxes are in approximate balance for larvae reared on either control or the NaCl-rich diet. Absorption across the caeca, ureter and the middle midgut of larvae on the NaCl-rich diet is of similar magnitude to the sum of the secretion of Na^+ by the Malpighian tubules and the hindgut. Similarly, for larvae reared on control diets, the absorption of K^+ across the ureter and middle midgut is counterbalanced by secretion of K^+ by the Malpighian tubules and hindgut.

It is also worth noting that the gut epithelia scanned in this study were bathed in control saline. Although our previous study measured ion secretion by tubules isolated in saline or in haemolymph from larvae reared on the control, KCl-rich or NaCl-rich diets, it has not been possible to perform SIET scans of isolated guts bathed in haemolymph because of the large volumes (>> 1 ml) of fluid required. Our previous study also examined the effects of secretagogues on fluid and ion secretion by isolated tubules bathed in haemolymph. These studies suggested that elevation of intracellular Ca^{2+} may be involved in stimulation of tubules in larvae reared on the KCl-rich diet. It would be of interest in future studies to examine the effects of neuropeptides (e.g. Drosokinin, CAPA; Dow and Davies, 2003) and treatments which elevate the concentrations of intracellular second messengers (e.g. thapsigargin, exogenous cAMP; Naikkhwah and O'Donnell, 2010) on K^+ and Na^+ transport by guts isolated from larvae reared on control versus salt-rich diets.

Figure captions:

Figure 1. Schematic diagram of the *Drosophila melanogaster* gut. The dashed brackets indicate the regions of each gut segment for which K^+ and Na^+ fluxes are presented in figures 3 – 7. The region of the anterior midgut scanned includes the acid zone. The insets show morphological features used to define the regions scanned.

Figure 2. SIET measurement of K^+ fluxes across the middle midgut of a larva raised on the KCl-rich diet. The direction of each arrow indicates movement of ions out of (absorption) or into (secretion) the gut, whereas the length of the arrow denotes the magnitude of the flux.

Figure 3. K^+ and Na^+ fluxes across the caeca of larval *D. melanogaster*. K^+ flux across caeca of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across caeca of larvae reared on (C) the control diet or (D) the NaCl-rich diet. The entire length of the caecum was scanned, with zero μm (origin) corresponding to the tip of the caecum. Values are means ± s.e.m.; *N=5-7*.

Figure 4. K^+ and Na^+ fluxes across the anterior midgut (acid zone) of larval *D. melanogaster*. K^+ flux across the acid zone of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across the acid zone of larvae reared on the (C) control diet or (D) the NaCl-rich diet. Approximately 1000 μm of the acid zone was scanned, with zero μm (origin) corresponding to the start of acid zone (after the textured region of the anterior midgut) as shown in figure 1. Values are means ± s.e.m.; *N=5-7*.

Figure 5. K^+ and Na^+ fluxes across the middle midgut of larval *D. melanogaster*. K^+ flux across middle midguts of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across middle midguts of larvae reared on (C) the control diet or (D) the NaCl-rich diet. Approximately 1000 μm of the anterior middle midgut was scanned, with zero μm (origin) corresponding to the end of the ring-like feature shown in figure 1. Values are means ± s.e.m.; *N=5-7*.

Figure 6. K^+ and Na^+ fluxes across the ureter of larval *D. melanogaster*. K^+ flux across ureters of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across ureters of larvae reared on (C) the control diet or (D) the NaCl-rich diet. The entire length of the ureter was scanned, with zero distance (origin) corresponding to the start of the ureter at the midgut and hindgut junction. Values are means ± s.e.m.; *N=5-7*.

Figure 7. K^+ and Na^+ fluxes across the anterior half of the ileum of larval *D. melanogaster*. K^+ flux across iliums of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across iliums of larvae reared on (C) the control diet or (D) the NaCl-rich diet. Approximately 1000 μm of the anterior ilium was scanned, with zero distance (origin) corresponding to the end of the pylorus (Fig. 1). Values are means ± s.e.m.; *N=5-7*.

Figure 8. Summary of K^+ and Na^+ transport across the gut and Malpighian tubules of larval *Drosophila*. The positive values represent movement of K^+ or Na^+ from lumen to the haemolymph (absorption) and the negative values represent movement of K^+ or Na^+ from haemolymph to the lumen (secretion). (A) K^+ transport across the gut of larvae reared on the control versus the KCl-rich diet. (B) Na^+ transport across the gut of larvae reared on the control versus the NaCl-rich diet. Fluxes for each segment in pmol s-1 have been calculated from SIET measurements (pmol cm^{-2} s^{-1}) and surface areas (cm^2) of each tissue segment, as described in methods. Values are means ± s.e.m.; *N=5-7*.

