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RESEARCH ARTICLE

Ammonium secretion by Malpighian tubules of *Drosophila melanogaster*: application of a novel ammonium-selective microelectrode

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SUMMARY

Ammonia is a toxic nitrogenous waste product of amino acid metabolism that may accumulate to high levels in the medium ingested by larvae of the fruit fly *Drosophila melanogaster*. Here we report measurements of haemolymph NH_4^+ concentration and the secretion of NH_4^+ by the Malpighian (renal) tubules. Measurement of NH_4^+ concentrations in secreted droplets is complicated either by the requirement for large sample volumes for enzymatic assays or by the inadequate selectivity of NH_4^+-selective microelectrodes based on nonactin. We have developed a novel liquid membrane NH_4^+-selective microelectrode based on a 19-membered crown compound (TD19C6), which has been used previously in ammonium-selective macroelectrodes. In conjunction with an improved technique for correcting for interference of potassium, NH_4^+-selective microelectrodes based on TD19C6 permit accurate measurement of ammonium concentration in haemolymph samples and nanolitre droplets of fluid secreted by the Malpighian tubules of *D. melanogaster*. The results indicate that active secretion of ammonium into the Malpighian tubule lumen is sufficient to maintain concentrations of ~1 mmol l^{-1} ammonium in the haemolymph of larvae reared on diets containing 100 mmol l^{-1} ammonium chloride.

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INTRODUCTION

Insects are predominantly uricotelic, consistent with the Baldwin–Needham hypothesis that terrestrial organisms excrete less-soluble nitrogenous excretion products, such as urea, uric acid or allantoin, as a means of water conservation (Baldwin and Needham, 1934). Ammonia is generally considered to be the most toxic, but also the most soluble, of the nitrogenous wastes (Withers, 1992). However, ammonia is excreted by cockroaches, blowflies and mosquitoes (Mullins and Cochran, 1973; Prusch, 1972; Scaraffia et al., 2005), and significant amounts of ammonium urate are excreted by the desert locust *Schistocerca gregaria* (Phillips et al., 1994). Some insects can excrete a range of nitrogenous end products simultaneously, including ammonia, urea, uric acid and allantoin (Cochran, 1985), and alterations in diet may shift the relative abundances of these nitrogenous wastes (Baldwin and Needham, 1934).

Ammonia (NH_3) is a weak base that exists in two forms: neutral ammonia (NH_3) and ionic ammonium (NH_4^+). Throughout this paper, ammonia refers to both NH_3 and NH_4^+. The pK_a is ~9.3, so that >99% of ammonia is in the form of NH_4^+ at physiological pH (~7) (Emerson et al., 1975). Because the hydrated radius of NH_4^+ is similar to that of K^+, NH_4^+ may compete with K^+ for permeation through membrane K^+ transporters, with consequent effects on transmembrane potentials and intracellular ion concentrations (Martinelle and Häggström, 1993; Moser, 1987). In addition, ammonia may perturb cellular function through effects on extracellular and intracellular pH. There are thus multiple mechanisms of ammonia toxicity, and concentrations of 0.5 to 5 mmol l^{-1} in the blood or haemolymph are lethal to many invertebrates and vertebrates (Withers, 1992).

Ammonia concentrations have been measured in the haemolymph of only a few insect species. Reported values range from 0.8 mmol l^{-1} for larvae of the tobacco hornworm, *Manduca sexta* (Weihrauch, 2006), to 16 mmol l^{-1} in larvae of the sheep blowfly *Lucilia cuprina* (Marshall and Wood, 1990). Ammonia is removed from the haemolymph by secretion across the Malpighian tubules and rectum of locusts and the rectum of larval blowflies (Marshall and Wood, 1990; Prusch, 1974; Stagg et al., 1991; Thomson et al., 1988). For example, Malpighian tubules of the desert locust, *Schistocerca gregaria*, secrete fluid containing 5 mmol l^{-1} ammonium, fourfold above the concentration in the haemolymph (Stagg et al., 1991). Adults of the yellow fever mosquito, *Aedes aegypti*, excrete ammonia following ingestion of blood or ammonia-rich solutions (Scaraffia et al., 2005), although the tissues responsible are unknown. In addition, serotonin-stimulated Malpighian tubules of *Rhodnius prolixus* isolated in saline containing ammonium as the sole cation secrete fluid at 40% of rate seen in (Na, K)-replete saline, suggesting that NH_4^+ can be a substrate for K^+ or Na^+ transporters in the basolateral and apical membranes (Maddrell, 1969). Data from multiple studies thus suggest that insect Malpighian tubules may secrete ammonium.

Insects that feed on decaying, protein-rich food likely ingest ammonia derived from ammonifying bacteria and/or fungi through a process known as ammonification (Payne, 1973). In addition, larval stages may also be exposed to and/or mature in a high ammonia environment, which may provide selective pressure for ammonia tolerance. For instance, ammonia concentrations in the diet of *Drosophila* cultures increase from ~10 mmol l^{-1} after 4 days to ~30 mmol l^{-1} after 20 days (Borash et al., 1998). Whole-body ammonia content of larvae reared on dietary medium containing

370 mmol l^{-1} ammonia was three times above that of larvae fed a control diet (Borash et al., 2000). Survival during exposure to high concentrations of ammonia in the diet suggests that both larvae and adults are tolerant of the toxic effects of ammonia. In addition, larvae fed on increasing doses of NH_4Cl (~0.25–0.5 mol l^{-1}) showed the greatest fitness when assayed on ammonia-supplemented food (Borash et al., 2000). These data suggest that such larvae increase the capacity to detoxify or excrete ammonia. *Drosophila* larvae may thus provide a useful model system for studies of ammonia transport by the excretory organs.

One challenge for such studies is the difficulty of measuring ammonia concentrations in the small droplets of fluid secreted by *Drosophila* tubules set up in the Ramsay fluid secretion assay. A single Malpighian tubule may secrete ~20 nl of fluid over a period of 45 min, but conventional colourometric or enzymatic techniques for measuring ammonia concentrations require sample volumes of 20 µl or more. Ion-selective microelectrodes are suitable for measurement of ion concentrations in nanolitre volumes, and we have previously measured concentrations of Na^+, K^+, $Ca^{2+}$and H^+ in fluid secreted by *Drosophila* tubules (O'Donnell and Maddrell, 1995). However, current ammonium-selective microelectrodes are based on liquid membranes containing the antibiotic nonactin and are subject to interference from both K^+ and Na^+. Nonactin-based microelectrodes are approximately four times more selective for NH_4^+ compared with K^+ and ~100 times more selective for NH_4^+ compared with Na^+. Detection limits of 0.1 mmol l^{-1} NH_4^+ in crayfish saline containing 5.4 mmol l^{-1} K^+ have been reported, but the limit in a simulated intracellular saline containing 194 mmol l^{-1} K^+ is ~5 mmol l^{-1} (Fresser et al., 1991). Given that fluid secreted by *Drosophila* tubules contains ~120 mmol l^{-1} K^+ and ~30 mmol l^{-1} Na^+ (O'Donnell and Maddrell, 1995), nonactin-based electrodes are of limited use for analysis of NH_4^+ secretion by Malpighian tubules.

