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RESEARCH ARTICLE

Phenotypic plasticity in response to dietary salt stress: Na^+ and K^+ transport by the gut of *Drosophila melanogaster* larvae

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SUMMARY

***Drosophila* provides a useful model system for studies of the mechanisms involved in regulation of internal ion levels in response to variations in dietary salt load. This study assessed whether alterations in Na^+ and K^+ transport by the gut of larval *D. melanogaster* reared on salt-rich diets contribute to haemolymph ionoregulation. Na^+ and K^+ fluxes across the isolated guts of third instar larvae reared on control or salt-rich diets were measured using the scanning ion-selective electrode technique (SIET). K^+ absorption across the anterior portion of the posterior 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 eightfold 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 posterior midgut. Na^+ was absorbed across the ileum of larvae reared on the control diet, but was secreted across the ileum 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 *D. melanogaster*. SIET measurements of K^+ and Na^+ fluxes along the length of the gut show that 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 ionoregulation.**

Key words: insect gut, salt stress, sodium, potassium, haemolymph ionoregulation, phenotypic plasticity.

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). Larvae of the brine fly *Ephydra hians* show an impressive capacity to osmoregulate in water containing more than 150 mmol l^{-1} sulphate (Shimizu and Bradley, 1994). Sulphate absorbed across the midgut is removed from the haemolymph by active transport across the anterior half of the colon. The fruit fly *Drosophila* is 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), or during dehydration and rehydration (Albers and Bradley, 2004). Most genes that are upregulated or downregulated by salt stress are highly enriched in the Malpighian tubules and/or the hindgut, suggesting that these tissues play major roles in the response to salt stress (Stergiopoulos et al., 2008). There is a robust increase in carbohydrate metabolism during salt stress, and 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* (CG2196) is 7000-fold higher in the Malpighian tubules than in whole flies (Stergiopoulos et al., 2008). In addition, two proteins 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). The *Drosophila* gene nuclear factor of activated T-cells (*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, 2011) examined changes in haemolymph Na^+ and K^+ levels as well as Malpighian tubule fluid and ion transport in salt-stressed flies. Haemolymph Na^+ concentration increased 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 was from 45 to ~80 mmol l^{-1}. Regulation of haemolymph K^+ was more precise. There was no significant change in K^+ concentration in the haemolymph collected from adults at 6, 12 or 24 h or 7 days after transfer from the control diet to the diet containing an additional 0.4 mol l^{-1} KCl. In the larvae, haemolymph K^+ concentration increased from 22 to 75 mmol l^{-1} 6 h after the transfer to the KCl-rich diet, but the control level was restored within 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, 2011).

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 because they are the sites of nutrient absorption and urine modification, respectively (Etienne et al., 2001). In this study we used the scanning ion-selective electrode technique (SIET) to measure transport of Na^+ and K^+ across the foregut, midgut and hindgut (ileum), as well as the ureter and the lower Malpighian tubule, of larvae reared on either a standard *Drosophila* diet or 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. Third instar larvae were used in all the experiments. The control diet was prepared as described previously (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 one part 85% o-phosphoric acid) and 7.45 ml of 10% *p*-hydroxybenzoic acid methyl ester (Tegosept, Sigma-Aldrich, St Louis, MO, USA) dissolved in ethanol were added to the mixture. Experimental diets were prepared by addition of 0.4 mol l^{-1} NaCl or 0.4 mol l^{-1} KCl to the standard diet.

Gut dissection and physiological salines

The entire gut from each larva was dissected under *Drosophila* saline containing (in 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. Saline pH was adjusted 7.0 with NaOH. A saline containing 20 mmol l^{-1} Na^+ was made by equimolar substitution of *N*-methyl-D-glucamine for NaCl. Each gut was placed under saline in a 35 mm Petri dish. Dishes were pre-coated with 100 µl droplets of 125 µg ml^{-1} poly-L-lysine (70–150 kDa, Sigma-Aldrich, St Louis, MO, USA) and air dried before filling with saline to facilitate adherence of the tissue to the bottom of the dish.

Ion-selective microelectrodes and SIET

Micropipettes were pulled on a P-97 Flaming-Brown pipette puller (Sutter Instruments Co., Novato, CA, USA) from 1.5 mm borosilicate glass (World Precision Instruments Inc., Sarasota, FL, USA). This gave a micropipette with a short shank and a tip opening of approximately 3–5 µm. The micropipettes were then silanized with *N*,*N*-dimethyltrimethylsilylamine at 200°C for 30 min, and were stored at room temperature in a desiccator. 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 filling them with a column (~500 µm) of K^+ ionophore I, cocktail B (Fluka, Buchs, Switzerland). Na^+-selective microelectrodes were made by first backfilling the micropipettes with 150 mmol l^{-1} NaCl and then tip filling them with a column (~200 µm) of Na^+ ionophore cocktail, which consisted of 10% Na^+ ionophore X (Fluka), 89.75% nitrophenyl octyl ether and 0.25% sodium tetraphenylborate (Messerli et al., 2008). The K^+ ionophore cocktail is more selective for K^+ relative to Na^+, Ca^{2+} and Mg^{2+} by factors of $10^{3.9}$, $10^{4.9}$ and $10^{4.9}$, respectively (Amman et al., 1987). The Na^+ ionophore cocktail is more selective for Na^+ relative to K^+, Ca^{2+} and Mg^{2+} by factors of $10^{2.6}$, $10^{3.5}$ and $10^{3.7}$, respectively (Messerli et al., 2008). 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 150 mmol l^{-1} NaCl and 15 mmol l^{-1} NaCl/135 mmol l^{-1} KCl, respectively. Slopes (mV) for a 10-fold change in ion concentration were (mean ± s.e.m.) 52.1±2 (N=6) for K^+-selective microelectrodes and 57±2 (N=6) for Na^+-selective microelectrodes. Reference electrodes were constructed from 10 cm borosilicate glass capillaries that were bent 1 cm from the end at a 45 deg 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, USA) and Automated Scanning Electrode Technique (ASET) software (version 2.0; Science Wares, Falmouth, MA, USA). The ion-selective microelectrodes 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 and three replicate measurements at each site could thus be completed in <30 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 30–50 µm. The transepithelial potential, apical and basal membrane potentials and basal unstirred layer pH of isolated midgut segments are stable for at least 25–30 min [e.g. fig. 5 in Shanbhag and Tripathi (Shanbhag and Tripathi, 2005) and fig. 14 in Shanbhag and Tripathi (Shanbhag and Tripathi, 2009)], indicating that the physiological health of the preparation is maintained for such periods. Our preliminary measurements indicated that Na^+ and K^+ fluxes were also stable for 20–30 min and we therefore designed our protocol so that each gut segment was scanned immediately after dissection and the scans were complete within 20–30 min. Regions of the gut that were examined in detail are shown in Fig. 1.