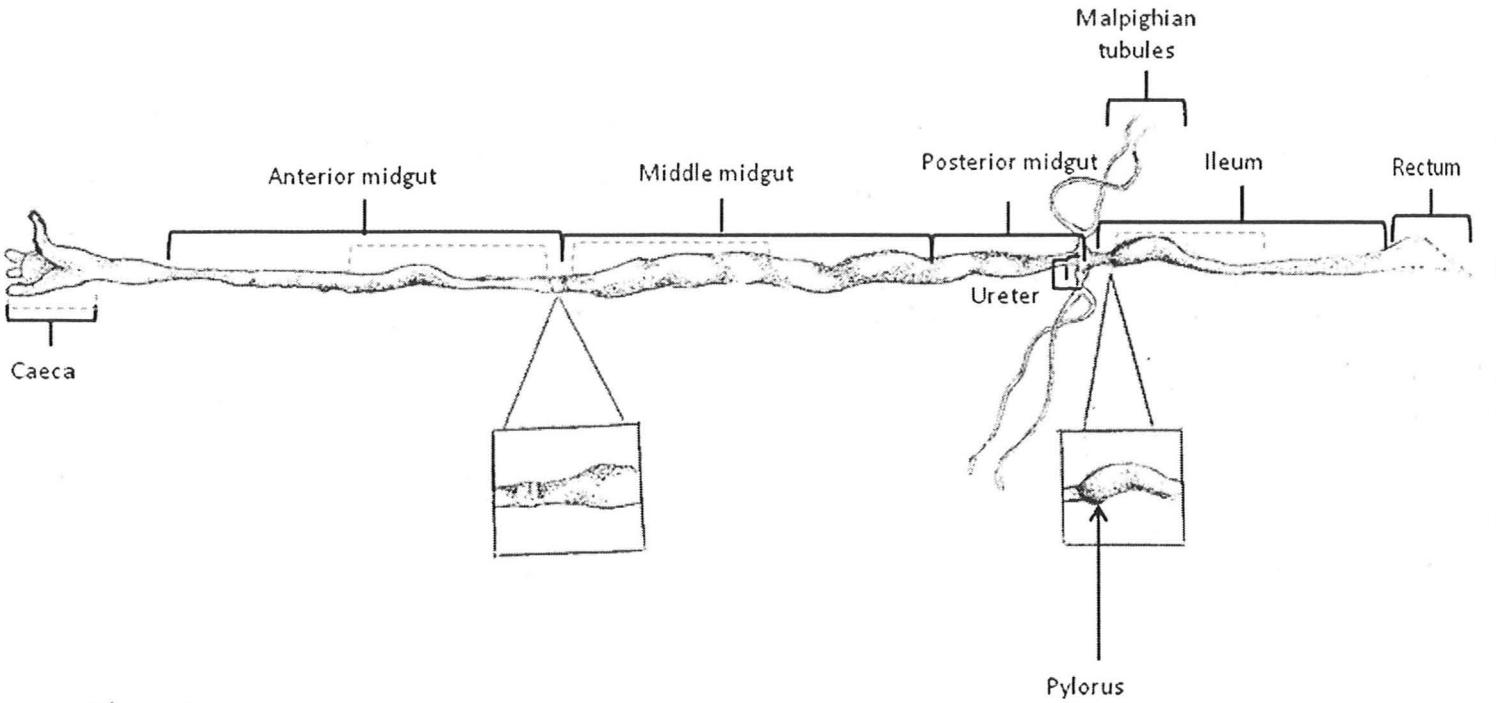


Figure 1

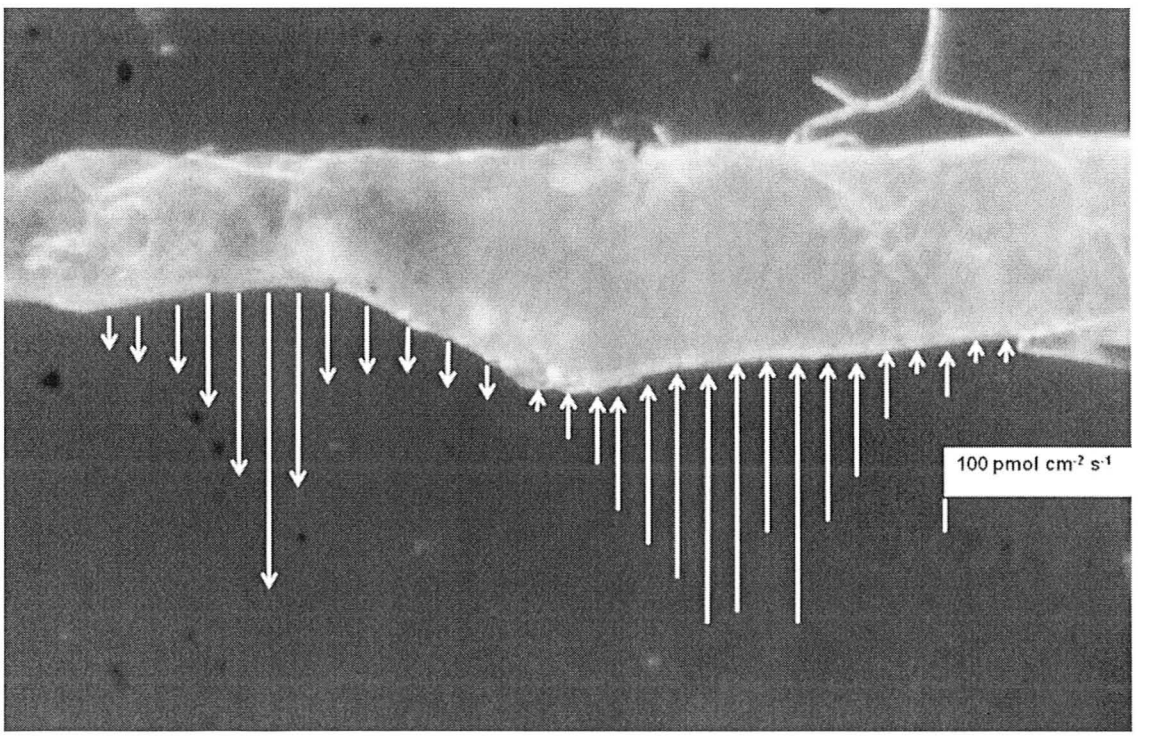
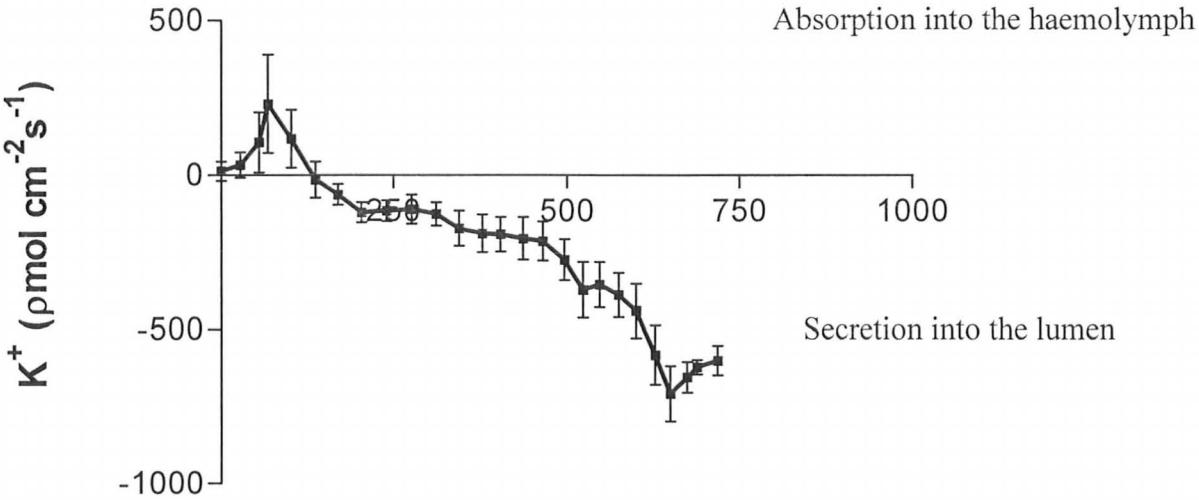
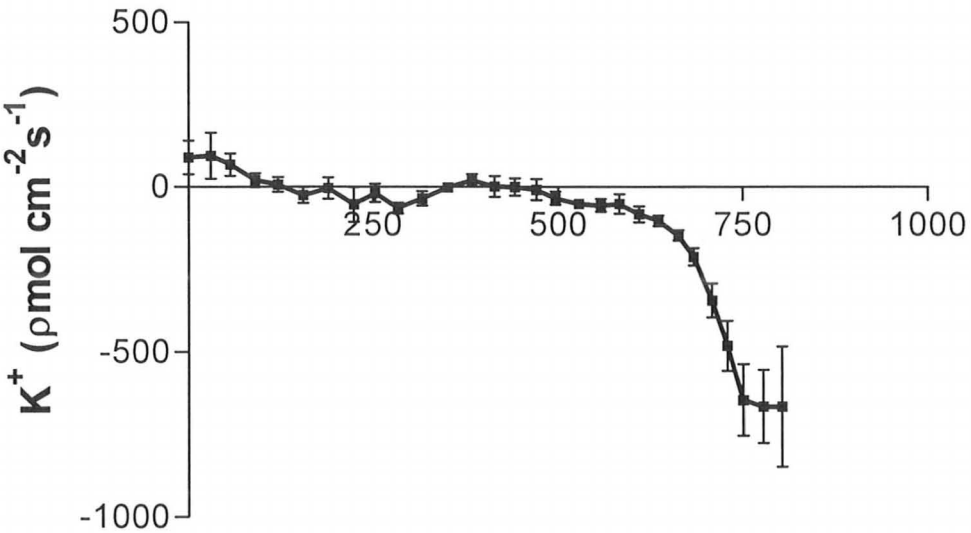


Figure 2

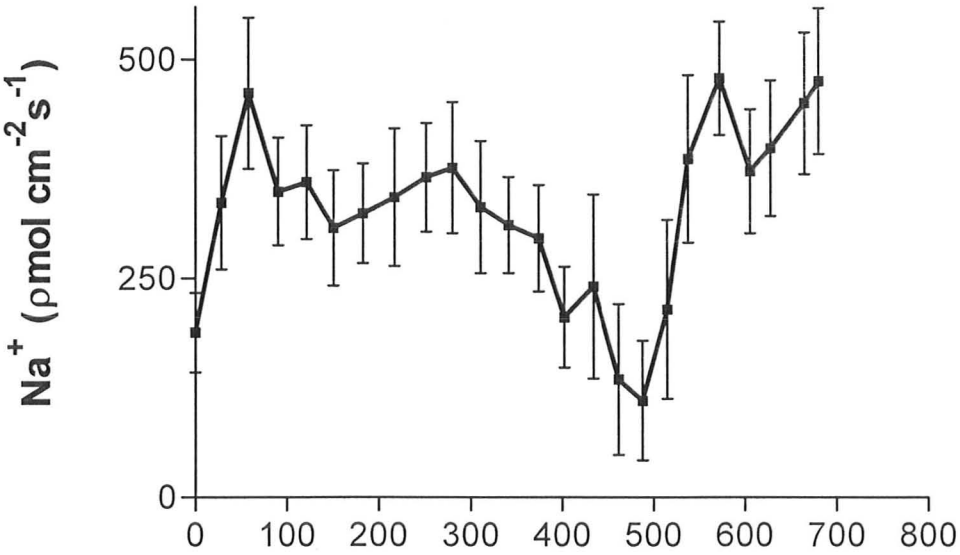
A)


Absorption into the haemolymph
Secretion into the lumen
K+ (ρmol cm-2s-1)
500
0
-500
-1000
500
750
1000

B)


K+ (ρmol cm-2s-1)
500
0
-500
-1000
250
500
750
1000

C)


500
250
0
0
100
200
300
400
500
600
700
800
Na+ (ρmol cm-2s-1)

D)