Use of electrodes based on nonactin in physiological solutions typically requires compensation for interference from both Na^+ and K^+. Studies with macroelectrodes have shown that membranes containing polyvinyl chloride and a novel ionophore based on a 19-membered crown compound 2,5,12,15,22,26-hexaoxaheptacyclo[25.4.4.$4^{6.11}4^{16.21}$.$0^{1.27}$.$0^{6.11}$.$0^{16.21}$]-tritetracontane (TD19C6) have much greater selectivity towards sodium than do membranes based on nonactin (Suzuki et al., 2000). Thus, use of NH_4^+-selective microelectrodes based on this compound offers the possibility that a complex calculation to correct for the effects of interfering ions could be reduced to one in which only interference from K^+ needs to be considered. In this paper, we describe both the development of a novel ammonium-selective microelectrode based on TD19C6 and an improved method of correcting for the effects of interference by physiological levels of potassium. We then use these microelectrodes to assess haemolymph ammonia concentrations and ammonia secretion by isolated Malpighian tubules of *Drosophila melanogaster*.

MATERIALS AND METHODS

Fly stocks and culture

Stocks of the Oregon R strain of *Drosophila melanogaster* Meigen 1830 were maintained at room temperature (21–23°C) on medium containing (in g): 100 sucrose, 18 agar, 1 potassium dihydrogen orthophosphate, 8 sodium potassium tartrate tetrahydrate, 0.5 NaCl, 0.5 $MgCl_2$, 0.5 $CaCl_2$, 0.5 ferric sulphate, 50 dry active yeast and 7.45 ml l^{-1} 10% tegosept in EtOH and 10 ml l^{-1} acid mix (11 parts H_2O, 10 parts propionic acid and 1 part of 85% *o*-phosphoric acid diluted with 1 litre of deionized water). Ammonia-rich medium was prepared by addition of NH_4Cl to the control diet.

Dissections and Ramsay assay

Ion concentrations were measured in droplets of fluid secreted by Malpighian tubules set up in a modified Ramsay assay (Dow et al., 1994). Briefly, pairs of Malpighian tubules connected by the common ureter were removed from third instar *D. melanogaster* larvae. Dissections were performed in saline containing (in mmol l^{-1}): 117.5 NaCl, 20 KCl, 2 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 8.6 HEPES, 20 glucose and 10 glutamine. Saline pH was titrated to pH 7.0 with NaOH. Salines containing ammonia were prepared by equimolar substitution of NH_4Cl for NaCl.

Preliminary measurements with K^+-selective and Na^+-selective microelectrodes indicated that the haemolymph of third instar *D. melanogaster* larvae contained 26.5±0.6 mmol l^{-1} K^+ and 34.9±0.8 mmol l^{-1} Na^+ (N=20), similar to values determined previously (Naikkhwah and O'Donnell, 2011). Malpighian tubules were therefore bathed in 'haemolymph-like' *Drosophila* saline, which contained 25 mmol l^{-1} K^+ and 40 mmol l^{-1} Na^+. In experiments using saline that mimicked the Na^+ and K^+ concentrations of haemolymph, ionic strength was maintained by equimolar substitution of NH_4^+ by *N*-methyl-D-glucamine (NMDG). Two stock solutions contained (in mmol l^{-1}): 29.8 NaCl, 25 KCl, 82.7 NMDG Cl, 2 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$ and 8.6 HEPES, and 40 NH_4Cl; 29.8 NaCl, 25 KCl, 42.7 NMDG Cl, 2 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$ and 8.6 HEPES, respectively. Salines containing 0–40 mmol l^{-1} NH_4^+ were prepared from mixtures of the two stock solutions. All salines were titrated to pH 7.0.

Each pair of isolated tubules was transferred into a 20 µl bathing saline droplet held in a shallow well in a Sylgard®-lined Petri dish filled with paraffin oil. One tubule remained in the saline droplet and the other was pulled out of the droplet and wrapped around a 0.15 mm diameter steel pin so that the secreted fluid emerged from the cut end of the common ureter (Dow et al., 1994). Secreted fluid droplets were removed after 45 min and the diameter (d) was recorded using an eye-piece micrometer. Secreted droplet volume was calculated as $\pi\ d^3/6$ and secretion rate (nl min^{-1}) was calculated by dividing droplet volume by the time (min) over which it had formed.

Ion-selective microelectrodes

K^+-selective microelectrodes were prepared using a cocktail based on the K^+ ionophore valinomycin (Potassium ionophore I – cocktail B, Fluka, St Louis, MO, USA). Na^+-selective microelectrodes were prepared using a cocktail based on 10% (w/v) sodium ionophore X, 0.25% Na tetraphenylborate and 89.75% *o*-nitrophenyl octyl ether (NPOE) (Messerli et al., 2008). Ammonium-selective microelectrodes were based on cocktails that contained different proportions of TD19C6, lipophilic salt and solvent, as described in the Results.