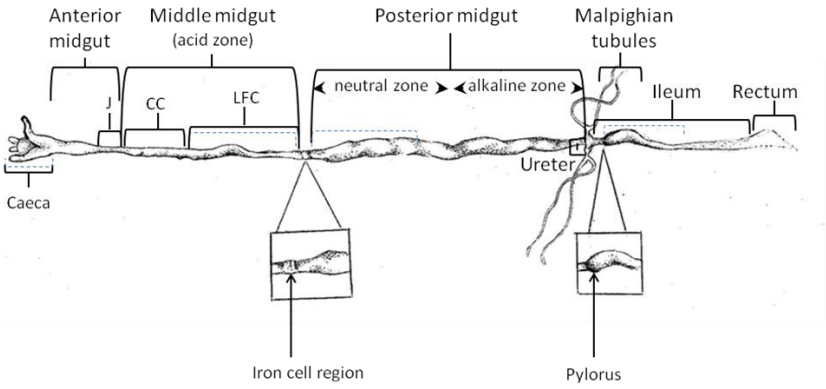

Fig. 1. Schematic diagram of the *Drosophila melanogaster* gut. The dashed brackets indicate the regions of each gut segment for which K^+ and Na^+ fluxes are presented in Figs 3–7. The insets show morphological features used to define the regions scanned. CC, copper cells; J, junction between anterior and middle midgut; LFC, large flat cells.

Calculation of ion fluxes

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

$$\Delta C = C_B 10^{(\Delta V / S)} - C_B, \quad (1)$$

where ΔC is the concentration gradient between the two points ($\mu mol\,cm^{-3}$); C_B is the background ion concentration ($\mu mol\,cm^{-3}$), calculated as the average of the concentrations at each point; ΔV is the voltage gradient obtained from ASET (μV); and S is the slope of the electrode (μV) for a 10-fold change in ion concentration bracketing the range of interest. 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 assumption is appropriate for the calibration solutions and salines used in the present study because they are of similar ionic strength.

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

$$J_I = D_I(\Delta C) / \Delta x, \quad (2)$$

where J_I is the net flux of the ion ($pmol\,cm^{-2}\,s^{-1}$), D_I is the diffusion coefficient of the ion [$1.55\times10^{-5}\,cm^2\,s^{-1}$ for Na^+, $1.92\times10^{-5}\,cm^2\,s^{-1}$ for K^+ (Lide, 2002)] and Δx is the distance between the two points measured (cm). Guts were bathed in control saline containing $20\,mmol\,l^{-1}$ K^+ for measurements of K^+ fluxes. Preliminary studies indicated that it was difficult to resolve Na^+ fluxes by SIET in control saline because of the high concentration of Na^+ ($132.5\,mmol\,l^{-1}$). Na^+ flux measurements were therefore made in saline in which the Na^+ concentration was reduced to $20\,mmol\,l^{-1}$ by replacement with *N*-methyl-D-glucamine.

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 measured from digital images of each preparation using ASET software, and 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 SigmaPlot 10 (Systat Software, San Jose, CA, USA). 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 $P<0.05$.

RESULTS

Preliminary scans along the entire length of the isolated gut were performed in order to identify the primary regions of K^+ and Na^+ absorption and/or secretion in larvae reared on the control diet. The regions with the largest fluxes of K^+ and Na^+ (N=3 and 2, respectively) were (in order of anterior to posterior position) the gastric caecum, the large flat cell region of the middle midgut [included within the acid zone of Shanbhag and Tripathi (Shanbhag and Tripathi, 2009)], the anterior half of the posterior midgut [corresponding to the neutral zone of Shanbhag and Tripathi (Shanbhag and Tripathi, 2009)], and the anterior half of the ileum (Fig. 1). Fluxes in other regions were much smaller. K^+ fluxes in the midregion and posterior region of the posterior midgut, for example, were ~11 and ~1%, respectively, of the flux in the anterior half of posterior midgut. Na^+ fluxes in the midregion and posterior region of the posterior midgut were ~33 and ~10%, respectively, of the flux in the anterior half of the posterior 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. We also scanned the ureters and the lower segment of the Malpighian tubule because these regions are placed in the paraffin oil outside of the bathing saline droplet in the Ramsay assay and their contribution to Na^+ and K^+ transport had thus not been assessed in our previous study (Naikkhwah and O'Donnell, 2011). An example of K^+ flux measurements across the posterior midgut is shown in Fig. 2 and Figs 3–7 summarize the fluxes in the selected regions of the gut, the ureters and the lower Malpighian tubule.