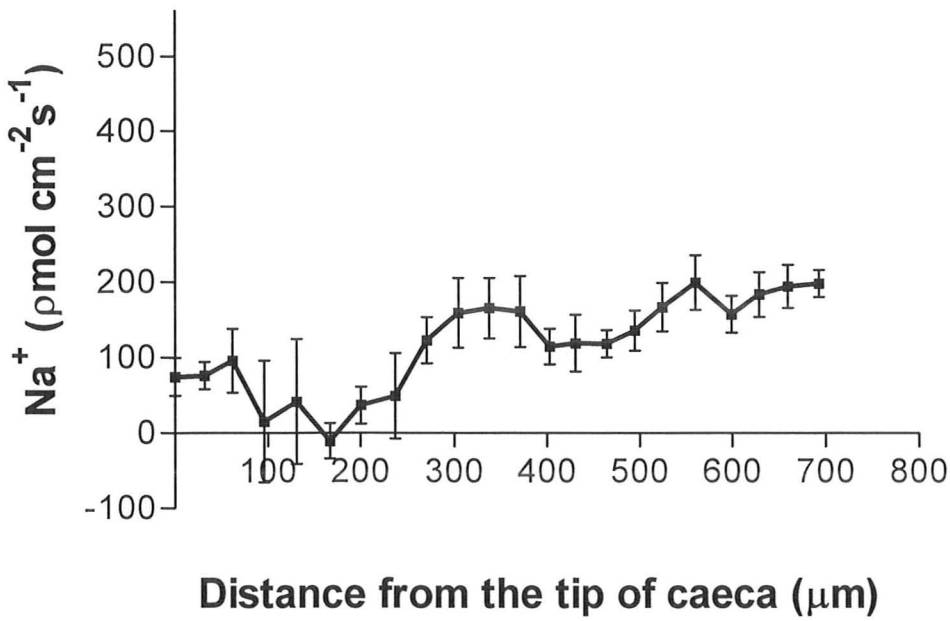
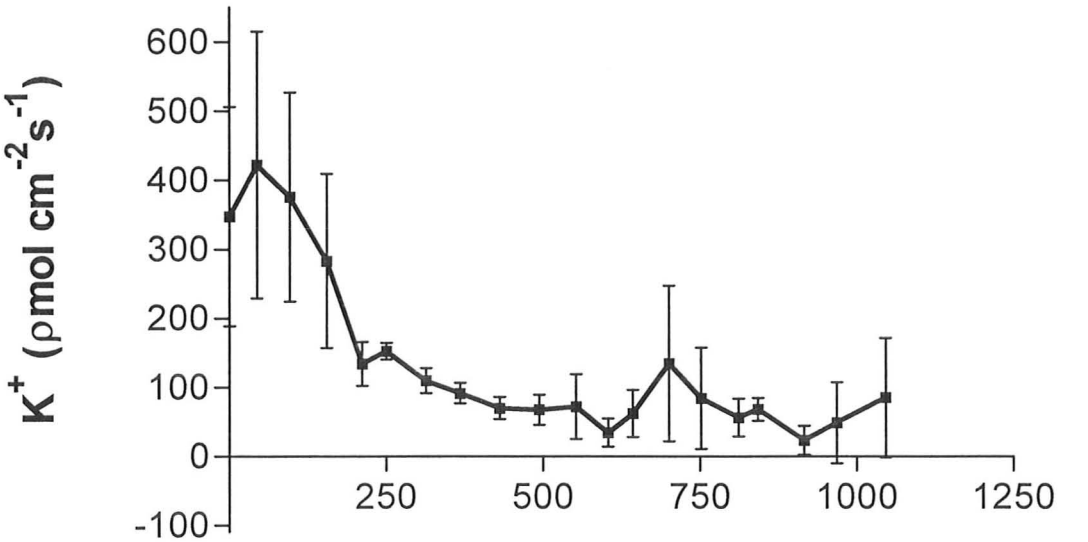

500
400
300
200
100
0
-100
100
200
300
400
500
600
700
800
Na+ (ρmol cm-2s-1)
Distance from the tip of caeca (μm)

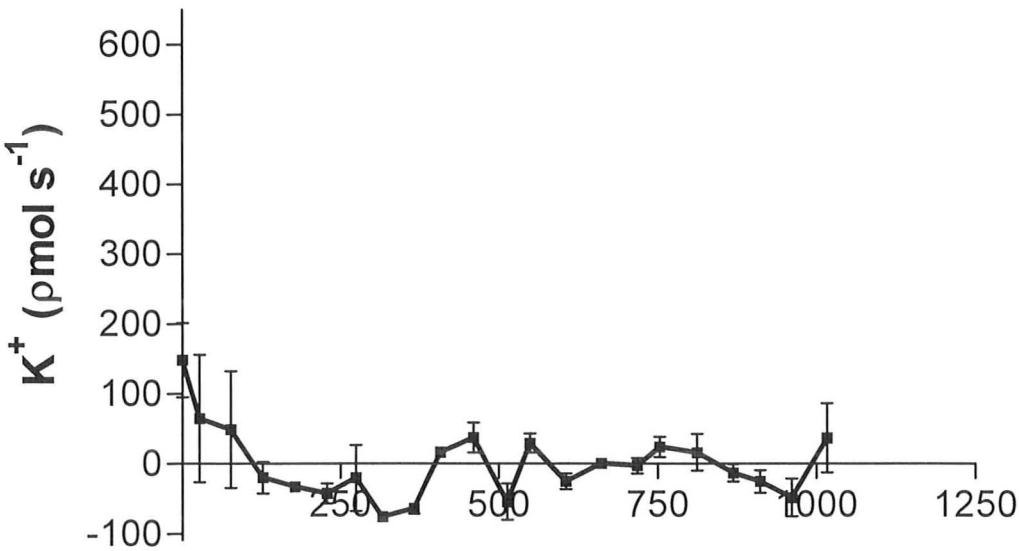
Figure 3

A)

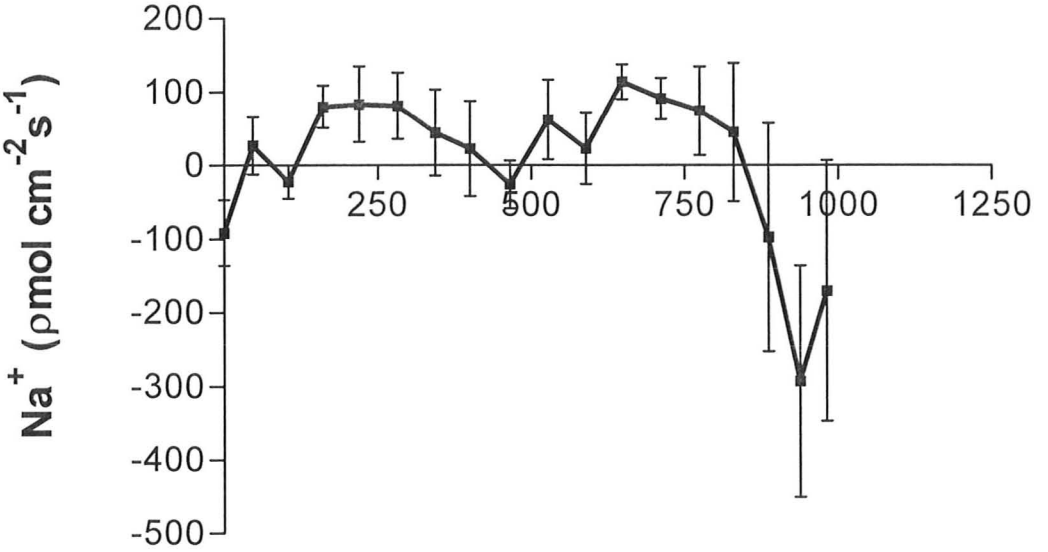


Secretion into the lumen

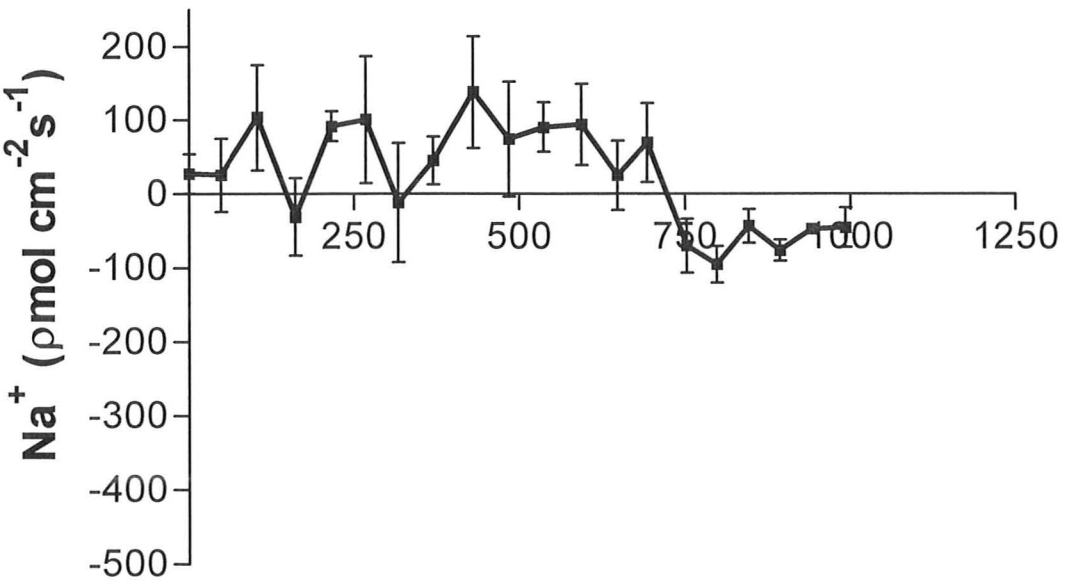
B)