Micropipettes were pulled from borosilicate glass capillaries using a vertical puller (Narishige, Tokyo, Japan) and lightly silanized with 0.2 µl dichlorodimethylsilane placed in a 10 cm glass Petri dish and inverted over batches of 14 micropipettes on a hot plate at 200°C. The low level of silanization rendered the glass sufficiently hydrophobic to retain the hydrophobic ionophore cocktail and prevent its displacement by capillary rise of aqueous solutions, but not so hydrophobic as to allow capillary rise of paraffin oil into the tip when the microelectrode was used for measurements of fluid droplets collected in the Ramsay assay. Silanized micropipettes were backfilled with 100 mmol l^{-1} NH_4Cl for ammonium microelectrodes, 150 mmol l^{-1} NaCl for sodium microelectrodes, and 150 mmol l^{-1} KCl for potassium microelectrodes and the reference microelectrodes. A column of ionophore cocktail ~200 µm long was

drawn into the tip of the microelectrode using negative pressure applied from a 25 ml syringe connected to the back of the microelectrode through plastic tubing and a MEH900S microelectrode holder (WPI, Sarasota, FL, USA). Microelectrodes were conditioned in solutions containing 15 mmol l^{-1} of the ion of interest for ~30 min prior to their use. Micromanipulators were used to insert ion-selective and reference microelectrodes into 20 µl droplets of calibration solutions under paraffin oil. The tip of each ion-selective microelectrode was broken to a diameter of ~5 µm by touching it against the shank of the reference microelectrode. Tip breakage reduced the 95% response time for the ion-selective microelectrodes, as described in the Results. Slopes of K^+-selective and Na^+-selective microelectrodes were determined in calibration solutions of constant ionic strength (150 mmol l^{-1}), similar to that of the saline and the fluid secreted by the Malpighian tubules (Linton and O'Donnell, 1999). K^+-selective and Na^+-selective microelectrodes were calibrated in mixtures of NaCl and KCl in which the sum of Na^+ and K^+ concentrations was 150 mmol l^{-1}. Slopes of K^+-selective microelectrodes were 54 mV between 150 and 15 mmol l^{-1} K^+ and 58 mV between 15 and 1.5 mmol l^{-1} K^+. Slopes of Na^+-selective microelectrodes were 62 mV between 150 and 15 mmol l^{-1} K^+ and 54 mV between 15 and 1.5 mmol l^{-1} Na^+. Slopes of NH_4^+-selective microelectrodes based on TD19C6 are reported in the Results.

Precise correction for the interfering effects of K^+ on the NH_4^+-selective microelectrodes required that concentrations of both ions be determined simultaneously. A chlorided silver wire inserted into the back of each barrel was connected to one of two channels on a high impedance (>10^{13} Ω) input amplifier (A-M Systems, Sequim, WA, USA) connected to a data acquisition system (PowerLab, ADInstruments, Sydney, Australia) running Chart software (version 5). The following equation was used to calculate concentrations from voltage traces for each ion-selective microelectrode (ISME):

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

where $[\mathrm{Ion}]_{\mathrm{sample}}$ represents the ion concentration in the sample, $[\mathrm{Ion}]_{\mathrm{cal}}$ represents an ion concentration in the calibration solution, ΔV represents the difference in voltage recorded between the sample and the calibration solution, and S represents the slope of the electrode. Although ion-selective electrodes measure ion activity and not concentration, data can be expressed in terms of concentrations if it is assumed that the ion activity coefficient is the same in calibration and experimental solutions. This assumption is appropriate for haemolymph samples and for fluids secreted by Malpighian tubules, both of which are of similar ionic strength to the calibration solutions used in this study. For ease of comparison with previous studies evaluating NH_4^+-selective microelectrodes (Fresser et al., 1991) and with studies in which ionic concentrations in fluids secreted by Malpighian tubules were measured by X-ray microanalysis or atomic absorption spectrophotometry (Clark and Bradley, 1996; Hopkin et al., 2001; Williams and Beyenbach, 1983), we have expressed our data in units of concentration.

Measurements of ion concentrations in the haemolymph and the diet

Third instar *D. melanogaster* larvae were rinsed with deionized water on an absorbent pad and allowed to dry for at least 5 min. Using forceps, the cuticle of each larva was torn under paraffin oil and haemolymph was immediately drawn into a pipette and expelled under paraffin oil in another dish for measurements using ISMEs.

Samples of diet that initially contained 0, 30 or 100 mmol l^{-1} NH_4^+ were collected from regions of the culturing vials in which ~100 larvae had been feeding for 8 days. Samples of the diet were placed under oil for measurements with K^+-selective and NH_4^+-selective microelectrodes.

Selectivity coefficients

Selectivity coefficients are a measure of the ability of an ISME to distinguish an interfering ion from the ion of interest. Selectivity coefficients of NH_4^+-selective microelectrodes based on TD19C6 for various interfering ions were determined initially using the separate solutions method. Solutions of each interfering ion (100 mmol l^{-1} of the chloride salts) were tested against a 100 mmol l^{-1} NH_4Cl calibration solution. The Nicolsky–Eisenman equation was then used to calculate selectivity coefficients (Ammann, 1986):

$$K_{ij} = 10^{\frac{(V_j - V_i)}{58.74}} \cdot \frac{a_i}{\left(a_j\right)^{z_i/z_j}}, \quad (2)$$

where K_{ij} is the selectivity coefficient for the primary ion i relative to the interfering ion j, V_j and V_i are the voltages measured in the 100 mmol l^{-1} solutions of j and i, respectively, a_i and a_j are the corresponding activities and z_i and z_j are the corresponding valences. Ion activity (a_i) was calculated from concentration (c_i) as $a_i = \gamma c_i$, where γ is the corresponding activity coefficient. The activity coefficients used in these calculations were as follows (γ ion): 0.765 NH_4^+, 0.766 K^+, 0.776 Na^+, 0.754 Li^+, 0.761 Rb^+, 0.752 Cs^+, 0.517 Ca^{2+}, 0.535 Mg^{2+}, 0.81 NMDG and 0.755 $CH_3NH_3^+$ (Chen et al., 2009; Goldberg and Nuttall, 1978; Macaskill and Bates, 1986; Robinson and Sinclair, 1934; Verrall, 1975).

Ammonium concentrations were corrected for the interference produced by potassium using the following equation:

$$[\mathrm{NH_4^+}]_{\mathrm{corrected}} = [\mathrm{NH_4^+}]_{\mathrm{uncorrected}} - (K_{\mathrm{NH4,K}})[\mathrm{K^+}], \quad (3)$$

where $[\mathrm{NH_4^+}]_{\mathrm{uncorrected}}$ is the ammonium concentration calculated using Eqn 1. Derivation of Eqn 3 from the Nicolsky–Eisenmann equation is described in the Appendix.

