The Journal of Experimental Biology 214, 3443-3454

doi:10.1242/jeb.057828

RESEARCH ARTICLE

Salt stress alters fluid and ion transport by Malpighian tubules of *Drosophila melanogaster*: evidence for phenotypic plasticity

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Accepted 25 July 2011

SUMMARY

***Drosophila* are tolerant of high levels of dietary salt and can provide a useful model for studies of the physiology of salt stress. The effects of NaCl- and KCl-rich diets on haemolymph ionoregulation and Malpighian tubule (MT) fluid secretion, Na^+ and K^+ secretion and transepithelial potential were examined in larval and adult *Drosophila melanogaster*. K^+ concentrations in the haemolymph of adults reared on the 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 K-rich diet, K^+ concentrations increased from 23 to 75 mmol l^{-1} after 6 h, then returned to the control value within 48 h. Na^+ concentrations in the haemolymph of adults or larvae reared for 1–7 days on the NaCl-rich (0.4 mol l^{-1}) diet increased by ~50% relative to values for insects reared on the control diet. Rates of secretion of fluid, Na^+ and K^+ by MTs isolated from larvae reared on the Na-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. Evidence of phenotypic plasticity was seen in the response of MTs isolated from larvae reared on the K-rich diet for >6 h and bathed in saline containing 60 mmol l^{-1} K^+; secretion of fluid and K^+ increased by >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 Na- or K-rich diets. Secretion was further increased by addition of exogenous cAMP but not by addition of thapsigargin to the haemolymph. The results show that haemolymph ionoregulation in larvae reared on salt-rich diets involves both alterations in the basal secretion rates of Na^+ and/or K^+ as well as stimulatory effects of diuretic factors present in the haemolymph. The results suggest that such factors stimulate tubule fluid and ion secretion through increases in intracellular Ca^{2+} in response to salt stress.**

Key words: Malpighian tubule, salt stress, sodium, potassium, haemolymph ionoregulation.

INTRODUCTION

Insects are known to survive a wide variety of desiccating or hypersaline environments that pose challenges to the maintenance of homeostasis. For example, larvae of mosquitoes, chironomids and the alkali fly *Ephydra hians* are known to live in osmotically stressful hypersaline conditions (Newman, 1976; Nayar, 1969; Scudder, 1969; Phillips and Maddrell, 1974). Both the gut, particularly the hindgut, and the Malpighian tubules 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 (Bradley and Phillips, 1977). Larvae of salt-tolerant mosquitoes *Ochlerotatus taenioryhncus* 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% seawater, relative to tubules of larvae reared in dilute media (Donini et al., 2006).

Recent research has highlighted the usefulness of *Drosophila melanogaster* for studies of salt stress. Fifty percent of adult flies reared on diets containing 0.85 mol l^{-1} NaCl survive 4 days (Stergiopoulos et al., 2008), and >75% of flies survive exposure to 0.4 mol l^{-1} NaCl or KCl (Huang et al., 2002). Although *D. melanogaster* is not normally exposed to such high levels of salt stress, its advantages as a genetic model and the availability of electrophysiological methods for measurement of ion transport by both the Malpighian tubules and the gut make it an attractive species to study. Most genes that are upregulated or downregulated by salt stress are highly enriched in the Malpighian tubules and/or the hindgut, implicating these tissues in the response to salt stress (Stergiopoulos et al., 2008). In this study we have examined the effects of salt stress on haemolymph ion regulation and 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. We have also measured tubule transepithelial potential. Lastly, we have examined the effects of secretagogues on the secretion of fluid, Na^+ and K^+ by tubules isolated from larvae reared on control or salt-rich diets.

MATERIALS AND METHODS

Insects and diet preparation

The Oregon R strain of *Drosophila melanogaster* Meigen were raised on artificial diets and maintained at 21–23°C in laboratory culture. The control diet was prepared as described by Roberts and Stander (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, Oakville, Canada) dissolved in ethanol were added to the mixture. The control diet contained 36 mmol l^{-1} Na^+ and 35 mmol l^{-1} K^+. Experimental diets were prepared by the addition of salts or sucrose to the control diet so that the final concentrations were 0.4 mol l^{-1} NaCl, 0.4 mol l^{-1} KCl or 0.8 mol l^{-1} sucrose. These are referred to hereafter as Na-rich, K-rich and sucrose-rich diets, respectively. For experiments involving larvae, we used third instar larvae which had been transferred as third instars for 6, 12, 24 or 48 h to the experimental diet. Larvae that were chronically exposed to the 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 third instar.

Measurement of haemolymph volume

Haemolymph volume was estimated by a blotting technique (Folk et al., 2001). This involved weighing third instar 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 mass from the initial mass and assuming that haemolymph density was 1 mg μl^{-1}.

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 third 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 ul) of haemolymph. Haemolymph was collected from larvae and adults that had fed chronically on control, K-rich, Na-rich or sucrose-rich diets. Care was taken to collect only clear samples of haemolymph, free of any haemocytes.

Malpighian tubule dissection and Ramsay assays

Tubules were 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. The pH was adjusted to pH 7.0 with NaOH. A saline containing 60 mmol l^{-1} K^+ (60K saline) was made by equimolar substitution of KCl for NaCl. Dissections were performed using forceps as described by Dow et al. (Dow et al., 1994) and each pair of Malpighian tubules joined by a common ureter was removed and transferred using a fine glass probe to a droplet of bathing saline (20 µl) or haemolymph (5 µl) under paraffin oil in the Ramsay assay dish. Haemolymph was collected <10 min before the assay and was stirred with a glass rod prior to addition of the tubule to remove clots.

Each tubule pair was arranged so that the end of one tubule was bathed in the saline well and the end of the other tubule 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 on the Sylgard-lined bottom of the dish. The diameter (d) of the droplet was measured with an ocular micrometer under at 80× magnification, and droplet volume was calculated as $\pi d^3/6$. Fluid secretion rate (nl min^{-1}) was calculated by dividing droplet volume by the time over which it formed.

Effects of secretagogues

Ramsay assays were set up in drops of haemolymph and secreted droplets were collected after 40 min. Either cAMP (100 µmol l^{-1}) or thapsigargin (10 µmol l^{-1}) was then added and a second droplet was collected after a further 40 min.

Measurement of K^+ and Na^+ concentrations 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 that 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 minimal level 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 (ADInstruments).

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. K^+-selective and 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:

$$[\mathrm{Ion}]_{\mathrm{sample}} = [\mathrm{Ion}]_{\mathrm{c}} 10^{(\Delta V/S)}, \quad (1)$$

where $[\mathrm{Ion}]_{\mathrm{sample}}$ is the ion concentration of the droplet of haemolymph or tubule secretion, $[\mathrm{Ion}]_{\mathrm{c}}$ is the concentration in a calibration drop, ΔV is the change in potential (mV) between the sample and the same 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 concentration 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 (pmol min^{-1} $tubule^{-1}$) was calculated as the product of fluid secretion rate (nl min^{-1}) and secreted fluid K^+ or Na^+ concentration (mmol l^{-1}).