Caecum

K^+ was secreted from haemolymph to lumen across much of the length of each caecum in guts isolated from larvae reared on the control diet, except for a small region of absorption within ~100 µm of the tip (Fig. 3A). This pattern was maintained in the 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 the lumen to the haemolymph across the entire length of each caecum of guts isolated from larvae reared on the control diet (Fig. 3C), but there was a twofold

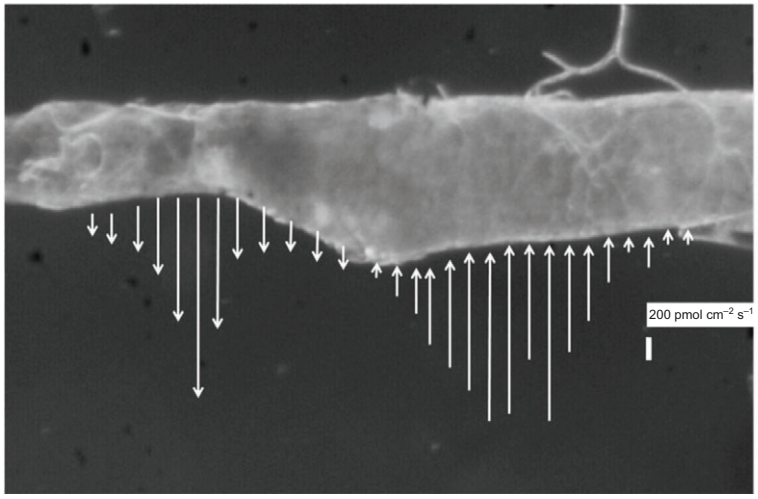

Fig. 2. Scanning ion-selective electrode technique measurement of K^+ fluxes across the anterior half of the posterior midgut of a *D. melanogaster* larva raised on the KCl-rich diet. The leftmost arrow is the end of the iron cell region, as shown in Fig. 1. The direction of each arrow indicates movement of ions out of (absorption) or into (secretion) the gut, whereas the length of the arrow denotes the magnitude of the flux. The scale is denoted by the length of the thick vertical bar below the 200 pmol cm^{-2} s^{-1} label.

to threefold reduction in the magnitude of the flux across caeca isolated from larvae reared on the NaCl-rich diet (Fig. 3D).

Middle midgut

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

Posterior midgut

K^+ was strongly absorbed across the anterior region of the posterior midgut of larvae reared on the control diet [corresponding to the neutral zone of Shanbhag and Tripathi (Shanbhag and Tripathi, 2009)], with the peak in absorption exceeding 4000 pmol cm^{-2} s^{-1} at a point approximately 450 µm from the start of the posterior midgut (Fig. 5A). K^+ was secreted across the remaining 300 µm of the posterior midgut of the controls. There was a dramatic reduction in K^+ absorption across the posterior midgut of larvae reared on the KCl-rich diet as well as an increase in the magnitude of K^+ secretion and the extent of the posterior midgut across which K^+ was secreted (Fig. 5B).

In contrast to the pattern of K^+ transport, Na^+ was secreted across the anterior region of the posterior midgut and absorbed across the

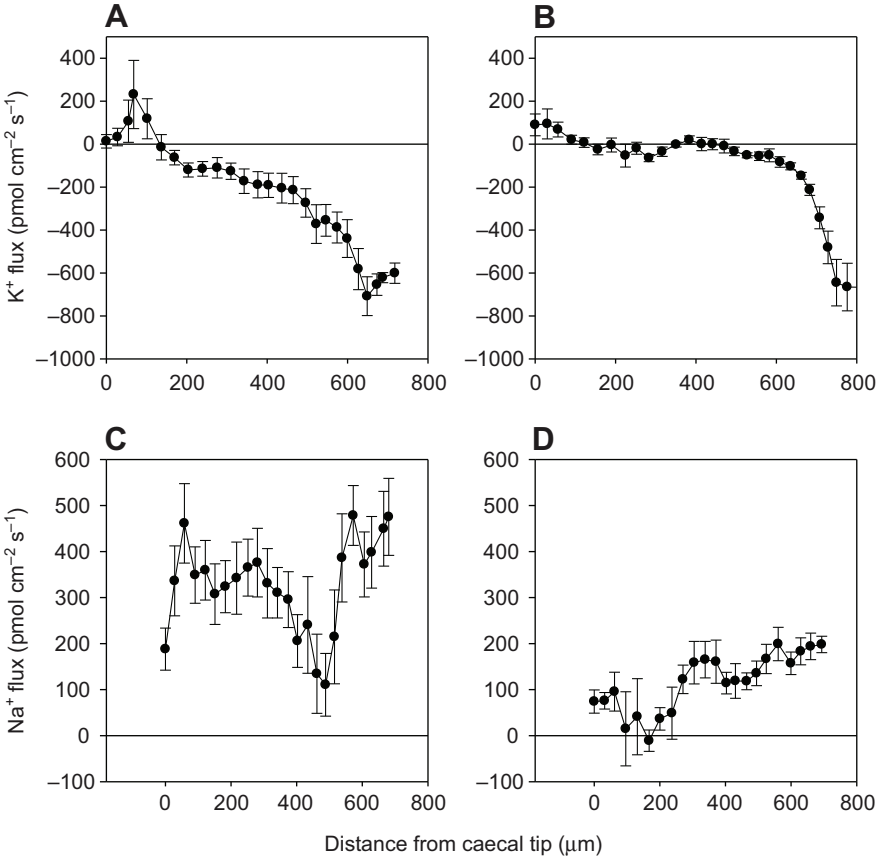

Fig. 3. K^+ and Na^+ fluxes across the caecum of larval *D. melanogaster*. K^+ flux across caecum of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across caecum of larvae reared on (C) the control diet or (D) the NaCl-rich diet. The entire length of the caecum was scanned, with 0 µm (origin) corresponding to the tip of the caecum. The positive values represent movement of K^+ or Na^+ from the lumen to the haemolymph (absorption) and the negative values represent movement of K^+ or Na^+ from the haemolymph to the lumen (secretion). Values are means ± s.e.m. (*N*=5–7 larvae).