C)



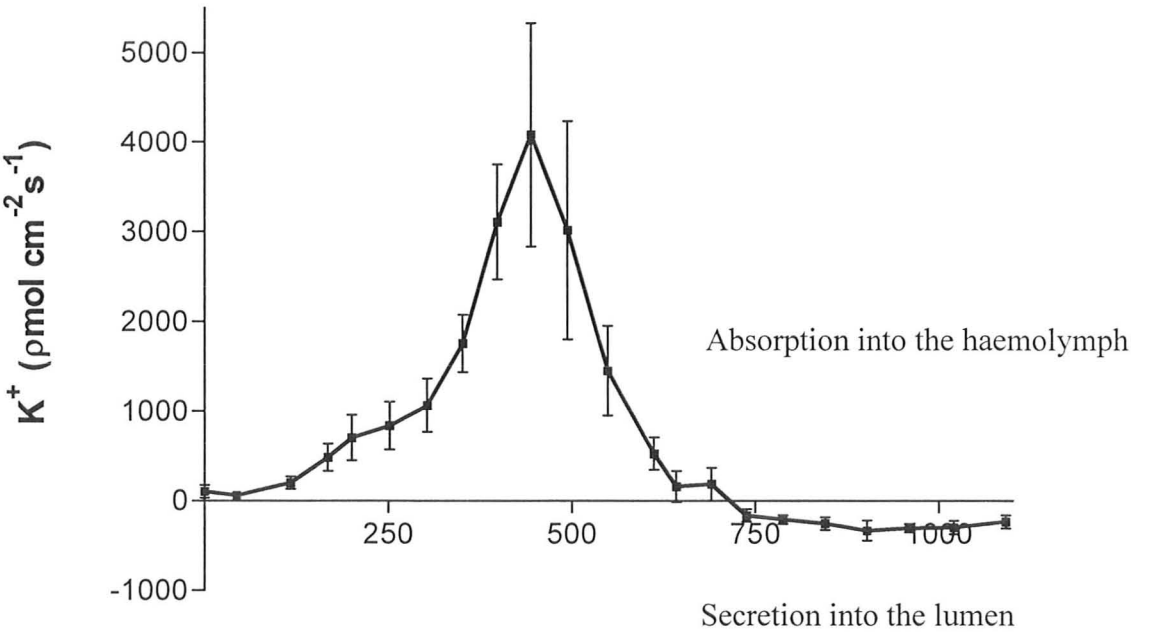
D)



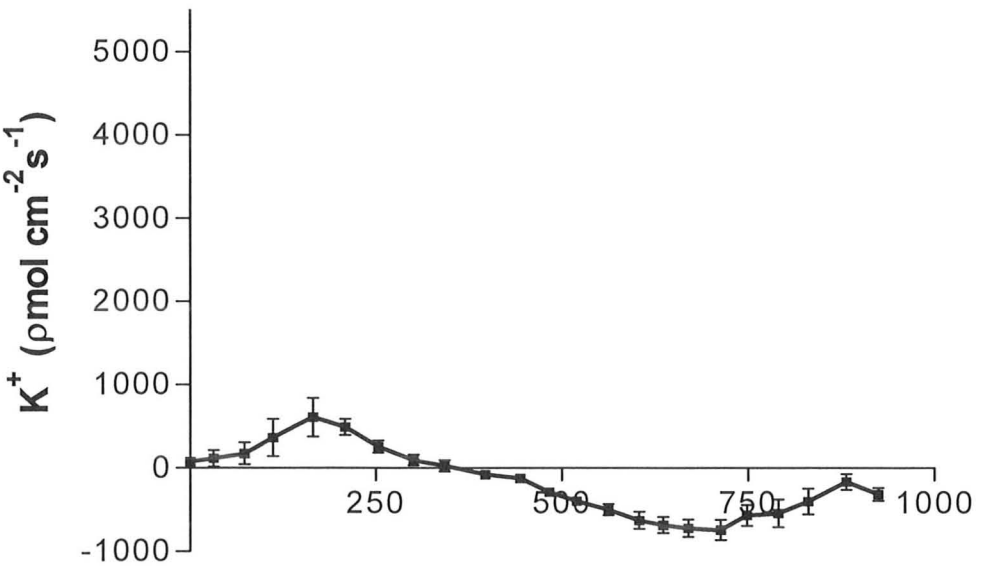
Distance from the start of acid zone (μm)

Figure 4

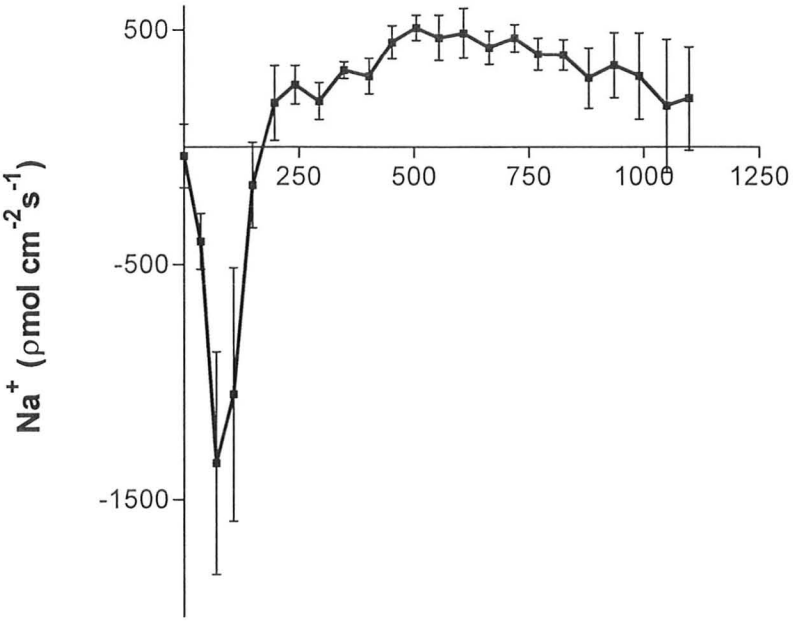
A)


5000
4000
3000
2000
1000
0
-1000
250
500
750
1000
K+ (ρmol cm-2 s-1)
Absorption into the haemolymph
Secretion into the lumen

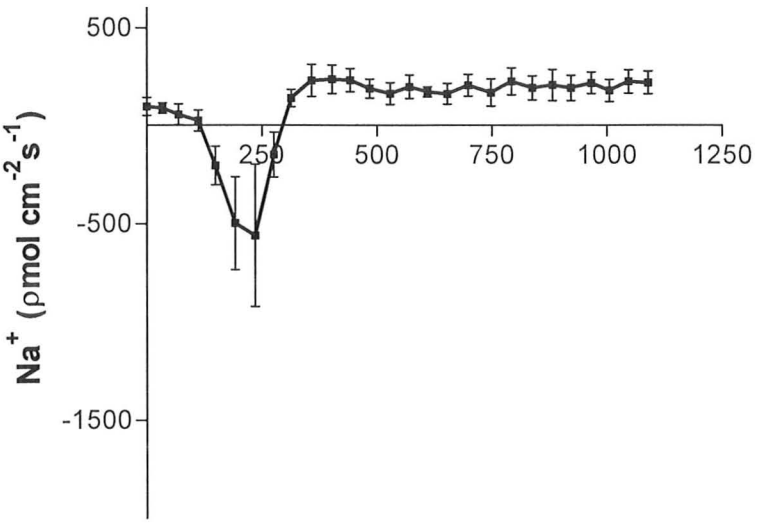
B)