Measurements of transepithelial potential

The lumen of the main segment of the Malpighian tubule was impaled with a sharp microelectrode (R>30 MΩ) pulled from theta-glass and filled with 3 mol l^{-1} KCl. Dissected tubules were placed in saline in Petri dishes in which 100 µl drops of 125 µg ml^{-1} poly-L-lysine had previously been placed and allowed to air-dry. Tubules readily adhered to the bottom of these dishes and did not move when the microelectrode tip was advanced against the tubule wall. Microelectrodes were advanced at an oblique angle using a hydraulic micromanipulator (Narishige). Electrical potentials were recorded using the hardware and software described above for use with ion-selective microelectrodes.

Data analysis

Data were plotted using SigmaPlot (Systat Software, San Jose, CA, USA). Values are expressed as means ± s.e.m. for the indicated number of tubules (*N*). Data in which measured parameters were plotted against dietary composition or time were analyzed by one-way ANOVA with Tukey's *post hoc* multiple comparison. 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 at $P<0.05$.

RESULTS

Effects of salt-rich diets on larval mass and haemolymph volume

The mean mass of a third 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 (Fig. 1A,B). There was a significant decrease of 26 and 30% in the mass of larvae maintained on K-rich or Na-rich diets, respectively (Fig. 1A). The haemolymph volume of larvae reared on the K-rich diet was maintained but haemolymph volume of larvae reared on the Na-rich diet decreased to 52% of the control value (20% of larval mass; Fig. 1B).

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^+ (Fig. 2A). There was no significant increase in K^+ concentration in the haemolymph of adult flies maintained for as long 7 days on the K-rich diet. By contrast, there was a transient increase in K^+ concentration in the haemolymph of larvae reared on the K-rich diet (Fig. 2B). Haemolymph K^+ concentration increased more than threefold to 75 mmol l^{-1} after 6 h, remained at approximately twice the control level for the first 24 h and then returned to the control level within 48 h.

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

The increases in K^+ and Na^+ concentrations in the haemolymph of larvae reared on K-rich and Na-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 the 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

Growing larvae face considerable ionoregulatory stress when reared on salt-rich diets because of their high rates of feeding. Although third instar larvae reared on K-rich and Na-rich diets are slightly smaller than those reared on the control diet, they continue to feed as larvae, undergo metamorphosis and emerge as adults. We were particularly interested in determining whether fluid and ion transport by the Malpighian tubules of the larvae were altered in response to dietary salt stress. Therefore, we used the Ramsay assay and ion-selective microelectrodes to assess the rates of fluid and ion (Na^+ and K^+) transport by tubules isolated from third instar larvae that were reared on control or salt-rich diets.

For larvae reared on Na-rich diets, there were no significant changes in fluid secretion rate (Fig. 3A) or secreted fluid concentrations of Na^+ or K^+ (Fig. 3B) relative to tubules isolated from larvae reared on the control diet. With the exception of a reduction in K^+ flux after 6 h, 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 K-rich diet for 48 h or longer (Fig. 4). Fluid secretion rates of tubules increased after 24 h or more on the K-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 h and by 278% in the larvae that were chronically exposed to the K-rich diet (Fig. 4C).

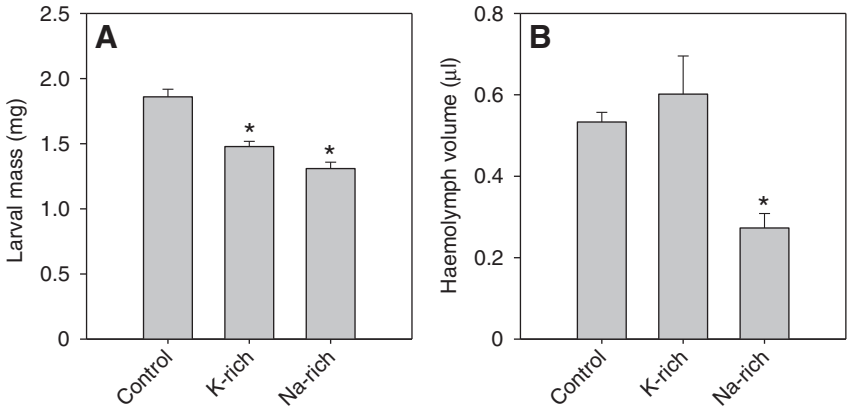

Fig. 1. *Drosophila melanogaster* larval mass and haemolymph volume. (A) Mass of third instar larvae reared on the control, K-rich (0.4 mol l^{-1}) or Na-rich (0.4 mol l^{-1}) diets. (B) Haemolymph volume in the larvae reared on control, K-rich or Na-rich diets. All data in this and subsequent figures are shown as means ± s.e.m. Asterisks denote significant differences ($P<0.05$) relative to control values. *N*=14 larvae for each diet.