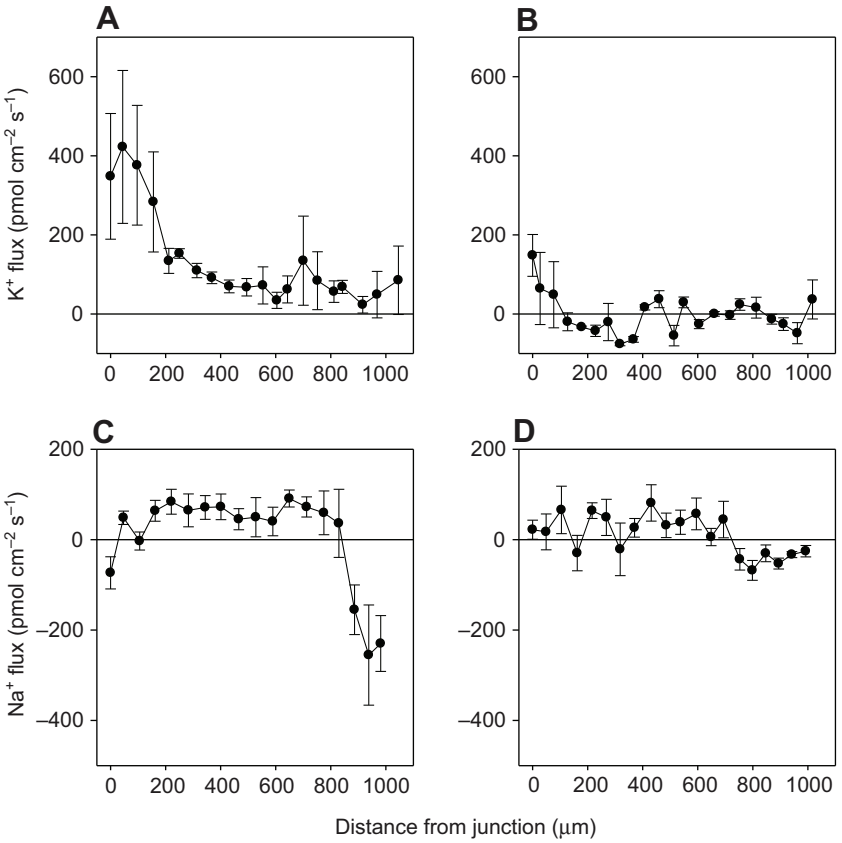

Fig. 4. K^+ and Na^+ fluxes across the middle midgut of larval *D. melanogaster*. K^+ flux across the middle midgut of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across the middle midgut of larvae reared on the (C) control diet or (D) the NaCl-rich diet. Approximately 1000 μm of the middle midgut was scanned, with 0 μm (origin) corresponding to the junction of the copper cells and the large flat cells of the middle midgut, as shown in Fig. 1. The positive values represent movement of K^+ or Na^+ from the lumen to the haemolymph (absorption) and the negative values represent movement of K^+ or Na^+ from the haemolymph to the lumen (secretion). Values are means ± s.e.m. (*N*=5–7 larvae).

more posterior regions (Fig. 5C,D). Relative to the posterior midgut of larvae reared on the control diets (Fig. 5C), Na^+ transport across both secretory and absorptive regions was reduced in the posterior midgut of larvae reared on the NaCl-rich diet (Fig. 5D).

Ureter

Our previous study examined Na^+ and K^+ transport across the main fluid secretory segment of the Malpighian tubules using the Ramsay assay (Naikkhwah and O'Donnell, 2011). 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 the lumen to the 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 (Fig. 6A). The rate of K^+ absorption was much higher (1000–2000 pmol cm^{-2} s^{-1}) within 100 μm of the gut–ureter junction. The pattern and magnitude of K^+ transport were similar in the ureters of guts isolated from larvae reared on the KCl-rich diet (Fig. 6B). Na^+ was absorbed at similar rates from the lumen to the haemolymph across the entire length of the ureters isolated from larvae reared on the control or NaCl-rich diets (Fig. 6C,D).

Lower Malpighian tubule

The lower Malpighian tubule of adult *Drosophila* has previously been shown to reabsorb fluid and K^+ from tubule lumen into the haemolymph (O'Donnell and Maddrell, 1995; Rheault and O'Donnell, 2001). We confirmed that the lower tubule of adult flies reabsorbs K^+. K^+ was reabsorbed at rates of 207±37 pmol cm^{-2} s^{-1} (*N*=4) when sampled at seven sites along the lower tubules of adult flies, comparable to the rates observed in an earlier study (Rheault and O'Donnell, 2001). By contrast, the lower Malpighian tubule of the larva secretes K^+ (Fig. 7A,B) but reabsorbs Na^+ (Fig. 7C,D). K^+ was secreted at rates of ~100 pmol cm^{-2} s^{-1} across the lower Malpighian tubule of larvae reared on control or KCl-rich diets (Fig. 7A,B). Na^+ was reabsorbed at rates of 300 to 500 pmol cm^{-2} s^{-1} across the lower tubule of larvae reared on the control or NaCl-rich diets.

Ileum

K^+ was secreted across the anterior half of the ileum of larvae reared on the control or KCl-rich diets (Fig. 8A,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 ileum of larvae reared on the control diet (Fig. 8C) but was secreted across most of the length of the anterior ileum of larvae reared on the NaCl-rich diet (Fig. 8D).