5000
4000
3000
2000
1000
0
-1000
250
500
750
1000
K+ (ρmol cm-2 s-1)

C)



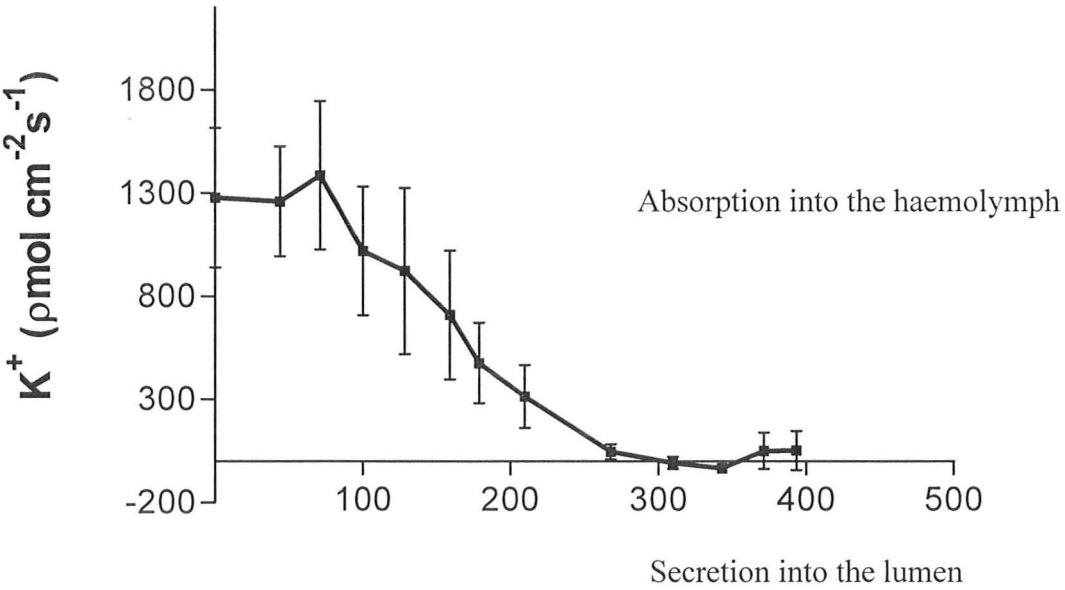
D)



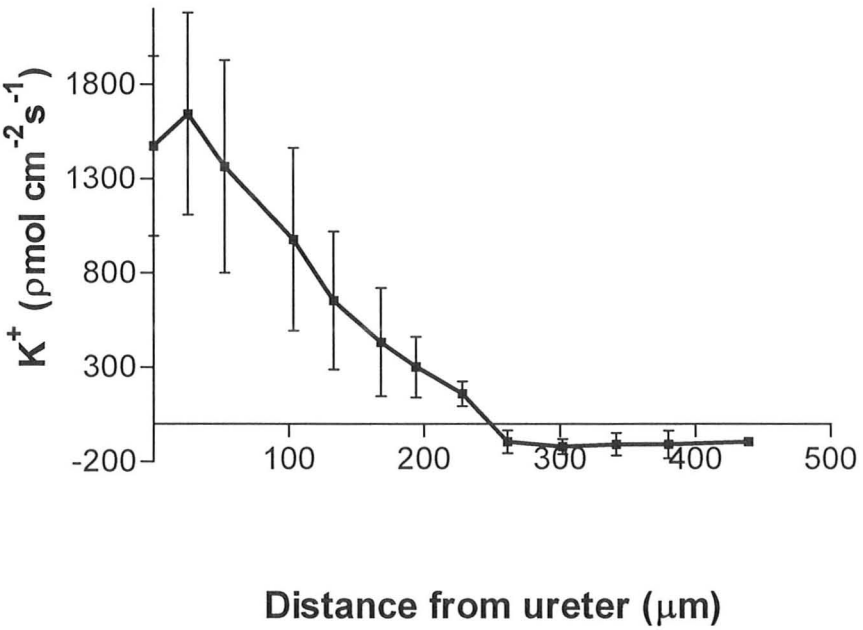
Distance from the start of Midgut (μm)

Figure 5

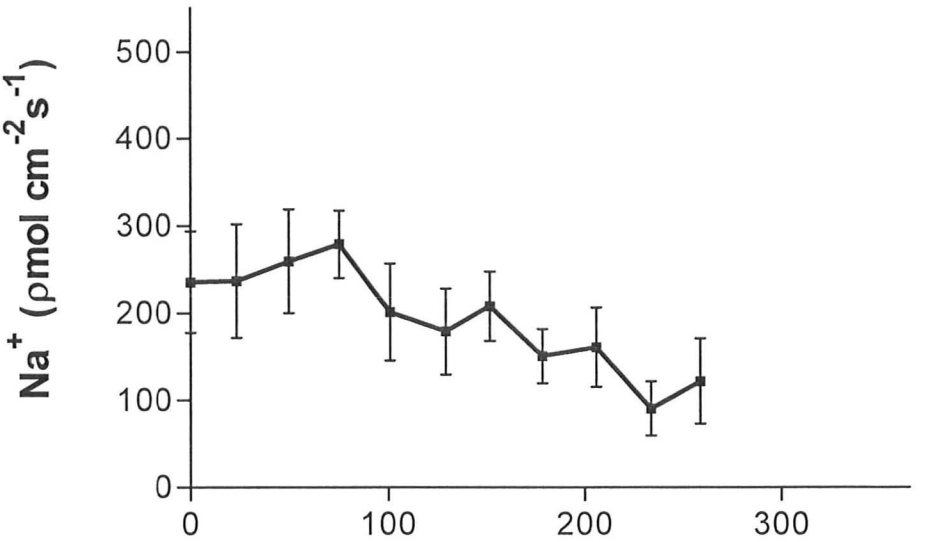
A)


K^+ (ρmol $cm^{-2}s^{-1}$)
1800
1300
800
300
-200
100
200
300
400
500
Absorption into the haemolymph
Secretion into the lumen

B)


K^+ (ρmol $cm^{-2}s^{-1}$)
1800
1300
800
300
-200
100
200
300
400
500
Distance from ureter (μm)

C)


500
400
300
200
100
0
0
100
200
300
Na+ (ρmol cm-2s-1)

D)