As described in the Results, the selectivity coefficient for NH_4^+-selective microelectrodes based on TD19C6 towards K^+ ($K_{\mathrm{NH4,K}}$) varied with the concentration of NH_4^+. This led to errors in $[\mathrm{NH_4^+}]_{\mathrm{corrected}}$ when a single value for $K_{\mathrm{NH4,K}}$ was used to correct for K^+ interference. We therefore developed an improved method of correcting for K^+ interference by determining $K_{\mathrm{NH4,K}}$ at each of five to nine different values of $[NH_4^+]$ in the presence of a fixed concentration of interfering ion (K^+). Ionic strength of calibration solutions was maintained at 170 mmol l^{-1} by equimolar substitution of NaCl for NH_4Cl. Because both the uncorrected and corrected ammonium concentration as well as potassium concentrations were known in the calibration solutions, Eqn 3 could be rearranged to solve for the selectivity coefficient:

$$K_{\mathrm{NH4,K}} = \left([\mathrm{NH_4^+}]_{\mathrm{corrected}} - [\mathrm{NH_4^+}]_{\mathrm{uncorrected}}\right)/[\mathrm{K^+}]. \quad (4)$$

The regression equation for the line fitting $\log K_{\mathrm{NH4,K}}$ to $[NH_4^+]$ was then used to calculate the appropriate value of $K_{\mathrm{NH4,K}}$ for use in Eqn 3 when analyzing samples of haemolymph or fluid secreted by Malpighian tubules of *Drosophila* larvae.

Statistics

Data are presented as means ± s.e.m. for the indicated number (N) of measurements. Statistical significance of differences was evaluated by one-way ANOVA. Significance of unpaired differences between measured and known ammonium concentrations, for each $[NH_4^+]$, was evaluated by the Student's t-test. Differences were considered significant if $P<0.05$.

RESULTS

Characteristics of NH_4^+-selective microelectrodes based on TD19C6

Preliminary measurements indicated that NH_4^+-selective microelectrodes based on a cocktail containing the solvent bis-(1-butylpentyl) adipate (BBPA) had slower response times but slightly higher selectivity compared with electrodes made with NPOE. All subsequent microelectrodes were therefore made using ionophore cocktails containing BBPA. The cocktail that had the highest selectivity contained 1% of the ionophore TD19C6, 0.25% of the lipophilic salt potassium tetrakis[3,5-bis-(trifluoromethyl) phenyl] borate (KtktFlMePB) and 98.75% BBPA. For microelectrodes with tip diameters of ~5 μm, the 95% response time of NH_4^+-selective microelectrodes under oil was slower (~7 s) than that of the K^+-selective or Na^+-selective microelectrodes (~1 s). Slopes of ≥50 mV decade^{-1} for NH_4^+-selective microelectrodes down to 0.1 mmol l^{-1} NH_4^+ in the presence of 150 mmol l^{-1} NaCl (Table 1) were maintained for >24 h. In *Drosophila* saline containing up to 2 mmol l^{-1} K^+, slopes ≥50 mV decade^{-1} were observed down to 1 mmol l^{-1} NH_4^+. In K^+-free *Drosophila* saline, concentrations of NH_4^+ ≥0.1 mmol l^{-1} could be resolved. In general, increasing concentrations of K^+ in *Drosophila* saline of up to 20 mmol l^{-1} progressively reduced the slope of the ammonium microelectrode (Table 1).

A previous study determined selectivity coefficients for the ionophore TD19C6 incorporated into macroelectrodes containing polyvinyl chloride (Suzuki et al., 2000). However, selectivity coefficients are not always similar in macroelectrodes and microelectrodes, or in liquid-membrane electrodes that do not contain polyvinyl chloride. We therefore measured selectivity coefficients for both the NH_4^+-selective and K^+-selective microelectrodes used in our experiments (Fig. 1). Selectivity coefficients are typically presented in log form (e.g. $\log K_{NH4,K}$). A selectivity coefficient of –1 thus indicates that the electrical potential corresponding to a given [NH_4^+] will also be produced by a 10-fold higher concentration of K^+. Positive values of the selectivity coefficient indicate that the ionophore is more selective for the interfering ion than for the principal ion.

Potential interfering ions of similar charge to those of NH_4^+ and K^+ were chosen along with ions found commonly in biological fluids. Although methylammonium produced the greatest interference ($K_{NH4,CH3NH3}$=–0.63), levels of this compound are negligible in biological fluids. Among physiological ions, K^+ produced the greatest interference ($\log K_{NH4,K}$=–0.94; Fig. 1A). Selectivity coefficients for interfering ions using a cocktail based on the classical NH_4^+-selective ionophore nonactin (Fresser et al., 1991) are shown for comparison in the right column of Fig. 1A. The selectivity coefficients for both K^+ (–0.6) and Na^+ (–2.0) indicate greater interference of these ions on nonactin-based microelectrodes relative to the novel NH_4^+-selective microelectrode based on TD19C6. Na^+, the predominant cation in extracellular fluids, does not interfere with NH_4^+-selective microelectrodes based on TD19C6 to any significant degree ($\log K_{NH4,Na}$=–3.58).

In general, physiological ions interfered less with the potassium-selective microelectrode (Fig. 1B). Selectivity coefficients of the K^+-selective microelectrode for several ions have been determined previously and are presented in the right column of Fig. 1B (Ammann et al., 1987). The value of $\log K_{K,NH4}$ was –1.76, as shown in the left column (Fig. 1B). As a result, interference due to equimolar NH_4^+ would increase apparent K^+ concentrations above the actual value by 1.74%. In summary, these results suggested that the novel NH_4^+-selective microelectrode based on TD19C6 suffered from significant K^+ interference, whereas the K^+ microelectrode is highly selective for K^+ *versus* all other potential interfering ions in extracellular fluids such as haemolymph or the fluids secreted by the Malpighian tubules.

[K^+] and [Na^+] in fluid secreted by Malpighian tubules of larval *D. melanogaster*

Isolated Malpighian tubules bathed in ammonia-free haemolymph-like *Drosophila* saline secreted fluid that contained 124±3.1 mmol l^{-1} K^+ (N=11) and 6.7±0.9 mmol l^{-1} Na^+ (N=13).

Table 1. Slopes (mV decade^{-1} change in [NH_4^+]) of NH_4^+-selective microelectrodes based on TD19C6 in 150 mmol l^{-1} NaCl or in *Drosophila* saline (DS) containing 0–20 mmol l^{-1} K^+