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

Fig. 9 shows the net flux across each of the gut segments described above, calculated by multiplying the flux measured by SIET (pmol cm^{-2} s^{-1}) by the surface area of the tissue (cm^2), as described in the Materials and methods. In addition, Fig. 9 presents previously published 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 four tubules (Naikkhwah and O'Donnell, 2011). 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 four tubules (Naikkhwah and O'Donnell, 2011).

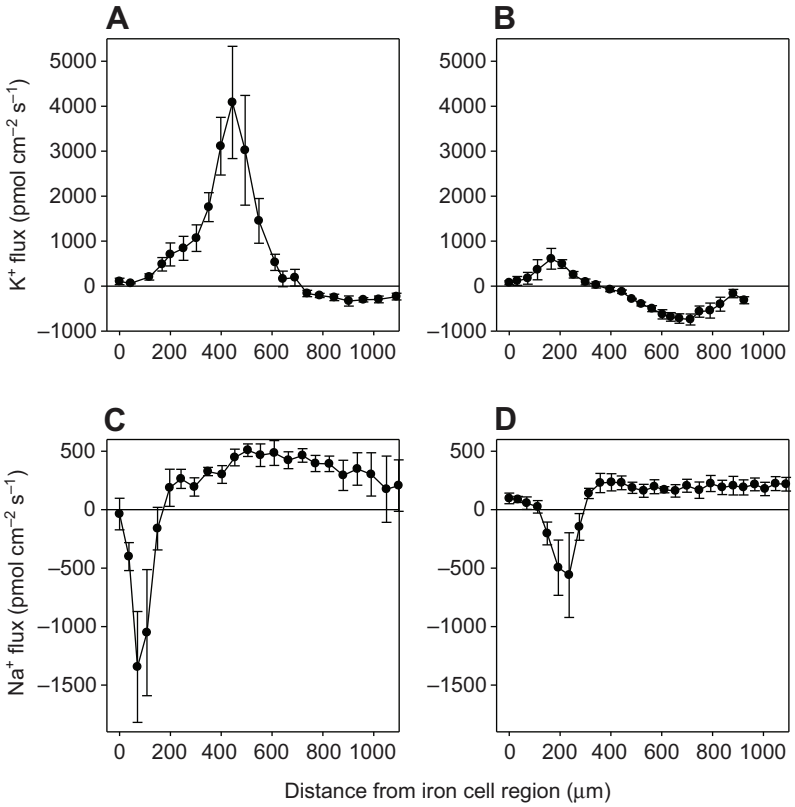

Fig. 5. K^+ and Na^+ fluxes across the posterior midgut of larval *D. melanogaster*. K^+ flux across the posterior midgut of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across the posterior midgut of larvae reared on (C) the control diet or (D) the NaCl-rich diet. Approximately 1000 μm of the anterior section of the posterior midgut was scanned, with 0 μm (origin) corresponding to the end of iron cell region shown in Fig. 1. The positive values represent movement of K^+ or Na^+ from the lumen to the haemolymph (absorption) and the negative values represent movement of K^+ or Na^+ from the haemolymph to the lumen (secretion). Values are means ± s.e.m. (*N*=5–7 larvae).

The significance of the patterns and magnitudes of K^+ and Na^+ transport shown in Fig. 9 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 *D. melanogaster*. The measurements of K^+ and Na^+ fluxes along the length of the gut show that 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 were the dramatic decreases in K^+ absorption across the middle midgut and the posterior midgut. There was an eightfold reduction in the height of the peak K^+ absorption across the anterior 400–600 μm of the posterior midgut of larvae reared on the KCl-rich diet relative to the posterior midgut of larvae reared on the control diet (Fig. 5A,B). There was also an increase in the magnitude and extent of K^+ secretion across the more posterior regions of the posterior midgut (Fig. 5A,B). As a result, K^+ was secreted across the posterior midgut as a whole in larvae reared on the KCl-rich diet (Fig. 9A). Although there was 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. In addition, K^+ secretion across the ileum was maintained or, in some larvae, dramatically increased.

In conjunction with our previous study (Naikkhwah and O'Donnell, 2011) 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 was increased in larvae reared on the KCl-rich diet and these changes involved both an increase in the capacity to secrete K^+ when haemolymph K^+ levels were increased and diuretic factors that increased K^+-secretion more than twofold. At the same time, K^+ absorption by the middle midgut was reduced and K^+ was secreted into the posterior midgut, so that there was less K^+ entering the haemolymph from the gut lumen. The latter change is noteworthy because there is presumably a chemical gradient tending to drive K^+ from the gut lumen into the haemolymph in larvae feeding on the KCl-rich diet.

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

Although the most dramatic alterations in K^+ transport in response to the KCl-rich diet were seen in the posterior midgut, the most significant qualitative alteration of Na^+ transport in response to dietary NaCl loading was seen in the anterior ileum. Whereas there was net absorption of Na^+ in the anterior ileum of larvae reared on the control diet, net secretion was seen in the anterior ileum of larvae reared on the NaCl-rich diet (Fig. 9B). Fluxes of Na^+ across the middle midgut were similar in larvae reared on control or NaCl-rich diets, and there was trend towards a reduction in Na^+ absorption across the caeca (P<0.18) and the posterior midgut (P<0.09), although the differences were not quite statistically significant (two-tailed *t*-test). It is also worth noting that Na^+ is recycled by the Malpighian tubules: the flux into the main segment was matched closely by the reabsorption of Na^+ by the lower segment (Fig. 9). It must be noted that the measurements with Na^+-selective microelectrodes were made in saline containing 20 mmol l^{-1} Na^+ in order to resolve ion fluxes using SIET. Haemolymph from larvae reared on control and NaCl-rich diets contains ~45 mmol l^{-1} Na^+ and ~80 mmol l^{-1}, respectively (Naikkhwah and O'Donnell, 2011). Our *in vitro* measurements may thus overestimate Na^+ absorption