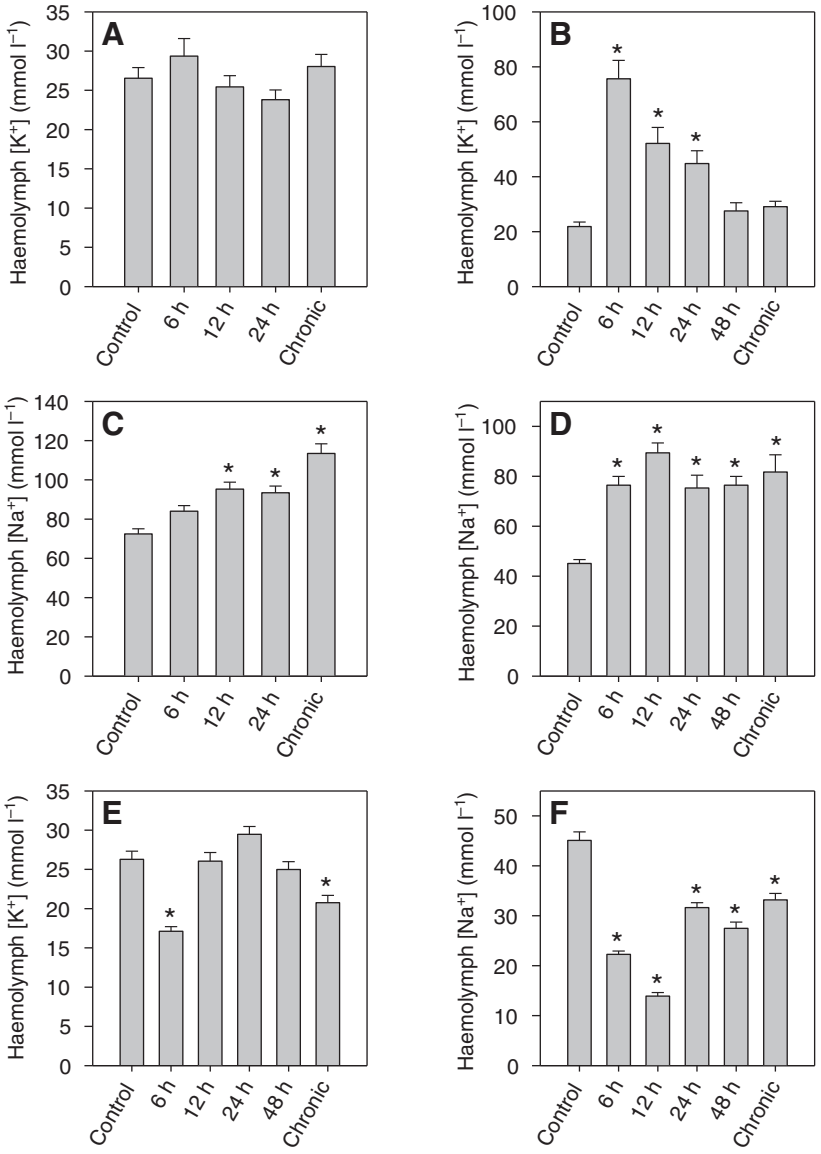

Fig. 2. K^+ or Na^+ concentrations in haemolymph of *D. melanogaster* larvae or adults reared on the control diet or after acute (6, 12, 24 and 48 h) or chronic (7 days) exposure to K-rich (0.4 mol l^{-1}), Na-rich (0.4 mol l^{-1}) or sucrose-rich (0.8 mol l^{-1}) diets. (A) K^+ concentration in haemolymph of adults on the K-rich diet. (B) K^+ concentration in haemolymph of larvae on the K-rich diet. (C) Na^+ concentration in haemolymph of adults on the Na-rich diet. (D) Na^+ concentration in haemolymph of larvae on the Na-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. Asterisks denote significant differences (P<0.05) relative to control values. N=19–22 adults or larvae per sample point.

Effects of K-rich diets on secretion of Na^+, K^+ and fluid by larval Malpighian tubules isolated in 60K saline

The effects of the K-rich diet on secretion by larval tubules were further explored by isolating the tubules in saline containing an elevated level of K^+. The rationale for these experiments was that the potassium level of the haemolymph of larvae reared on the K-rich diet is elevated for the first 24 h (Fig. 2) and that the tubules may secrete higher levels of K^+ relative to controls when the saline contains increased levels of K^+. We therefore designed a saline containing 60 mmol l^{-1} K^+, mimicking the levels present in the haemolymph of larvae reared for 6 to 24 h on the K-rich diet. The results (Fig. 5) show that the rate of fluid secretion by tubules isolated before the transfer to the K-rich diet (0 h) and set up in 60K saline did not differ from that of tubules set up in control (20 mmol l^{-1} K^+) saline (Fig. 5A). There was an increase in secreted fluid K^+ concentration and a corresponding decrease in Na^+ concentration and Na^+ flux in 60K saline in tubules isolated before the transfer to the K-rich diet (0 h) relative to tubules bathed in control saline (Fig. 5B). Importantly, there was an increase in fluid secretion rate in 60K saline for tubules isolated from larvae maintained on the K-rich diet for 6 h or longer, relative to the values at 0 h (i.e. before transfer to the K-rich diet). As a consequence, the K^+ flux of tubules isolated from larvae maintained on the K-rich diet for 6 h or longer was much greater than for tubules isolated from larvae before transfer to the K-rich diet and bathed in either control or 60K saline (Fig. 5C). K^+ flux in 60 K saline increased by 47–56% in tubules isolated from larvae maintained for 6 h or more on the K-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 K-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 pooled from larvae reared on the control diet or from larvae that had been exposed to the K-rich or Na-rich diet for 7 days or more (chronic exposure).

Fluid secretion rates of tubules isolated from larvae reared on the control diet and bathed in haemolymph collected from larvae

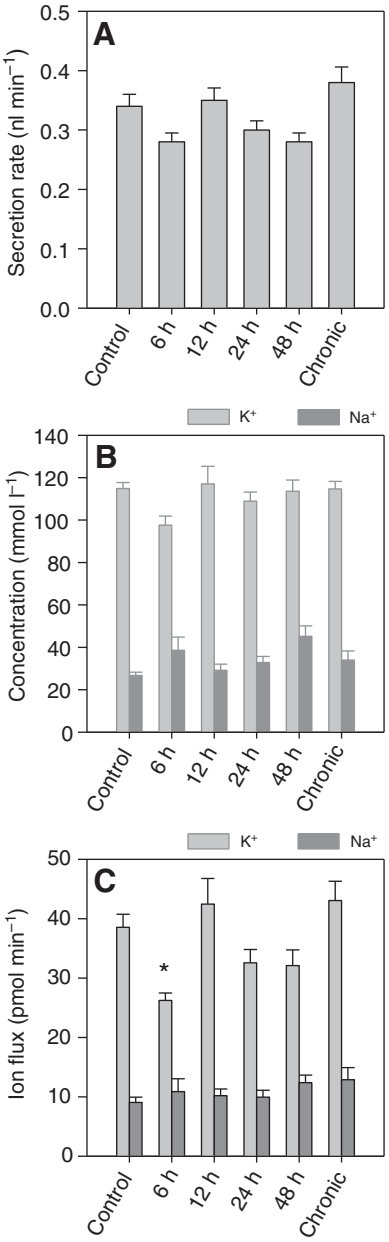

Fig. 3. The effects of acute (6, 12, 24 and 48 h) or chronic (7 days) exposure of *D. melanogaster* larvae to the Na-rich diet on: (A) Malpighian tubule fluid secretion rate, (B) the concentration of K^+ and Na^+ in the secreted fluid and (C) transepithelial flux of K^+ and Na^+ for tubules bathed in control saline. In the chronic exposure, eggs were laid on the Na-rich diet and third instar larvae were dissected ~7 days later. Asterisks denote significant differences (P<0.05) relative to values for tubules isolated from larvae reared on the control diet. N=10 tubules per sample point.