Ionophore	Solution	[NH_4^+] (mmol l^{-1})	Slope	s.e.m.	N	Number of microelectrodes
3% TD19C6	150 mmol l^{-1} NaCl	10–1	−55.79	0.29	48	12
		1–0.1	−49.28	0.39	63	12
1% TD19C6	150 mmol l^{-1} NaCl	10–1	−56.93	0.46	17	11
		1–0.1	−51.13	0.83	11	9
		0.1–0.01	−29.69	2.90	5	5
1% TD19C6	0 mmol l^{-1} K^+ DS	10–1	−58.21	0.85	3	3
		1–0.1	−52.58	1.37	3	3
		0.1–0.01	−25.54	0.75	3	3
1% TD19C6	2 mmol l^{-1} K^+ DS	10–1	−50.00	1.75	3	3
		1–0.1	−30.53	1.23	3	3
		0.1–0.01	−7.80	0.71	3	3
1% TD19C6	5 mmol l^{-1} K^+ DS	10–1	−48.76	2.10	3	3
		1–0.1	−22.55	0.72	3	3
		0.1–0.01	−4.71	0.28	3	3
1% TD19C6	10 mmol l^{-1} K^+ DS	10–1	−49.52	4.12	3	3
		1–0.1	−25.91	5.96	3	3
		0.1–0.01	−7.56	1.52	3	3
1% TD19C6	20 mmol l^{-1} K^+ DS	10–1	−32.62	1.64	3	3
		1–0.1	−7.35	1.15	3	3
		0.1–0.01	−1.04	0.48	3	3

[K^+] was varied by equimolar substitution of KCl for NaCl. The mean slope, s.e.m., number of samples (N) and number of microelectrodes are shown. NH_4^+-selective microelectrodes were based on a cocktail containing either 1% TD19C6, 0.25% potassium tetrakis[3,5-bis-(trifluoromethyl) phenyl] borate (KtktFlMePB) and 98.75% Bis(1-butylpentyl) adipate (BBPA) or 3% TD19C6, 0.25% KtktFlMePB and 96.75% BBPA.

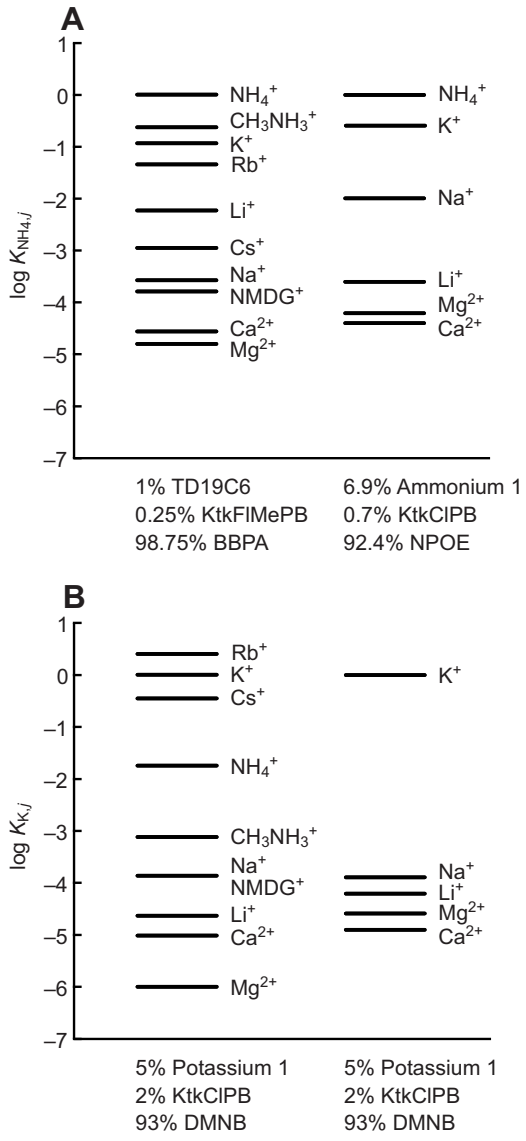

Fig. 1. Selectivity coefficients ($\log_{10}$) of (A) NH_4^+-selective and (B) K^+-selective microelectrodes. Values of log $K_{NH4,j}$ and log $K_{K,j}$ were obtained by the separate solutions method using 0.1 mol l^{-1} chloride salt solutions of the principal ions (NH_4^+ or K^+) and interfering ions (*j*). Membrane compositions of each microelectrode are listed below their respective columns. For comparison, selectivity coefficients obtained using nonactin-based NH_4^+-selective microelectrodes (Fresser et al., 1991) are shown in the right column of A and selectivity coefficients obtained using K^+-selective microelectrodes based on the same ionophore cocktail as we have used (Ammann, 1986) are shown in the right column of B. KtktFlMePB, potassium tetrakis[3,5-bis-(trifluoromethyl) phenyl] borate; KtkClPB, potassium tetrakis(4-chlorophenyl)borate; BBPA, bis-(1-butylpentyl) adipate; NPOE, 2-nitrophenyl octyl ether; DMNB, 1,2-dimethyl-3-nitrobenzene; Ammonium 1, ammonium ionophore 1 (nonactin); Potassium 1, potassium ionophore 1 (valinomycin).

When tubules were bathed in haemolymph-like *Drosophila* saline containing 30 mmol l^{-1} NH_4^+, there was a significant reduction in secreted fluid K^+ (113.6±3.9 mmol l^{-1}, N=15; P<0.05) but no significant change in secreted fluid Na^+ (10.0±1.7 mmol l^{-1}, N=12). Competition between K^+ and NH_4^+ for transport by the Malpighian tubule is examined further in the concluding paragraph of the Results section.

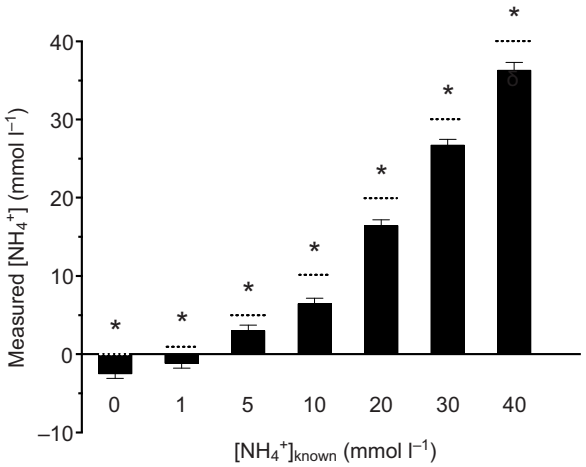

Fig. 2. Ammonium detection in solutions containing 150 mmol l^{-1} K^+. The interference of K^+ on the response of the NH_4^+-selective microelectrode was corrected using a selectivity coefficient (log$K_{NH4,K}$) of −0.94. Measured NH_4^+ concentrations (means ± s.e.m.) are shown on the *y*-axis, and the known NH_4^+ concentrations of the solutions are shown on the *x*-axis. Each dashed line indicates the known NH_4^+ concentration for the corresponding bar. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. Significant differences (P<0.05) between measured and known concentrations are indicated by asterisks (*t*-test, N=10).