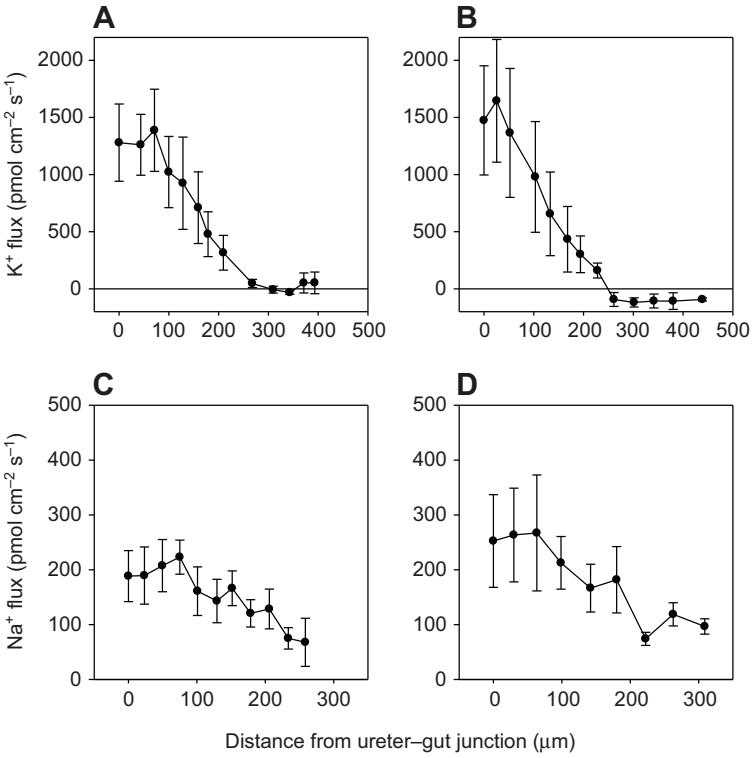

Fig. 6. K^+ and Na^+ fluxes across the ureter of larval *D. melanogaster*. K^+ flux across the ureter of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across the ureter of larvae reared on (C) the control diet or (D) the NaCl-rich diet. The origin (0 μm) corresponds to the start of the ureter at the midgut and hindgut junction. The positive values represent movement of K^+ or Na^+ from the lumen to the haemolymph (absorption) and the negative values represent movement of K^+ or Na^+ from the haemolymph to the lumen (secretion). Values are means ± s.e.m. (*N*=5–7 larvae).

and underestimate Na^+ secretion by the gut, relative to the gut *in situ*.

Our previous study (Naikkhwah and O'Donnell, 2011) 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 approximately four times the rate of Na^+ secretion by tubules of larvae reared on the NaCl-rich diet and bathed in saline. Secretion of Na^+ by the ileum (–0.19 pmol s^{-1}) 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 the 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

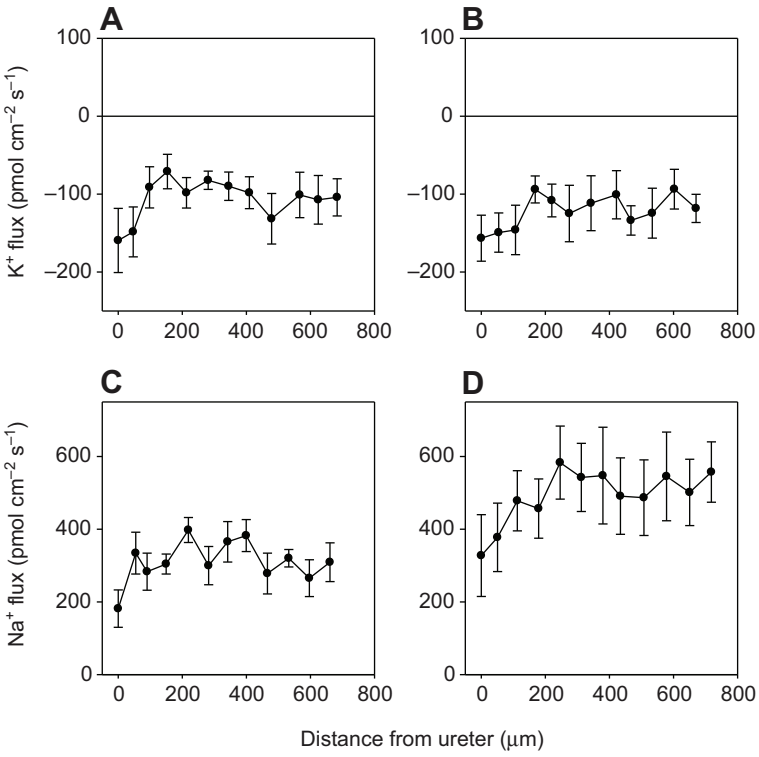

Fig. 7. K^+ and Na^+ fluxes across the lower Malpighian tubule of larval *D. melanogaster*. K^+ flux across lower tubule of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across the lower tubule of larvae reared on (C) the control diet or (D) the NaCl-rich diet. The entire length of the lower tubule was scanned, with 0 μm (origin) corresponding to the start of the junction of the lower tubule and the common ureter. The positive values represent movement of K^+ or Na^+ from the lumen to the haemolymph (absorption) and the negative values represent movement of K^+ or Na^+ from the haemolymph to the lumen (secretion). Values are means ± s.e.m. (*N*=5 larvae).