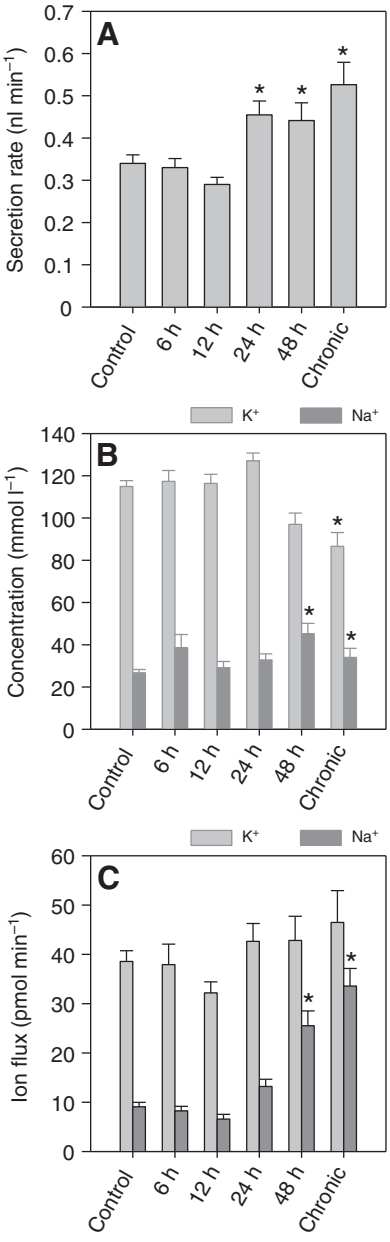

Fig. 4. The effects of acute (6, 12, 24 and 48 h) or chronic (7 days) exposure of *D. melanogaster* larvae to the K-rich diet on: (A) Malpighian tubule fluid secretion rate, (B) the concentration of K^+ and Na^+ in the secreted fluid and (C) transepithelial flux of K^+ and Na^+ for tubules bathed in control saline. Asterisks denote significant differences (P<0.05) relative to values for tubules isolated from larvae reared on the control diet. N=10 tubules per treatment.

reared on Na-rich or K-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 Na^+ concentration for tubules from larvae reared on the control diet and bathed in haemolymph collected from larvae reared on the Na-rich or K-rich diet. However, there was a small increase in the K^+ concentration of fluid secreted by tubules bathed in haemolymph collected from larvae reared on the K-rich diet (Fig. 6B). The changes in fluid secretion rate and secreted fluid K^+ concentration resulted in increases in K^+ secretion (Fig. 6C) of 95 and 111%, respectively, for tubules bathed in haemolymph collected from larvae reared on the Na-rich and K-rich diets relative to K^+ secretion by tubules bathed in haemolymph collected from larvae reared on the control diet. The changes in fluid secretion rate resulted in an increase in Na^+ secretion of 175% relative to the controls for tubules bathed in haemolymph collected from larvae reared on Na-rich diet (Fig. 6C).

The fluid secretion rate of tubules isolated from larvae reared on the Na-rich diet and bathed in haemolymph collected from larvae reared on the Na-rich or K-rich diets increased relative to the rate

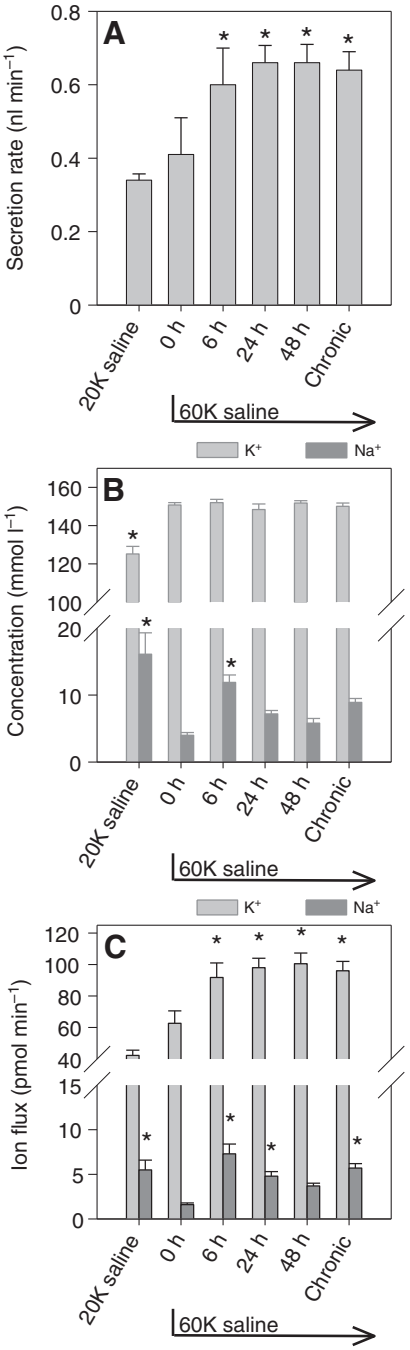

Fig. 5. The effects of acute (6, 12, 24 and 48 h) or chronic (7 days) exposure of *D. melanogaster* larvae to the K-rich diet on: (A) Malpighian tubule fluid secretion rate, (B) the concentration of K^+ and Na^+ in the secreted fluid and (C) transepithelial flux of K^+ and Na^+ for tubules bathed in control saline containing 20 mmol l^{-1} K^+ (20K saline) or 60 mmol l^{-1} K^+ saline (60K saline). Asterisks denote significant differences (P<0.05) relative to tubules isolated before transfer of the larvae from control to K-rich diet (0 h) and bathed in 60K saline. N=10 tubules per treatment.

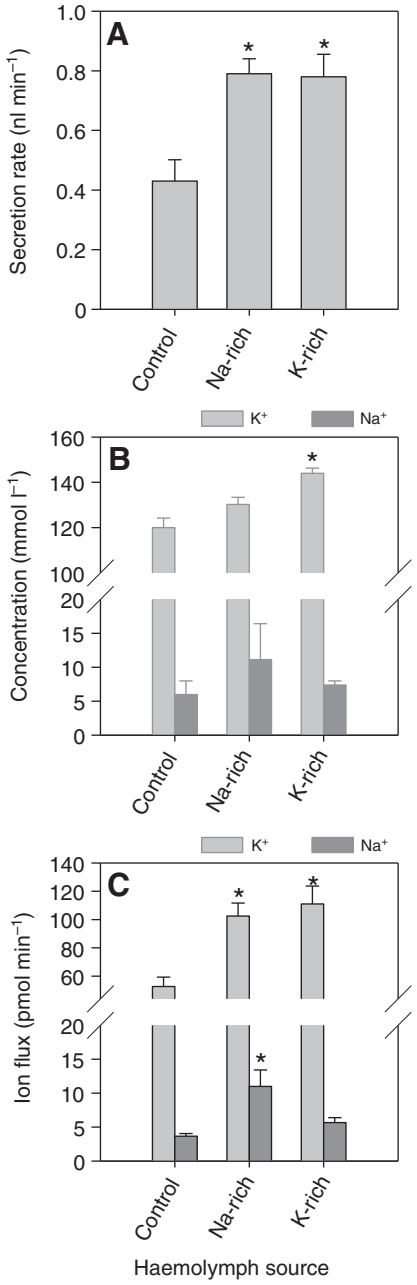

Fig. 6. The effects of haemolymph source on tubules isolated from *D. melanogaster* larvae reared on the control diet. Tubules were bathed in haemolymph collected from third instar larvae reared on the control diet or after chronic exposure to Na-rich or K-rich diet. (A) Malpighian tubule fluid secretion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

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 Na-rich or K-rich diets (Fig. 7B). For tubules bathed in haemolymph collected from larvae reared on the Na-rich diet, the changes in fluid secretion rate resulted in increases in the secretion of Na^+ and K^+ of 200 and 110%, respectively, relative to tubules bathed in haemolymph collected from larvae reared on the control diet (Fig. 7C). For tubules bathed in haemolymph from larvae reared on the K-rich diet, the corresponding increase in K^+ secretion was 57% (Fig. 7C).