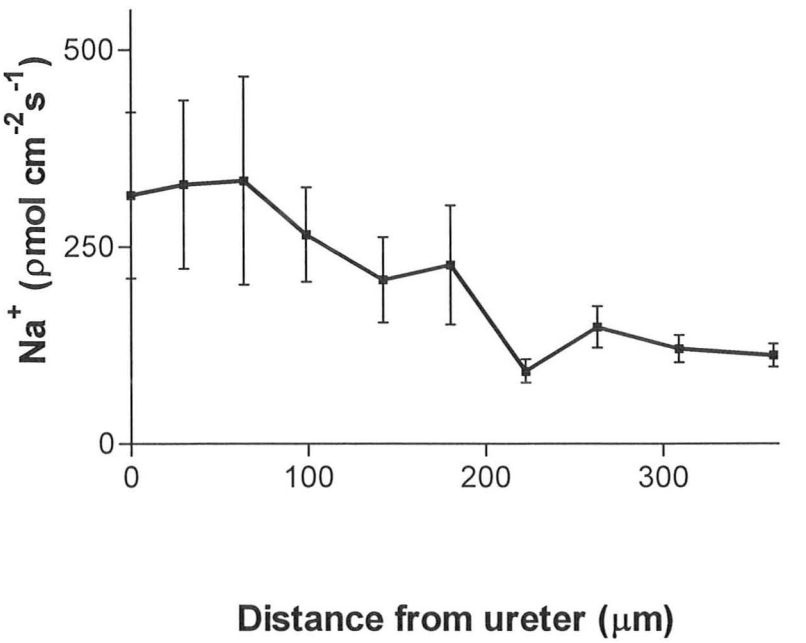
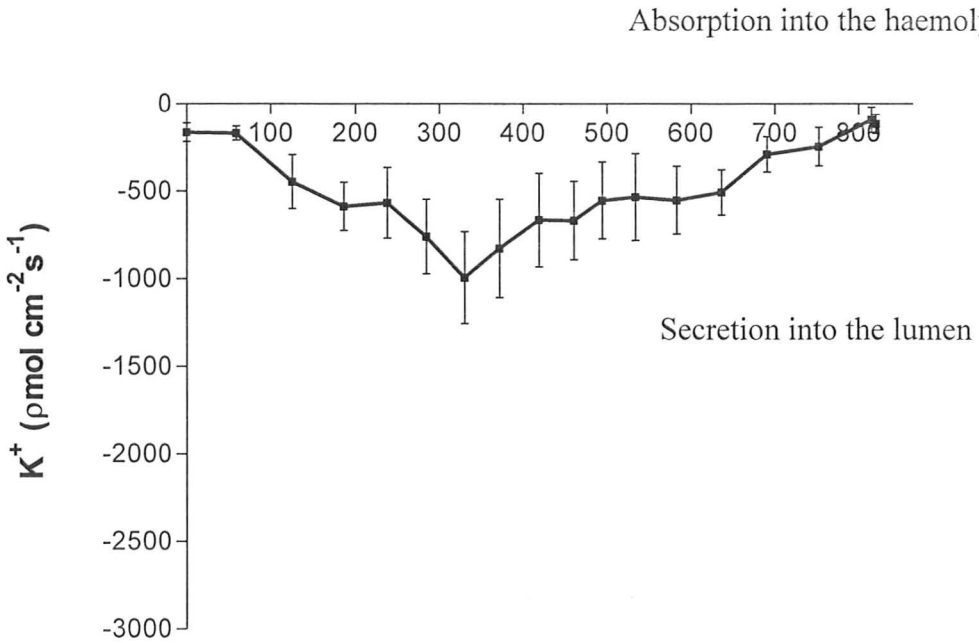
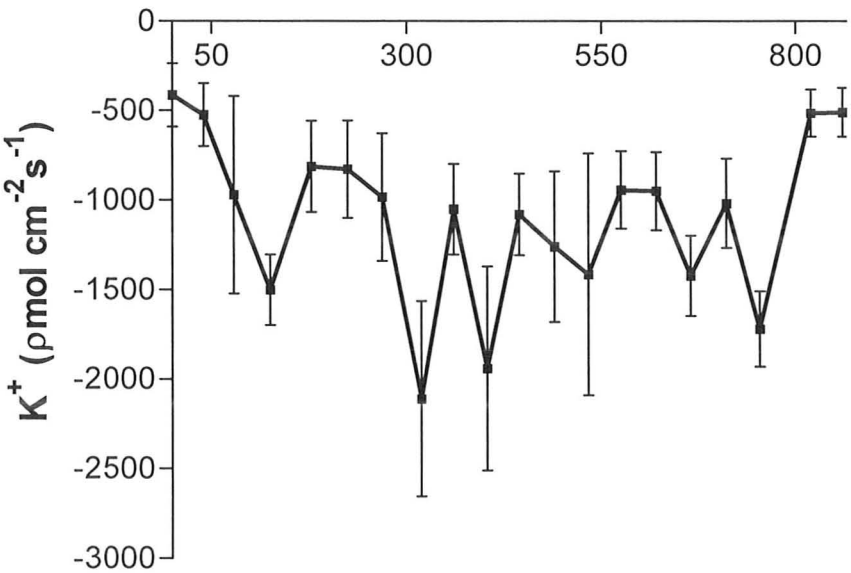

500
250
0
0
100
200
300
Na+ (ρmol cm-2s-1)
Distance from ureter (μm)

Figure 6

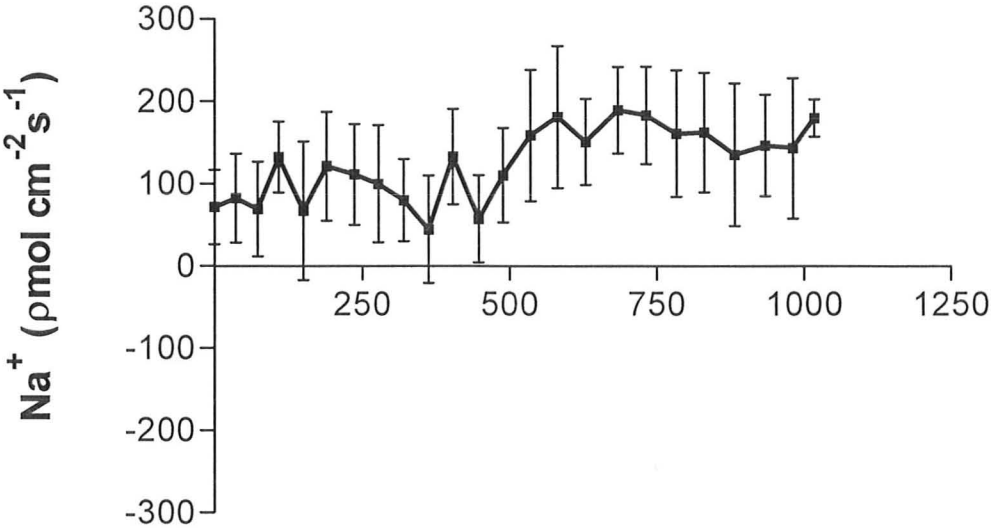
A)


Absorption into the haemolymph
Secretion into the lumen
K+ (ρmol cm-2 s-1)
0
-500
-1000
-1500
-2000
-2500
-3000
100
200
300
400
500
600
700
800

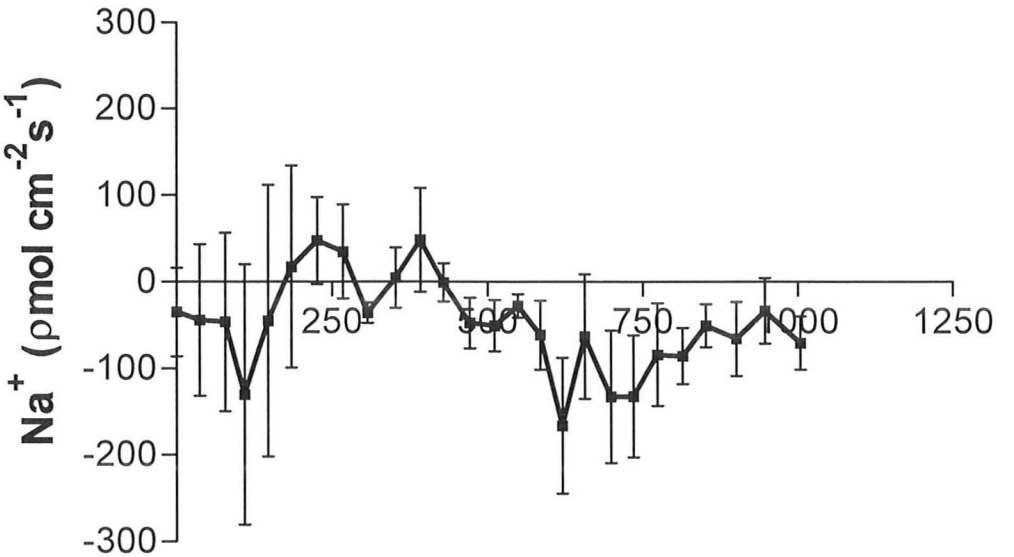
B)


K+ (ρmol cm-2 s-1)
0
-500
-1000
-1500
-2000
-2500
-3000
50
300
550
800

C)