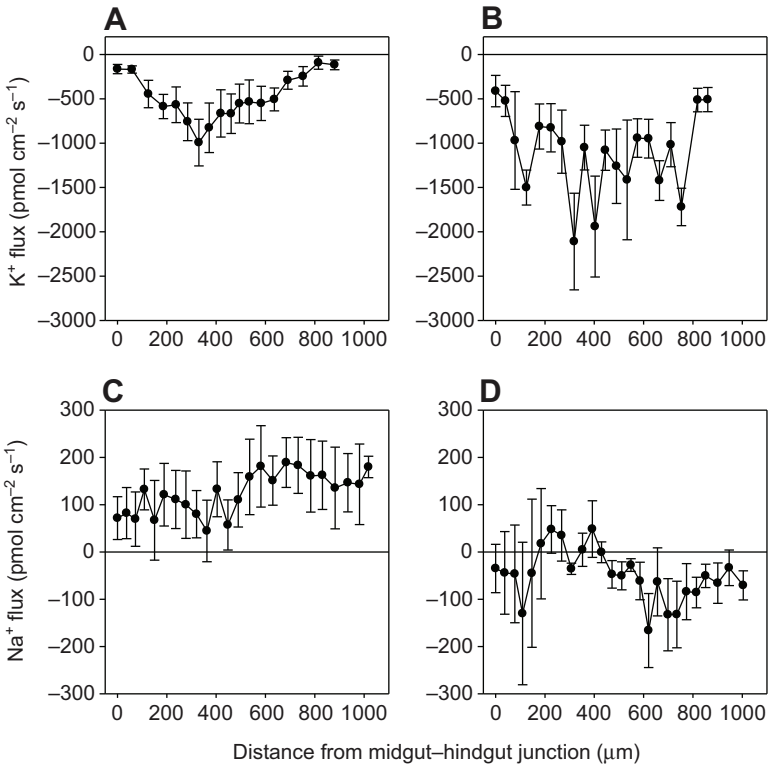

Fig. 8. K^+ and Na^+ fluxes across the anterior half of the ileum of larval *D. melanogaster*. K^+ flux across the ileum of larvae reared on (A) the control diet or (B) the KCl-rich diet. Na^+ flux across the ileum of larvae reared on (C) the control diet or (D) the NaCl-rich diet. Approximately 1000 μm of the anterior ileum was scanned, with 0 μm (origin) corresponding to the end of the pylorus (Fig. 1). The positive values represent movement of K^+ or Na^+ from the lumen to the haemolymph (absorption) and the negative values represent movement of K^+ or Na^+ from the haemolymph to the lumen (secretion). Values are means ± s.e.m. (*N*=5–7 larvae).

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 four 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 *Drosophila* larva.

In larvae of saline-tolerant *Aedes* mosquitoes, the rectum is responsible for production of hyperosmotic urine by active transport of Na^+, Cl^- and Mg^{2+} (Bradley, 1987). By contrast, the Malpighian tubules and rectum are not involved in excretion of excess Na^+ by larvae of the mosquito *Culiseta inornata* and a possible role for the anal papillae has been proposed (Garrett and Bradley, 1984a; Garrett and Bradley, 1984b). However, a role for the anal papillae of *Drosophila* larvae in salt excretion has been ruled out on the basis of measurements of papillae dimensions and on estimates of Cl^- transport inferred from patterns of silver staining (Te Velde et al., 1988). Chloride transport decreased with increasing salt concentration in the food, suggesting that the function of the papillae is absorption from dilute media rather than salt excretion.

Multiple epithelia contribute to ionoregulation

Fig. 9 summarizes the secretion and absorption of Na^+ and K^+ across the gut and Malpighian tubules of larvae reared on control or salt-rich diets. With the exception of the lower Malpighian tubule, K^+ flux across each tissue is approximately three to four times greater than the corresponding Na^+ flux. This difference may be a consequence of the high K^+ concentration of the normal diet of larval *Drosophila*, whereas Na^+ concentration in the typical diet of rotting fruit is low. Larvae may thus be faced with dietary K^+-loading as rotting fruit dries out and the concentration of K^+ increases. It should also be noted that the Na^+ flux measurements had to be made in low-Na^+ medium (20 mmol l^{-1} Na^+), so that Na^+ absorption may be higher and Na^+ secretion lower than *in situ*, when the haemolymph contains ~45 mmol l^{-1} Na^+ in larvae reared on the control diet and ~80 mmol l^{-1} in larvae reared on the Na^+-rich diet (Naikkhwah and O'Donnell, 2011).

It is also worth noting that the gut epithelia scanned in this study were bathed in saline and were not exposed, therefore, to signaling molecules in the haemolymph that might alter the rates of Na^+ and/or K^+ transport by the gut and Malpighian tubules. 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 (Naikkhwah and O'Donnell, 2011), 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 results also emphasize the differences between the same ion-transporting epithelia in the larva and the adult fly. The lower Malpighian tubule of the larva secretes K^+ and reabsorbs Na^+, whereas the lower tubule of the adult reabsorbs K^+ (O'Donnell and Maddrell, 1995). Expression of a tachykinin receptor is four times higher in tubules of the larva relative to the adult tubule, whereas expression of the receptors for the peptide diuretic hormone 31 (DH31), the products of the *capability* gene (CAPA peptides) and neuropeptide F (NPF) are significantly lower in the larva (Veenstra, 2009; Veenstra et al., 2008). The Malpighian tubule may thus perform quite different functions in the larva *versus* the adult fly.