The fluid secretion rate of tubules isolated from larvae reared on the K-rich diet also increased when bathed in haemolymph collected from larvae reared on the Na-rich or K-rich diets relative to the rate for tubules bathed in haemolymph collected from larvae reared on

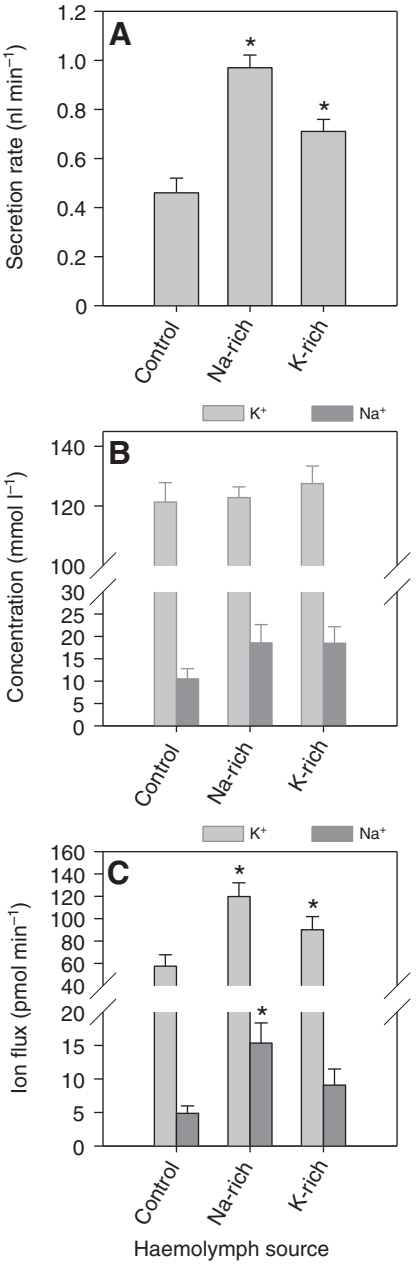

Fig. 7. The effects of haemolymph source on tubules isolated from *D. melanogaster* larvae reared on the Na-rich diet. Tubules were bathed in haemolymph collected from third instar larvae reared on the control diet or after chronic exposure to Na-rich or K-rich diet. (A) Malpighian tubule fluid secretion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

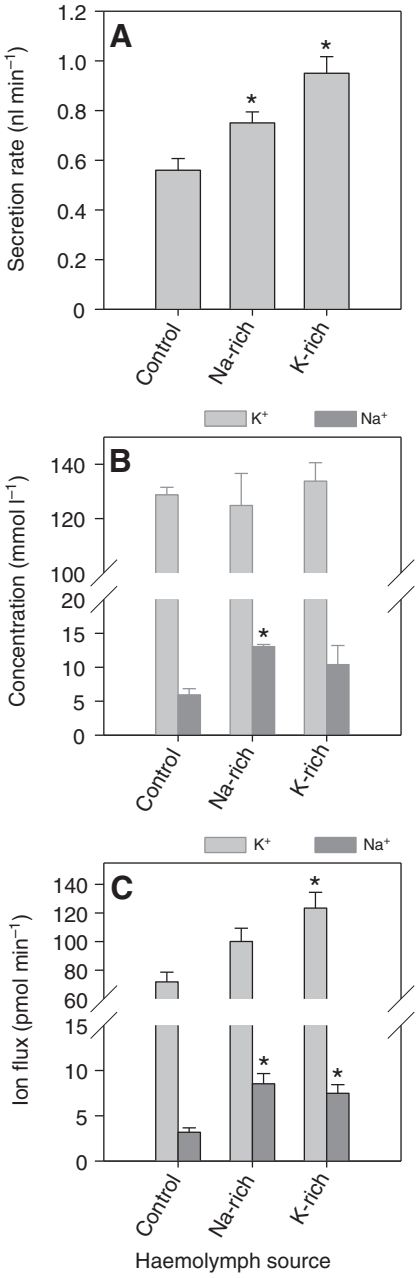

Fig. 8. The effects of haemolymph source on tubules isolated from *D. melanogaster* larvae reared on the K-rich diet. Tubules were bathed in haemolymph collected from third instar larvae reared on the control diet or after chronic exposure to Na-rich or K-rich diet. (A) Malpighian tubule fluid secretion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

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 Na-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% by tubules bathed in haemolymph collected from larvae reared on the Na-rich or K-rich diets, respectively, relative to tubules bathed in haemolymph collected from larvae reared on the control diet (Fig. 8C). K^+ secretion increased by 72% when tubules isolated from larvae reared on the K-rich diet were bathed in haemolymph collected from larvae reared on the same 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 tubules of adult *Drosophila* are mediated through the second messengers cAMP, cGMP and Ca^{2+} (Dow and Davies, 2003). cAMP and cGMP 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. In the stellate cells, the primary effect of increases in cytosolic Ca^{2+} is to increase Cl^- permeability mediated by Cl^- channels (O'Donnell et al., 1998). Sustained elevation of intracellular Ca^{2+} in the principal cells in response to stimulation with the neuropeptide capa-1 has been linked to activation of apical mitochondria and an increased supply of ATP to the V-ATPase (Terhzaz et al., 2006). Increases in cytosolic Ca^{2+} levels can be induced experimentally through addition of thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA).

We hypothesized that if V-ATPase activity or stellate cell Cl^- permeability had been increased by diuretic factors present in the haemolymph, then subsequent addition of cAMP or thapsigargin, respectively, would have little or no additional stimulatory effect (O'Donnell et al., 1996). The fluid secretion rates and fluxes of both K^+ and Na^+ increased in response to addition of cAMP to tubules isolated from larvae reared on K-rich diets and bathed in haemolymph collected from larvae reared on any of the three diets (Fig. 9A,C). The fluid secretion rate and K^+ flux increased and Na^+ flux was unchanged in response to the addition of cAMP to tubules isolated from larvae reared on the Na-rich diet and bathed in haemolymph collected from larvae reared on any of the three diets (Fig. 9B,D). The increases in flux (Fig. 9C,D) were due primarily to increases in fluid secretion rate (Fig. 9A,B) rather than increases in secreted fluid concentrations of Na^+ and/or K^+ (data not shown). These findings indicate that the tubules retain responsiveness to $0.1\,mmol\,l^{-1}$ cAMP when bathed in haemolymph isolated from larvae reared on Na-rich or K-rich diets, and suggest that such haemolymph does not contain diuretic factors that are mediated through increases in intracellular cAMP.