Potassium interference and correction

The concentrations of K^+ in the haemolymph and fluid secreted by the Malpighian tubules were well above the levels that produce significant interference for NH_4^+-selective microelectrodes. Use of salines containing physiological levels of K^+ thus required correction for this interference. K^+ concentrations in all samples of haemolymph or secreted fluid were therefore measured with K^+-selective microelectrodes and the corrected NH_4^+ concentration was calculated by subtracting the product of the measured K^+ concentration and the selectivity coefficient $K_{NH4,K}$ (Eqn 3). We initially used the value of $K_{NH4,K}$ determined by the separate solutions method ($10^{-0.94}$). For example, if $[NH_4^+]_{uncorrected}$ was 7 mmol l^{-1} in a solution containing 17.5 mmol l^{-1} K^+, then the corresponding $[NH_4^+]_{corrected}$ was: $7–(17.5)(10^{-0.94})=5$ mmol l^{-1}.

We measured ammonium concentrations in solutions containing 150 mmol l^{-1} K^+, a level expected to be the maximum found in fluid secreted by insect Malpighian tubules (Maddrell and Klunsuwan, 1973). However, when a single value for the selectivity coefficient (log$K_{NH4,K}$=–0.94) was used to correct for K^+ interference, a consistent absolute error of approximately 3 mmol l^{-1} NH_4^+ below the known concentration was found across all the test solutions over the range 0–40 mmol l^{-1} (Fig. 2).

Correcting for K^+ interference on NH_4^+-selective microelectrodes using the variable selectivity coefficient method

The results shown in Fig. 2 indicated that use of a single selectivity coefficient determined using the separate solutions method resulted in significant errors in correcting for K^+ interference. We therefore used Eqn 4 to determine values of log$K_{NH4,K}$ at each ammonium concentration in solutions resembling both haemolymph (25 mmol l^{-1} K^+; Fig. 3A) and fluid secreted by Malpighian tubules (120 mmol l^{-1} K^+; Fig. 3B). The relationship between log$K_{NH4,K}$ and $[NH_4^+]$ was then fitted by linear regression. In both linear regressions, log$K_{NH4,K}$ values declined with increasing ammonium

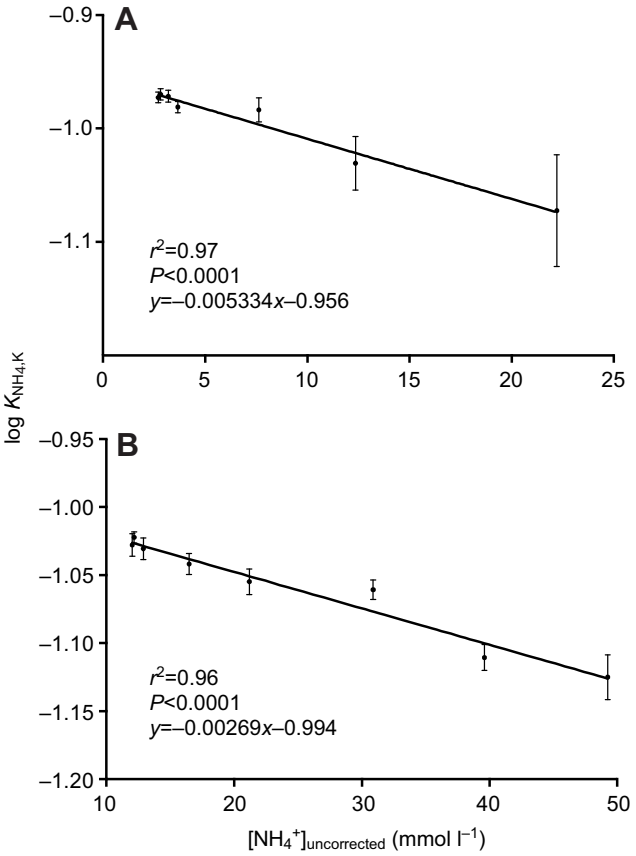

Fig. 3. Empirical adjustment of potassium selectivity coefficients (log_{10}) determined with the ammonium-selective microelectrode in (A) haemolymph-like saline (25 mmol l^{-1} K^+) and (B) secreted fluid-like saline (120 mmol l^{-1} K^+) calibration solutions. Values were obtained using the fixed interference method. Data are expressed as means ± s.e.m. Equations of the linear regression of the means and the corresponding correlation coefficients (r^2) and *P*-values are shown on the figure.

concentrations. Hereafter, the method used in this paper to correct for the effects of K^+ interference on NH_4^+ microelectrodes is referred to as the variable selectivity coefficient method (VSCM).

We first tested the VSCM by preparing haemolymph-like solutions containing 25 mmol l^{-1} K^+ and secreted fluid-like solutions containing 120 mmol l^{-1} K^+. We then compared the known $[NH_4^+]$ and the $[NH_4^+]$ measured by the microelectrode and corrected for K^+ interference. There were no significant differences between $[NH_4^+]_{corrected}$ and $[NH_4^+]_{known}$ for all concentrations over the ranges 0–20 and 0–40 mmol l^{-1} NH_4^+ (Fig. 4A,B, supplementary material Table S1). We were particularly interested in measuring NH_4^+ at low concentrations in the presence of high concentrations of K^+. Importantly, although the selectivity coefficients for the secreted fluid-like solutions were measured in a single set of solutions containing 120 mmol l^{-1} K^+, there was no significant difference between known and measured NH_4^+ concentrations between 0 and 5 mmol l^{-1} when the K^+ concentration was increased to 140 mmol l^{-1} (Fig. 4D, supplementary material Table S1). When the K^+ concentration was reduced to 100 mmol l^{-1}, the difference between known and corrected NH_4^+ concentrations over the range 0–5 mmol l^{-1} was small (0.3–0.4 mmol l^{-1}) but statistically significant (Fig. 4C, supplementary material Table S1).

Application of the NH_4^+-selective microelectrode

Measurement of NH_4^+ in the diet

A previous study has indicated that the NH_4^+ concentration of the diet in which *Drosophila* larvae are reared increases progressively to ~30 mmol l^{-1} after 21 days (Borash et al., 1998). We measured the concentrations of K^+ and NH_4^+ in diets that initially contained 42.2±0.7 mmol l^{-1} K^+ (*N*=30) and 0, 30 or 100 mmol l^{-1} NH_4^+. After 8 days, the K^+ concentration had declined to 29.4±0.8 mmol l^{-1} (*N*=30); there were no significant differences in K^+ concentrations in the diets with initial NH_4^+ concentrations of 0, 30 or 100 mmol l^{-1} and the data were therefore pooled. Uncorrected NH_4^+ concentrations measured in the three diets after 8 days were corrected for K^+ interference using selectivity coefficients ($logK_{NH4,K}$) calculated from Eqn 4. After the larvae fed for 8 days on diets that initially contained 0, 30 and 100 mmol l^{-1} NH_4^+, corrected NH_4^+ concentrations in samples of the diets were 21.7±1.0, 52.1±0.6 and 137.7±1.8 mmol l^{-1}, respectively (*N*=10).