It would be of interest in future studies to examine the effects of neuropeptides and treatments which elevate the concentrations of intracellular second messengers [e.g. thapsigargin and exogenous cAMP (Naikkhwah and O'Donnell, 2011)] on K^+ and Na^+ transport by guts isolated from larvae reared on control *versus* salt-rich diets. Immunohistochemical studies suggest that endocrine cells in the larval midgut contain seven regulatory peptides: the allatostatins A, B and C, short neuropeptide F, NPF, DH31 and the tachykinins

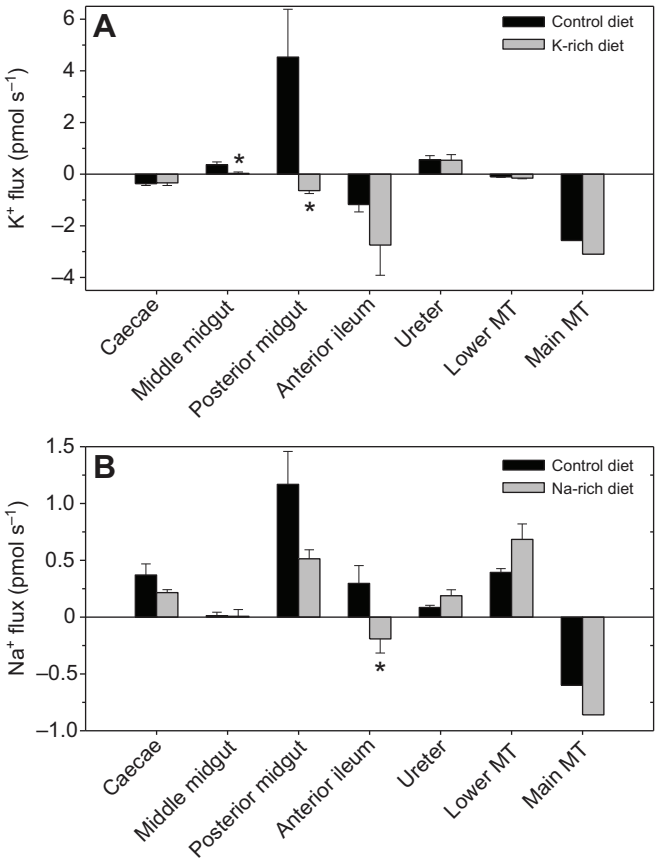

Fig. 9. Summary of K^+ and Na^+ transport across the gut and Malpighian tubules (MTs) of larval *D. melanogaster*. The positive values represent movement of K^+ or Na^+ from the lumen to the haemolymph (absorption) and the negative values represent movement of K^+ or Na^+ from the haemolymph to the lumen (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 scanning ion-selective electrode technique measurements (pmol cm^{-2} s^{-1}) and surface areas (cm^2) of each tissue segment, as described in the Materials and methods. Values are means ± s.e.m. (N=5–7 larvae). Asterisks denote significant differences (t-test, P<0.05) relative to the corresponding control. Fluxes for the main segment of the MTs were measured using the Ramsay assay (Naikkhwah and O'Donnell, 2011) and have been included for comparison.

(Veenstra, 2009). The same cells which contain short neuropeptide F in the anterior and posterior midgut are also strongly immunoreactive to antisera for the leucokinin receptor. In addition, the FlyAtlas project (Chintapalli et al., 2007) reveals that genes for the Drosokinin (leucokinin) receptor as well as the receptor encoded by CG12370, which is probably a diuretic hormone 44 (DH44) receptor (Veenstra, 2009), are greatly enriched in the larval hindgut. Similarly, the hindgut shows high expression levels for a receptor for a *Drosophila* homolog of glycoprotein A2 (GPA2) and glycoprotein B5 (GPB5) that activates the thyroid stimulating hormone (TSH) receptor in vertebrates but may act through cAMP as an antidiuretic hormone in insects (Sellami et al., 2011). Although Sellami et al. (Sellami et al., 2011) note that the small size of *Drosophila* 'makes the application of classical bioassays for anti-diuretic hormone very difficult if not impossible to perform', ion transport across the gut during diuresis or antidiuresis should be detectable by SIET. *In vitro* studies using SIET may thus allow us to determine whether specific endocrine factors modulate the transport of physiological ions (Na^+, K^+, H^+, Ca^{2+} and Cl^-) in specific regions of the larval gut. Such studies could provide information in addition to that gained through microperfusion of the gut and measurements of transepithelial potential and short circuit current (Onken et al., 2006; Onken et al., 2008). Similarly, determining the effects of drugs such as bafilomycin A1 and ouabain on ion fluxes could help to elucidate the roles of the vacuolar-type H^+-ATPase and Na^+/K^+-ATPase, respectively, in energizing Na^+ and K^+ transport across the larval gut. Measurements with extracellular pH-microelectrodes have revealed mechanisms of H^+-transport across the posterior larval midgut and the role of a bafilomycin-sensitive V-type H^+-ATPase in the basal membrane in driving luminal alkalinization in *Drosophila* (Shanbhag and Tripathi, 2005; Shanbhag and Tripathi, 2009). A lumenal concanamycin-sensitive proton pump is also important in driving acidification in the posterior midgut of the larval mosquito *Aedes aegypti* (Jagadeshwaran et al., 2010).

Recent studies have shown that *Drosophila* provides a simple and genetically tractable model for investigation of the autonomic nervous system and its visceral functions, particularly with regards to homeostatic regulation of the gastrointestinal tract (Cognigni et al., 2011; Buch and Pankratz, 2009). pH-sensitive dyes have been useful for estimating changes in acid–base status of gut segments in *Drosophila*, such as the acidification of the rectal contents in flies fed sugar-rich *versus* protein-rich diets (Cognigni et al., 2011). Future studies using the ion-selective microelectrode techniques presented in this paper and in other studies (Shanbhag and Tripathi, 2005; Shanbhag and Tripathi, 2009) will allow more detailed quantitative analysis of the interrelationships between nutritional status and the mechanisms and control of ion transport by specific regions of the *Drosophila* gut.

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