The fluid secretion rate and the flux of K^+ increased in response to addition of thapsigargin to tubules isolated from larvae reared on

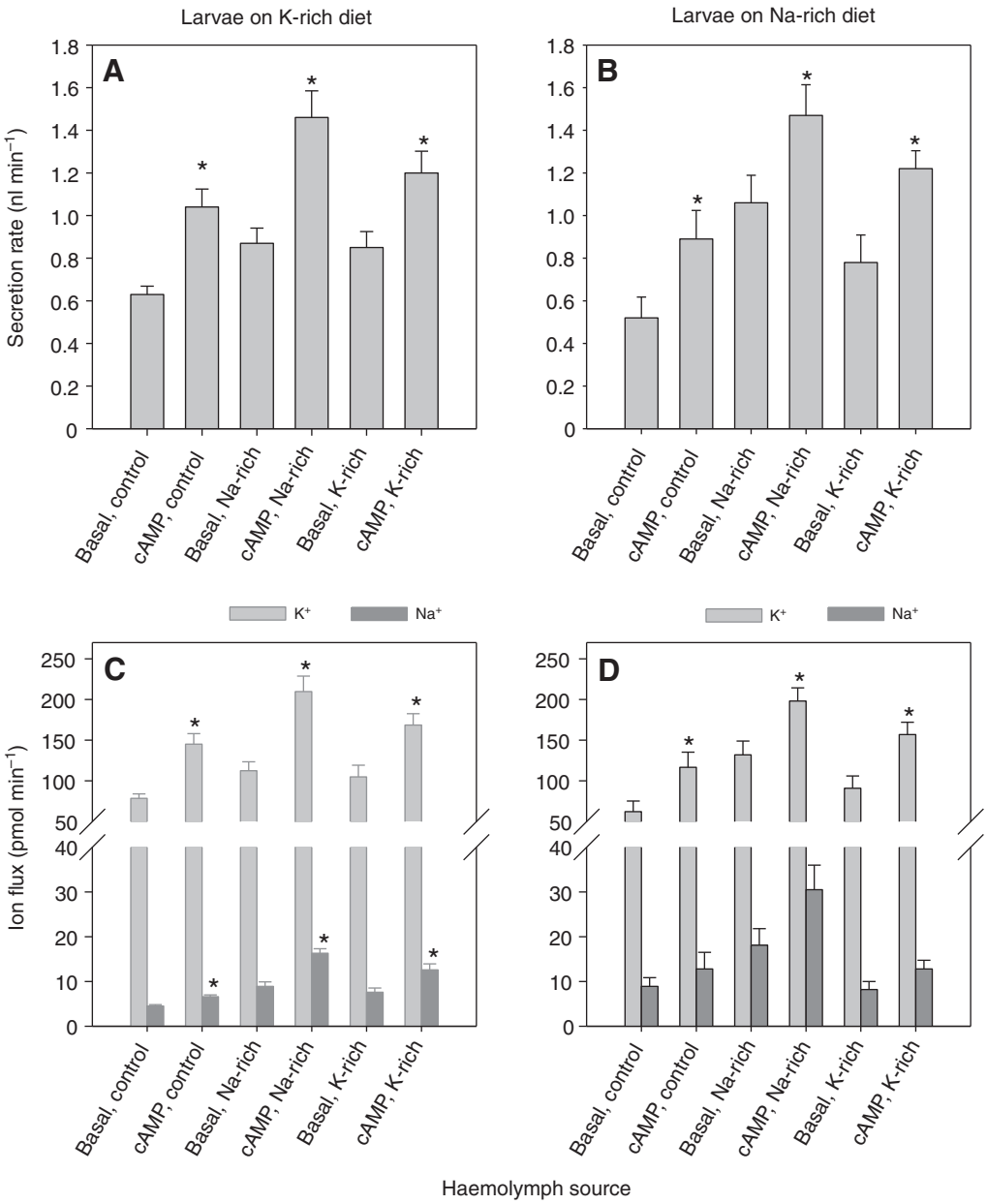

Fig. 9. The effects of cAMP and haemolymph source on fluid secretion rate (A,B) and transepithelial flux of K^+ and Na^+ (C,D) for tubules isolated from *D. melanogaster* larvae reared on the K-rich diet (A,C) or the Na-rich diet (B,D) and bathed in haemolymph collected from larvae reared on the control, K-rich or Na-rich diets. Fluxes were determined before (basal) or after stimulation with $0.1\,mmol\,l^{-1}$ cAMP (cAMP). Asterisks denote significant differences ($P<0.05$; paired *t*-test) relative to basal values. $N=6$ tubules per treatment.

K-rich or Na-rich diets and bathed in haemolymph collected from larvae reared on the control diet (Fig. 10). Fluid secretion rate and K^+ flux also increased and Na^+ flux was unchanged in response to the addition of thapsigargin to tubules isolated from larvae reared on the K-rich diet and bathed in haemolymph collected from larvae reared on the Na-rich diet (Fig. 10A,C). In addition, fluid secretion rate and K^+ flux increased in response to the addition of thapsigargin to tubules of larvae reared on the Na-rich diet and bathed in haemolymph collected from larvae reared on K-rich diets (Fig. 10B,D). The increases in flux in Fig. 10 were primarily due to an increase in fluid secretion rate rather than increases in secreted fluid ion concentrations (data not shown). By contrast, there were no increases in fluid secretion rate or K^+ flux when thapsigargin was added to tubules from larvae reared on the K-rich diet and bathed in haemolymph from the same group, or when thapsigargin was added to tubules from larvae reared on the Na-rich diet bathed in haemolymph from the same group. The results suggest that the increases in fluid secretion rate and K^+ flux in tubules of larvae reared on K-rich or Na-rich diets and bathed in haemolymph from the same group reflects the presence in the haemolymph of a factor promoting increases in intracellular Ca^{2+} in the tubule cells. As discussed below, thapsigargin may not stimulate the tubules if Ca^{2+} has previously been elevated in response to a factor in the haemolymph.

Effects of salt-rich diets on Malpighian tubule transepithelial potential

When tubules were bathed in control saline, a one-way ANOVA indicated that the transepithelial potential of tubules from larvae reared on the K-rich diet (47.1±2.9 mV; N=11) was significantly more positive than that of tubules reared on the control diet (25.0±2.0 mV; N=38). By contrast, when tubules were bathed in haemolymph collected from larvae reared on the K-rich diet, the transepithelial potential of tubules isolated from larvae reared on the control diet (34.7±3.8 mV; N=11) did not differ significantly from that of tubules of larvae reared on the K-rich diet (37.4±3.9 mV;