Measurement of NH_4^+ in haemolymph – effects of dietary ammonia

There was no significant change in NH_4^+ concentrations in the haemolymph of larvae that developed for 8 days in media that initially contained 0, 30 or 100 mmol l^{-1} NH_4Cl (Fig. 5). Potassium concentrations in the haemolymph increased slightly (<10%) but significantly in haemolymph of larvae reared on diet that initially contained 100 mmol l^{-1} NH_4Cl. Taken together, these results indicate that *D. melanogaster* larvae maintain haemolymph NH_4^+ and K^+ concentrations in response to high levels of dietary ammonia.

Measurement of NH_4^+ in fluid secreted by Malpighian tubules

We hypothesized that a possible mechanism underlying the tolerance to dietary ammonia may be active clearance of ammonium from the haemolymph by secretion of ammonia into the lumen of the Malpighian tubules. Lumenal pH in Malpighian tubules of larvae of the related species *Drosophilia hydei* is 7.1 (Bertram and Wessing, 1994), suggesting that NH_4^+ comprises the vast majority (>99%) of ammonia in the secreted fluid. When Malpighian tubules were bathed in haemolymph-like saline containing 25 mmol l^{-1} KCl and 40 mmol l^{-1} NaCl, the rate of fluid secretion was unaffected by increasing concentrations of NH_4^+ of up to 40 mmol l^{-1} (Fig. 6A). Mean fluid secretion rate (0.21 nl min^{-1}) was lower than the rate of ~0.35 nl min^{-1} for Malpighian tubules of larvae bathed in *Drosophila* saline containing 20 mmol l^{-1} K^+ and 137 mmol l^{-1} Na^+ (Naikkhwah and O'Donnell, 2011). Ammonium concentrations in the secreted fluid did not differ significantly from those in the bathing salines in all treatments, although they trended (*P*=0.06 to 0.09) towards concentrations slightly below those in the bath over the range 20–40 mmol l^{-1} NH_4^+. K^+ concentration in the secreted fluid declined slightly in saline containing 40 mmol l^{-1} NH_4^+ (Fig. 6B). In saline containing similar concentrations of K^+ (25 mmol l^{-1}) and NH_4^+ (30 mmol l^{-1}), the secreted fluid contained a fourfold higher concentration of K^+ than NH_4^+, indicating that the epithelial transporters have a preference for K^+. Fluxes of NH_4^+ and K^+ (pmol min^{-1}) shown in Fig. 6C were calculated as the product of fluid secretion rate (nl min^{-1}) and secreted fluid ion concentration (mmol l^{-1}). NH_4^+ flux increased with increasing concentrations of NH_4^+ in the bathing saline, whereas potassium flux was independent of [NH_4Cl]. The mean potassium flux (23.6 pmol min^{-1}) was higher than ammonium flux in salines containing 0–40 mmol l^{-1} NH_4^+. In aggregate, these results suggest that Malpighian tubules transport ammonium but that potassium transport is unaffected by ammonium at concentrations up to 30 mmol l^{-1}.

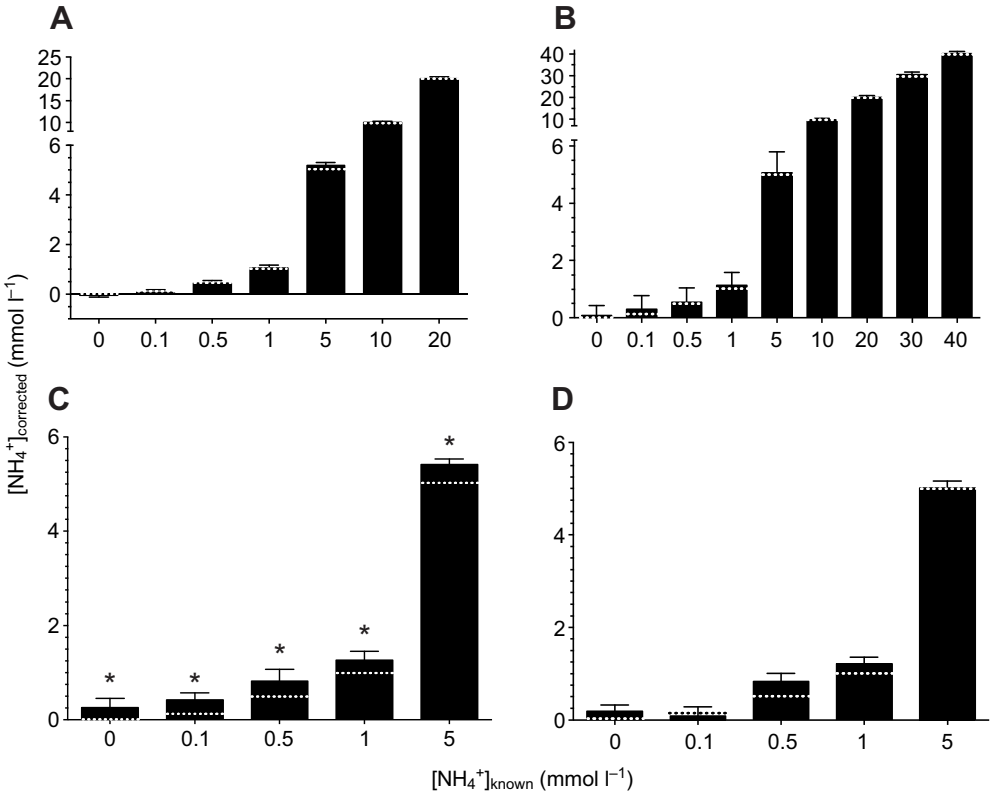

Fig. 4. Measured *versus* known $[NH_4^+]$ in solutions containing (A) 25 mmol l^{-1} K^+, (B) 120 mmol l^{-1} K^+, (C) 100 mmol l^{-1} K^+ and (D) 140 mmol l^{-1} K^+. Measured NH_4^+ concentrations after correction for K^+ interference ($[NH_4^+]_{corrected}$) are shown on the *y*-axis, and the known NH_4^+ concentrations of the solutions are shown on the *x*-axis. Each dashed line indicates the known NH_4^+ concentration for the corresponding bar. Ionic strength of all solutions was maintained by equimolar substitution of NH_4Cl for NaCl. Selectivity coefficients ($\log K_{NH4,K}$) used to correct for potassium interference were obtain from the equations: (A) $y=-0.0053x-0.956$ and (B–D) $y=-0.0027x-0.994$, where x represents uncorrected ammonium concentrations. Data are expressed as means ± s.e.m. N=6 (A), 7 (B), 12 (C) and 7 (D). Significant differences ($P<0.05$) between measured and known concentrations are indicated by asterisks (*t*-test).