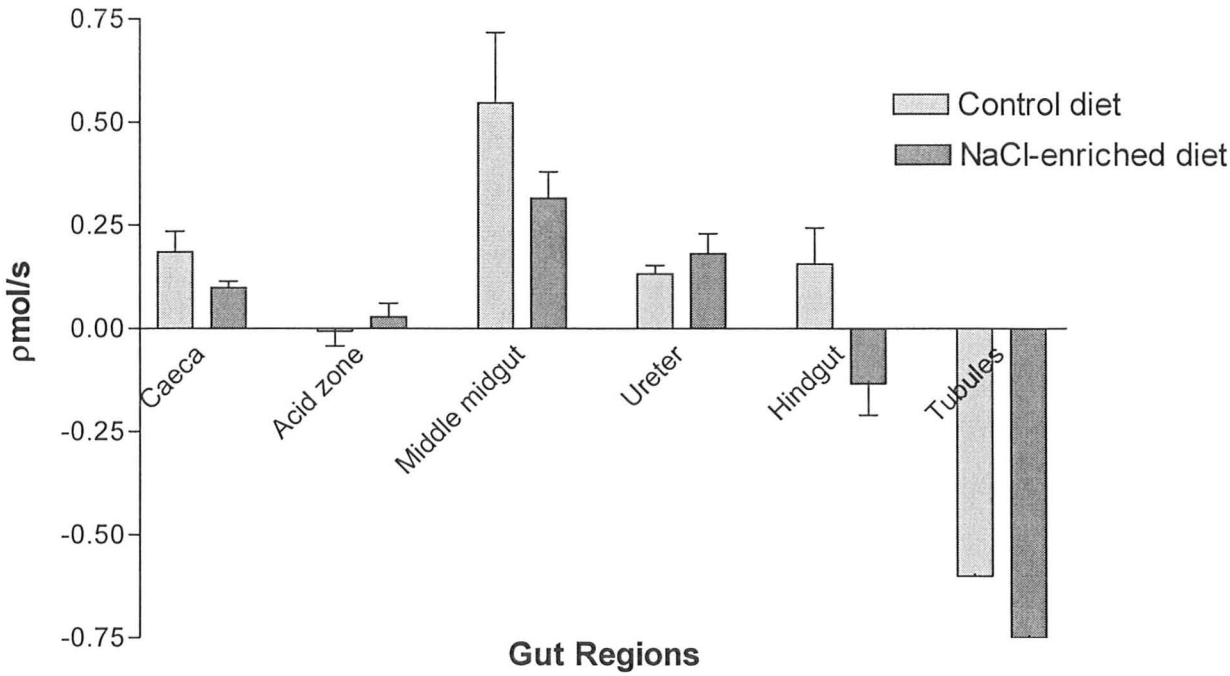
D)



Distance from the start of hindgut (μm)

Figure 7

A)


0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
pmol/s
Control diet
NaCl-enriched diet
Caeca
Acid zone
Middle midgut
Ureter
Hindgut
Tubules
Gut Regions

B)

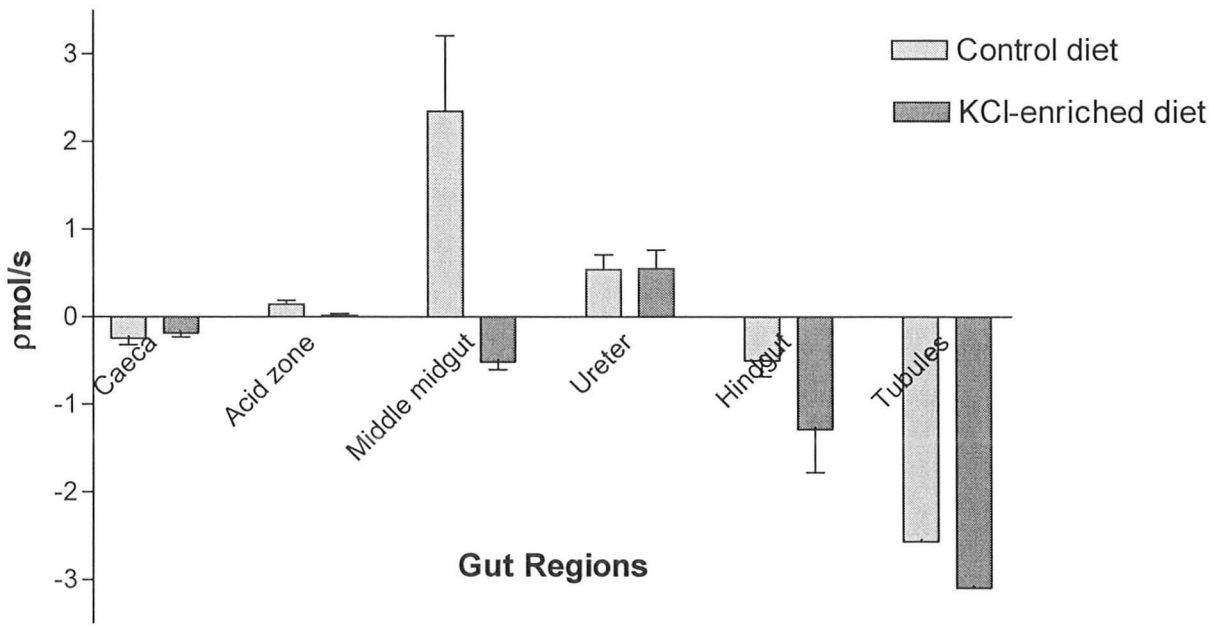

3
2
1
0
-1
-2
-3
pmol/s
Control diet
KCl-enriched diet
Caeca
Acid zone
Middle midgut
Ureter
Hindgut
Tubules
Gut Regions

Figure 8

Chapter 4

General discussion

In this study of dietary salt stress I have examined haemolymph ionoregulation in larval and adult *Drosophila* and the transport of K^+ and Na^+ by multiple epithelia of the larva. *Drosophila* provides an attractive model for studies of salt stress for such studies because of the ease with which the composition of the diet can be altered. However, a disadvantage is that the flies are relatively small and that their ionoregulatory tissues are of microscopic dimensions. The associated technical challenges have been overcome through the use of ion-selective microelectrodes, which allow ion concentrations to be measured in nanoliter samples of haemolymph or in fluids secreted by isolated Malpighian tubules set up in the Ramsay assay. Application of the scanning ion-selective microelectrode technique has permitted non-invasive analysis of Na^+ and K^+ transport by the isolated larval gut. Taken together, the results of chapters 2 and 3 have revealed how the actions of multiple epithelia are coordinated in maintaining haemolymph ionic homeostasis during dietary salt stress.

The results provide evidence for phenotypic plasticity of epithelial ion transport during salt stress. Specifically, the rates of fluid and ion transport by Malpighian tubules and the magnitudes and directions (secretion versus absorption) of Na^+ and K^+ transport across specific segments of the gut were altered by rearing the larvae on salt-rich diets. These experiments were done when the tissues were bathed in saline, and were not altered, therefore, by stimulatory or inhibitory effects produced by the actions of neurohormonal factors. In addition, experiments in which fluid and ion secretion were measured for Malpighian tubules isolated in larval haemolymph indicate that factors circulating in the haemolymph of larvae reared on salt-rich diets also modulate fluid and ion secretion by the Malpighian tubules.

Haemolymph ionoregulation during salt stress: Na^+ versus K^+

Third instar larvae reared are able to maintain their haemolymph volume at the control level and restore haemolymph K^+ concentration to the control level within 48 hours of transfer to the KCl-rich diet . By contrast, the haemolymph volume of the larvae reared on the NaCl-rich diet decreased to 52% of the control value and the haemolymph Na concentration remained 56% above the control level. Differences in the extent of regulation of haemolymph volume and K^+ and Na^+ concentrations during salt stress may relate to the biological relevance of the salt stress that has been imposed.

In the wild, increases in K^+ concentration may occur as the rotting fruit upon which eggs are laid dries out. *Drosophila* may therefore be exposed to relatively rapid changes in the K^+ concentration of their food sources. *Drosophila* are much less likely to be exposed in the wild to increases in the dietary Na^+ concentration since their food sources tend to be K^+-rich plant material. Nonetheless, the species does provide a useful model system for analysis of how ion transport by specific epithelia may be altered in other dipterans (*e.g.* salt marsh mosquitoes) exposed to salt stress, as discussed below.

Phenotypic plasticity in larval Drosophila melanogaster *during salt stress.*

Phenotypic plasticity refers to environmental effects of the expression of the phenotype. Plastic changes to the phenotype can, in some instances, represent adaptations to temporarily varying environments. Examples of phenotypic plasticity in insects include enhanced thermotolerance after prior exposure to low or high temperatures (Bowler and Anstee, 2008), the phase transition by which crowding leads to alterations in locust morphology during the solitary

to gregarious phase transition (Badisco et al., 2009) and the enhanced expression of detoxification enzymes in insects in the presence of plant allelochemicals (Li et al., 2002).