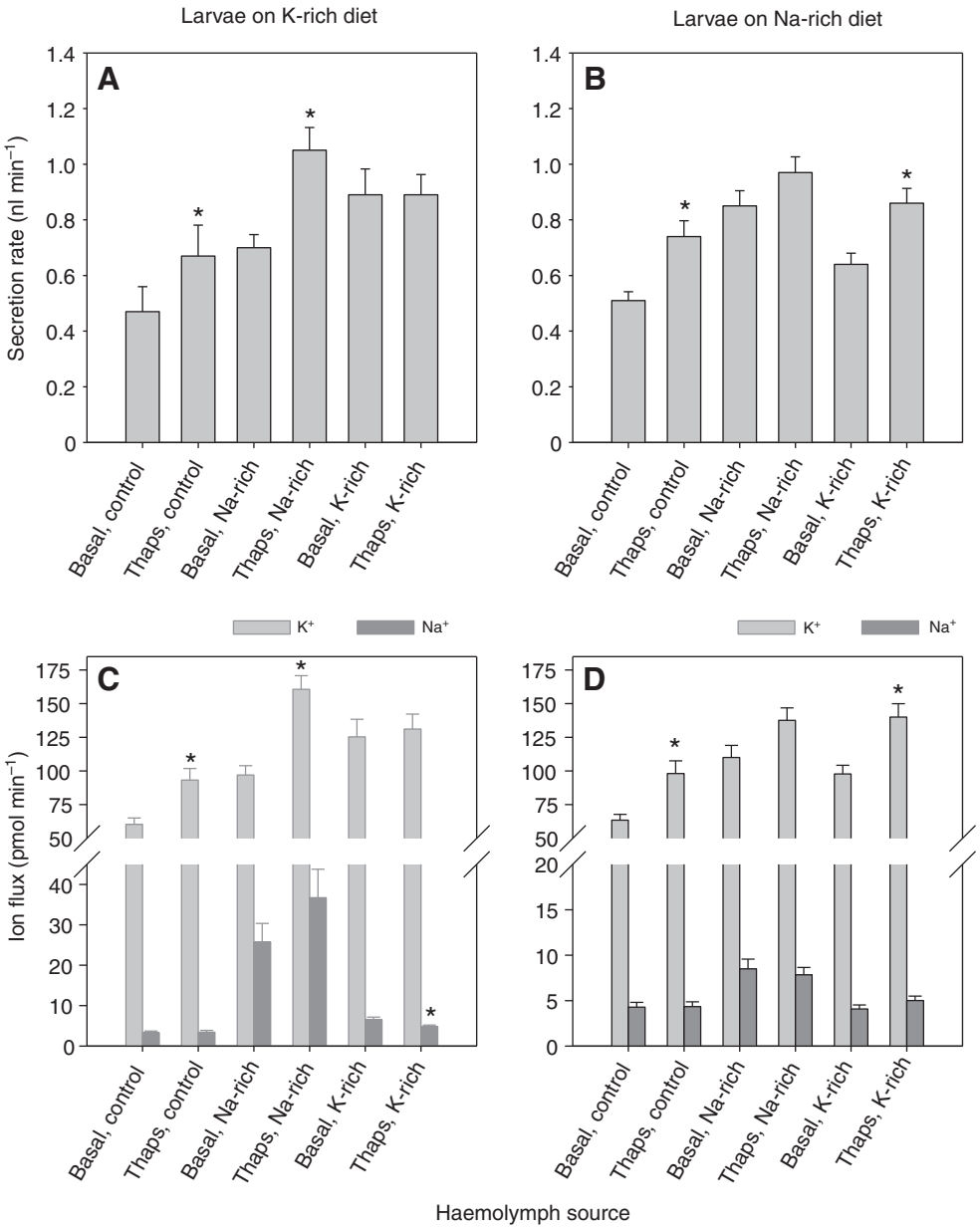

Fig. 10. The effects of thapsigargin and haemolymph source on fluid secretion rate (A,B) and transepithelial flux of K^+ and Na^+ (C,D) for tubules isolated from *D. melanogaster* larvae reared on the K-rich diet (A,C) or the Na-rich diet (B,D) and bathed in haemolymph collected from larvae reared on the control, K-rich or Na-rich diets. Fluxes were determined before (basal) or after stimulation with 10 μmol l^{-1} thapsigargin (thaps). Asterisks denote significant differences (P<0.05; paired t-test) relative to basal values. N=10–13 tubules per treatment.

N=10). The latter value is significantly more positive than the value noted above for control tubules bathed in saline.

In tubules of adult *Drosophila*, application of the peptide leucokinin I is known to collapse the transepithelial potential within a few seconds (O'Donnell et al., 1996). We wished to determine whether the larval tubules responded in a similar manner to leucokinin. The transepithelial potential of tubules isolated from larvae reared on the K-rich diet and bathed in saline was reduced from 33.2±3.1 mV (*N*=6) in control saline to 4.3±1.3 mV within 5 min of the addition of leucokinin 1 (10 μmol l^{-1}). The transepithelial potential for tubules in control saline in this experiment was slightly lower than for the tubules under the same conditions described in the preceding paragraph, presumably due to a lower room temperature (21 *versus* 23–24°C).

DISCUSSION

The results of the present study 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

Our 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}) of Na^+ or K^+. The potassium concentration was maintained at the control level in the haemolymph of adults throughout the period (up to 7 days) on the K-rich diet. In the larvae, haemolymph K^+ increased above the control level during the first 24 h on the K-rich diet and then returned to the control level by 48 h. Haemolymph volume was maintained at the control level in larvae reared on the K-rich diet. The increase in haemolymph K^+ concentration to 75 mmol l^{-1} at 6 h 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 *G. austeni*, addition of a K-rich (40 mmol l^{-1}) solution that 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 but not the neuorhaemal areas 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 12-fold increase in dietary Na^+ concentration (from 36 mmol l^{-1} in the control diet to 436 mmol l^{-1} in the diet containing an additional 0.4 mol l^{-1} NaCl), haemolymph Na^+ concentration increased after 48 h by 80% in the larvae and 53% in the adults.

Phenotypic plasticity of ion transport by Malpighian tubules of salt-stressed larvae

There were no changes in the rates of secretion of fluid, Na^+ or K^+ in Malpighian tubules isolated from larvae reared on the Na-rich diet. Although there was a sustained increase in haemolymph Na^+ concentration in these larvae, the maximum concentration was 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 (W.N. and M.J.O., unpublished observations).

By contrast, there were several dramatic changes in transport by the Malpighian tubules of larvae reared on the K-rich diet. Firstly, there was a sustained increase in fluid secretion rate after larvae were reared on the K-rich diet for 24 h or longer. Given that phenotypic plasticity can be broadly defined as the ability of an organism to change its phenotype in response to changes in the environment, our results show plasticity of tubule transport (the phenotype) in response to salt stress (the environmental change). Secondly, there was an unexpected decrease in secreted fluid K^+ concentration and a corresponding increase in Na^+ concentration when the tubules were bathed in saline containing 20 mmol l^{-1} K^+. However, the homeostatic benefits of the changes in tubule ion transport capacity were seen when the tubules were bathed in saline containing 60 mmol l^{-1} K^+, mimicking the increase in haemolymph K^+ concentration during the first 24–48 h on K-rich diet. The dramatic increase in fluid secretion rate of tubules isolated after 6 h or more on the K-rich diet and bathed in 60K saline is again indicative of phenotypic plasticity. There was an associated increase in secretion of both Na^+ and K^+ relative to tubules isolated prior to transfer to the K-rich diet. These changes indicate that there is a sustained increase in secretion of fluid, Na^+ and K^+ in tubules from larvae reared on K-rich diets. Because any endocrine factors present in the haemolymph are washed off during the 40 min 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 unstimulated tubules to eliminate K^+. The increase in the magnitude of the lumen-positive transepithelial potential in tubules isolated from larvae reared on the K-rich diet and bathed in control saline is consistent with an increase in the activity of the electrogenic V-type H^+-ATPase in the apical membrane of the tubules. An earlier microarray study did not show changes in gene expression of the V-ATPase in tubules of adult flies maintained for up to 32 h on the Na-rich diet (Stergiopoulos et al., 2008). However, expression of V-ATPase genes may be enhanced after chronic exposure (7 d) of larvae to the K-rich diet. Alternatively, the enhanced V-ATPase activity suggested by our transepithelial potential measurements may indicate post-translational modification of the protein. Chronic exposure to the K-rich diet might also promote the recruitment of V-ATPase to the apical membrane.

The more precise maintenance of K^+ concentration in the haemolymph of adults and larvae and the maintenance of haemolymph volume in larvae reared on K-rich diets indicate that *Drosophila* are more able to maintain homeostasis in response to excess K^+ relative to excess Na^+. We suggest that K stress is more physiologically relevant, in that *Drosophila* feeding on rotting K-rich fruit may be faced with increased K intake as the fruit dries out. Whereas the tubules secrete high levels of K^+ in response to increases in K^+ in the haemolymph for the first 24 h–48 h after transfer to the K-rich diet, other tissues such as the gut can play important roles in haemolymph K^+ homeostasis after 48 h (W.N. and M.J.O., unpublished observations).

The role of diuretic and/or 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* (Coast et al., 2002; Orchard and 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 haemocoel of these beetles shows that the most of the dye transported by the Malpighian tubules moves anteriorly into the midgut, indicating fluid recycling by this route. The most likely function for a diuretic factor in desert beetles, then, is clearance of metabolic wastes from the haemolymph (Nicolson, 1991). As such, the factors that stimulate fluid secretion by the Malpighian tubules *in vitro* may function as 'clearance hormones' *in vivo* (Nicolson, 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 K-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 reabsorption in the hindgut. Elevated secretion of Na^+ by larvae reared on the K-rich diet suggests that the haemolymph may contain a diuretic factor that stimulates cation transport non-selectively, consistent with the activation of the apical V-ATPase or the opening of a chloride conductive pathway. For larvae reared on Na-rich diets, there is enhanced secretion of Na^+ and also K^+ when bathed in haemolymph collected from larvae reared on the Na-rich diet. In this case, the K^+ balance may be maintained either through enhanced absorption by the midgut or reabsorption of K^+ by the hindgut.

Tubules isolated from *Drosophila* larvae reared on Na-rich or K-rich diets retained responsiveness to cAMP when bathed in haemolymph collected from larvae reared on control, Na-rich or K-rich diets. This finding suggests that the actions of the diuretic factor present in the haemolymph of larvae reared on Na-rich or K-rich diets are not mediated by cAMP. 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) selectively increases tubule secretion of Na and water, leading to the elimination of the Na load resulting from ingestion of the blood meal by the adult female mosquito. MNP is a member of the family of calcitonin-like peptides and its actions are mediated through the effects of cAMP (Coast et al., 2005).

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 Na-rich or K-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 Na-rich diet and bathed in haemolymph collected from larvae reared on the Na-rich diet. Thapsigargin did increase K^+ secretion by tubules of larvae reared on the K-rich diet when the tubules were bathed in haemolymph collected from larvae reared on control or Na-rich diets, but not when bathed in haemolymph collected from larvae reared on the K-rich diet. Thus, rearing the larvae on the Na-rich diet and bathing them in haemolymph from the same group stimulates fluid and K^+ secretion, and thapsigargin does not produce further stimulation. Similarly, rearing the larvae on the K-rich diet and bathing them in haemolymph from the same group stimulates fluid and K^+ secretion, and thapsigargin does not produce further stimulation. 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 salt-rich diets may act through increases in intracellular Ca^{2+}. The effects of both leucokinins (O'Donnell et al., 1998; Terhzaz et al., 1999) and tyramine (Blumenthal, 2003) are mediated through an increase in intracellular Ca^{2+} in the stellate cells. The increase in intracellular Ca^{2+} in turn leads to an increase in transepithelial chloride permeability and a consequent collapse of the transepithelial potential. Our results showed that transepithelial potential of tubules from larvae reared on the K-rich diet also collapses in response to leucokinin. It seems unlikely that the stimulatory effect of haemolymph from larvae reared on the K-rich diet involves kinins or tyramine because the transepithelial potential of isolated tubules bathed in haemolymph remains more positive than that of control tubules bathed in saline. Our working hypothesis, therefore, is that some factor in the haemolymph of larvae reared on the K-rich diet increases fluid secretion rates through an increase in Ca^{2+} levels primarily in the principal cells, although a small increase in Ca^{2+} within the stellate cells may also occur. Possible candidates for this stimulatory factor include two peptides that are encoded by the capability gene in *Drosophila* (capa-1 and capa-2) (Kean et al., 2002). It is worth noting that the capa peptides increase intracellular Ca^{2+} levels in both the principal cells and the stellate cells (Kean et al., 2002). Thapsigargin also leads to an elevation of Ca^{2+} in both principal and stellate cells (Rosay et al., 1997). The lack of stimulation of fluid secretion and K^+-secretion by thapsigargin in tubules bathed in haemolymph of larvae reared on the K-rich diet may indicate that Ca^{2+} has been elevated sufficiently for maximal stimulation of the tubules by the haemolymph factor and that any further increases in Ca^{2+} in response to thapsigargin thus produce no further stimulation.

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

The rate of transport of K^+ by the Malpighian tubules isolated from larvae reared on the K-rich diet and bathed in haemolymph from the same group is ~500 pmol min^{-1} (125 pmol min^{-1} $tubule^{-1}$ × four tubules). The haemolymph K^+ content in third instar larvae is approximately 18 nmol (25 mmol l^{-1} × 0.71 µl). If the tubules in larvae reared on K-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 four tubules in ~36 min. The rate of Na^+ transport by the four tubules of larvae reared on Na-rich diets is ~60 pmol min^{-1} (15 pmol min^{-1} $tubule^{-1}$ × four tubules) and the haemolymph Na^+ content is ~53 nmol (75 mmol l^{-1} × 0.71 µl). For Na^+ then, the clearance time is ~880 min. These calculations suggest that, although 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 Na-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. It will also be of interest to examine the role of compatible osmolytes such as proline during salt stress in *Drosophila*, given the important role such compounds play in mosquito larvae exposed to hyperosmotic media (Patrick and Bradley, 2000).

ACKNOWLEDGEMENTS

This study was supported by the Natural Sciences and Engineering Research Council of Canada Discovery and equipment grants to M.J.O.

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