My results provide clear evidence for phenotypic plasticity in epithelial ion transport during salt stress. For tubules isolated from larvae reared on the KCl-rich diet for 24 hours or more and bathed in control saline containing 20 mmol l^{-1} K^+, there was an increase of fluid secretion rate of ~ 56% relative to that of the controls. Unexpectedly, there was a decline in secreted fluid K^+ concentration and an increase in secreted fluid Na^+ concentration. As a consequence of the changes in fluid secretion rate and secreted fluid ion concentration, the flux of K^+ was similar to that of tubules from larvae reared on the control diet but the flux of Na^+ increased more than 3-fold relative to the controls. The adaptive value of an increase in Na^+ secretion of tubules isolated from larvae reared on the KCl-rich diet is unclear, but it does provide evidence that the ion transport mechanisms of the tubules are altered by prior exposure of the larvae to the KCl-rich diet. The tubules from larvae reared on the control and the KCl-rich diets were bathed in the identical saline, and differences in rates of secretion of fluid, Na^+ and K^+ must therefore reflect differences in the activity of ion transporters in the Malpighian tubules derived from different sources. In addition, when the tubules from larvae reared on KCl-rich diet were bathed in saline containing 60 mmol l^{-1} K^+, thereby mimicking the increase in K^+ in the haemolymph over the first 6 – 24 hours of exposure to KCl-rich diet, there was 47% - 56% increase in K^+ secretion by the tubules compared to tubules bathed in the same saline but isolated from larvae that had not been exposed to the KCl-rich diet. The increase of the basal (i.e. unstimulated) rate of fluid and ion secretion is again evidence of phenotypic plasticity. Moreover, under these conditions (i.e. in saline mimicking the haemolymph K^+ concentrations in larvae shortly after transfer to the KCl-rich diet) the enhanced secretion of K^+ is clearly adaptive

since it will tend to reduce the levels of K^+ in the haemolymph and thus contribute to haemolymph ionoregulation.

Chapter 3 of the thesis provides additional evidence for phenotypic plasticity of ion transport by gut epithelia during salt stress. Specifically, there was less absorption of K^+ and Na^+ across the midgut and increased secretion of K^+ and Na^+ by the hindgut of larvae reared on diets rich in K^+ and Na^+, respectively.

Taken together, my studies of ion secretion by the tubules and gut provide evidence that the suite of ion transporters are altered during exposure to excess dietary salt so that less salt is absorbed and more is secreted. The increase in fluid secretion rate by the tubules of salt-stressed larvae suggest that there may be increases in the transport by the apical V-type H^+-ATPase which energizes transepithelial ion transport in Malpighian tubules. Future studies might therefore use techniques such as quantitative RT-PCR to examine the levels of genes for the V-type H^+-ATPase and NHA (Na^+/H^+ antiporter) in tubules of larvae exposed to the KCl-rich or NaCl-rich diets (Wang et al., 2004; Rheault et al., 2007). In addition, comparable approaches might be used to assess which transporters in the gut are altered by salt stress. In the long-term, development of a gut specific DNA microarray might provide valuable clues as to the nature of the ion transporters which are altered in the gut of salt stressed-larvae. A tubule-specific microarray has been available for several years (Wang et al., 2004).

Effects of haemolymph-borne factors on ion transport by ionoregulatory epithelia of larval Drosophila *during salt stress*

Chapter 2 of this thesis showed that factors present in haemolymph collected from larvae reared on salt-rich diets stimulate the secretion of fluid and ions (Na^+, K^+) by isolated

Malpighian tubules. My experiments suggested that a factor which acts through elevation of intracellular Ca^{2+} may play a role in this stimulation. In this context it is worth noting the results of experiments by Blumenthal on the changes in ion transport by Malpighian tubules of adult *Drosophila* during dehydration. Blumenthal (2005) has provided evidence for autonomous regulation of MT function by tyramine and the modulation of this signaling pathway by osmolality. Most diuretic factors in insects are produced within neuroendocrine cells in the central nervous system and released into the haemolymph. Tyramine, however, is also produced constituitively within the Malpighian tubules and is then released into the haemolymph to stimulate fluid secretion through a Ca^{2+}-dependent mechanism. Blumenthal has proposed that tyramine production is reduced when an adult fly becomes dehydrated, leading to lower rates of fluid secretion by the Malpighian tubules. The resultant reduction in fluid flow into the rectum will then aid absorption of water and concentration of the feces.

Given the evidence for a reduction in Malpighian tubule fluid secretion during the increase in haemolymph osmolality and ion concentrations associated with dehydration, it is somewhat surprising, therefore, that dietary salt stress is correlated with the presence of haemolymph factors which increase fluid secretion rates. It is important to note that my experiments concerned the larva whereas Blumenthal examined adult *Drosophila.* In addition, an increase in dietary salt levels in the semi-liquid diet of the larvae may impose different stresses than dehydration associated with a reduction in haemolymph volume and consequent increases in haemolymph ion concentrations in the adult.

It would be of interest in future studies to identify the factor or factors responsible for the diuresis observed in the larval tubules bathed in haemolymph collected from larvae reared on the NaCl-rich or KCl-rich diets. In addition, it would be of interest to determine if the ion transport

by the gut is altered by factors released into the haemolymph of salt-stressed larvae. However, performing SIET scans of isolated guts bathed in haemolymph is not feasible because of the large volumes of fluid (> 0.5 ml) required. An alternative approach is to examine the effects of neuropeptides known to alter Malpighian tubule ion transport (e.g. Drosokinin, CAPA) and treatments which elevate the concentrations of intracellular second messengers (e.g. thapsigargin, exogenous cAMP) on K^+ and Na^+ transport by guts isolated from larvae reared on control versus salt-rich diets.

Previous studies have shown that the Malpighian tubules of mosquito larvae are altered in two ways in response to increases in ambient salinity (Donini et al., 2006). Firstly, changes in salinity of the medium in which mosquito larvae are reared lead to sustained changes in ion transport mechanisms in unstimulated tubules. Specifically, alterations of K^+ transport may lead to either conservation of Na^+ under freshwater (Na^+-deprived) conditions or elimination of more Na^+ in saline (Na^+-rich) conditions. Secondly, changes in rearing medium salinity affect the nature and extent of stimulation of fluid and ion secretion by secretagogues. For example, cAMP decreases secreted fluid Na^+ concentration of tubules isolated from 100%-seawater-reared larvae but does not alter the secreted fluid Na^+ concentration of tubules isolated from freshwater- or 30%-seawater-reared larvae. These studies of Malpighian tubules of mosquito larvae raise the possibility that neuroendocrine factors may produce different effects on Na^+ and K^+ transport by the gut of larval *Drosophila*, depending upon the degree and nature of dietary salt stress. Such effects would be in addition to the changes in Na^+ and K^+ transport by the unstimulated guts isolated from larval *Drosophila* exposed to dietary salt stress, as described in Chapter 3.

Significance of the results for other insects and future research

Larvae of mosquitoes such as the salt-tolerant species *Ochlerotatus taeniorhynchus* may be exposed to salt stress resulting from evaporative concentration of the media around the larvae or tidal influx of sea water into the salt marshes in which they live. SIET has been used to examine H^+ transport by the gut and Malpighian tubules of mosquito larvae (Boudko et al., 2001) and future work might thus use SIET and Na^+ microelectrodes to examine changes in Na^+ absorption and secretion in different segments of the gut when salt-tolerant mosquito larvae are bathed in media of different salinities. In addition, the techniques employed in this thesis could be used to examine ion transport associated with nutrient absorption by the gut of larval mosquitoes. Amino acid transport across the gut wall of mosquito larvae involves cotransport with Na^+ or K^+ across the apical and basal surfaces in different regions of the gut (Okech et al., 2008). It would be of interest, therefore, to determine whether nutrient absorption is altered during salt stress if the availability and transport of these alkali cations is changed as a consequence of the need to maintain haemolymph ionic homeostasis.

The availability of genetic tools such as the use of mutants, RNAi knockdowns and quantitative PCR for assessment of gene expression has made *Drosophila* a valuable model for studies of developmental biology and physiology. The Gal4/UAS system has been particularly useful for determining the function of particular cells. In this system, the GAL4 gene is placed under the control of a native gene promoter (the 'driver' gene), while the upstream activation sequence (UAS) controls the expression of a target gene. GAL4 is then only expressed in cells where the driver gene is usually active (Duffy, 2002). This system has been exceptionally powerful for examining the functioning and control of specific cells and regions of the Malpighian tubule. Expression of an aequorin transgene in specific cells has been used to show

that the leucokinin peptides mediate their effects through increases in intracellular Ca^{2+} in the stellate cells (Rosay et al., 1997; O'Donnell et al., 1998). Such studies were possible because of earlier work in which a set of 700 P{GAL4} enhancer trap lines was screened for patterned reporter gene expression in the Malpighian tubules and fly lines that showed genetic compartmentalization by tubule region and by cell type were selected (Sozen et al., 1997). By analogy with the development of the GAL4/UAS system for the Malpighian tubules, enhancer trap lines might be generated so that the functions and control of specific cells or regions of the gut could be studied. Whereas the Malpighian tubule has been studied profitably using the Ramsay assay, SIET provides the means to study ion transport by specific regions of the gut.

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