DISCUSSION

This study has shown that the use of an improved NH_4^+ ionophore, in conjunction with a novel method for correcting for the interfering effects of K^+ and simultaneous measurement of K^+ concentration, permits precise measurement of NH_4^+ concentration in nanolitre samples of haemolymph or fluid secreted by isolated Malpighian tubules. We believe that the methods used in this paper may have relevance for studies of NH_4^+ transport in other epithelia, such as perfused vertebrate kidney tubules.

The primary advantage of NH_4^+-selective microelectrodes based on TD19C6 rather than nonactin is much greater selectivity for NH_4^+ relative to Na^+. The value of the selectivity coefficient ($\log K_{NH4,Na}$) of –3.58 means that TD19C6-based microelectrodes are 3800 times more selective for NH_4^+ relative to Na^+. As a result, variations in Na^+ concentration of up to 10 mmol l^{-1} alter the uncorrected NH_4^+ concentration determined from TD19C6-based microelectrode measurements by only 3 μmol l^{-1}.

The selectivity of microelectrodes based on TD19C6 for NH_4^+ relative to K^+ ($\log K_{NH4,K}$=–0.94) slightly exceeds that for microelectrodes based on nonactin [$\log K_{NH4,K}$=–0.6 (Ammann, 1986)]. Furthermore, the accuracy with which NH_4^+ concentration can be measured is much improved by using the variable selectivity coefficient method to determine the value of $K_{NH4,K}$ over a range of NH_4^+ concentrations. In solutions mimicking the composition of fluid secreted by *Drosophila* Malpighian tubules, there was no significant difference between known and measured NH_4^+ concentrations over the range (0–5 mmol l^{-1}) when K^+ concentration was varied between 120 and 140 mmol l^{-1}. The error was 0.3–0.4 mmol l^{-1} over the range 0.1–5 mmol l^{-1} NH_4^+ when the K^+ concentration was lowered to 100 mmol l^{-1}. Taken together, these findings indicate that the variable selectivity coefficient method based on measurements in 120 mmol l^{-1} K^+ allows precise measurement of NH_4^+ even when K^+ concentration varies substantially. Similarly, in fluid mimicking the composition of haemolymph, there was no significant difference between known and measured NH_4^+ concentrations over the range 0–20 mmol l^{-1} NH_4^+. Our methods thus permit more precise measurement of NH_4^+ than can be achieved with nonactin-based microelectrodes, for which the detection limit in the presence of 194 mmol l^{-1} K^+ is ~5 mmol l^{-1} (Fresser et al., 1991). NH_4^+-selective microelectrodes based on nonactin are suitable, however, for use in freshwater, where the concentrations of the interfering ions K^+ and Na^+ are low (Rahaman-Noronha et al., 1996; Shih et al., 2008). It is also worth noting that although we used seven or eight different NH_4^+ concentrations to determine the regression lines used in the variable selectivity coefficient method, re-analysis of Fig. 3 indicates that three to four NH_4^+ concentrations bracketing the range of interest would provide a sufficiently high value for the regression coefficient (r^2>0.95, data not shown)

We have used NH_4^+-selective microelectrodes based on TD19C6 to analyze both haemolymph samples collected from larval *D. melanogaster* and the fluid secreted by their Malpighian tubules. The larvae maintain the concentration of NH_4^+ in their haemolymph at ~1 mmol l^{-1} even when feeding for 8 days on diets in which the initial concentration of NH_4^+ was 100 mmol l^{-1}. Maintenance of haemolymph ammonia concentrations at this level is presumably adaptive for larvae feeding on initially ammonia-free diets in which the concentration of NH_4^+ rises progressively to ~30 mmol l^{-1} (Borash et al., 1998). Several dipteran insects are known to contain high concentrations of ammonia in the haemolymph. Larvae of the sheep blowfly *L. cuprina*, for example, are exposed to elevated ammonia levels as they develop in urine-soaked wool, proteinaceaus sera and blood from the lesions produced by the larvae. Their haemolymph contains 13–23 mmol l^{-1} ammonia as the NH_4^+ concentration in the rearing medium is varied between 3 and 560 mmol l^{-1} (Marshall and Wood, 1990). The haemolymph of black

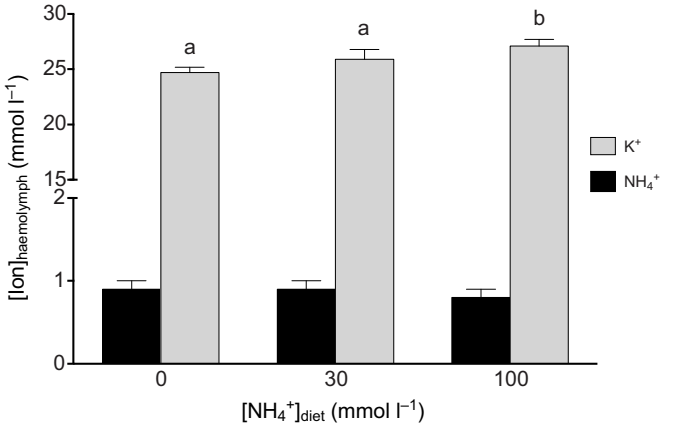

Fig. 5. Effect of dietary NH_4^+ on haemolymph K^+ and NH_4^+ concentrations. Selectivity coefficients ($\log K_{NH_4,K}$) used to correct for potassium interference in each sample were obtained from the equation $y=-0.0053x-0.956$, where x is the uncorrected ammonium concentration. Data are expressed as means ± s.e.m. Bars labelled with different letters are significantly different (one-way ANOVA, $P<0.05$; K^+, $N=9$; NH_4^+, $N=9$).

flies (*Simulium venustum*) also contains high levels (5 mmol l^{-1}) of ammonia (Gordon and Bailey, 1974).

Our data indicate that the tubules of larval *D. melanogaster* secrete fluids containing concentrations of NH_4^+ equal to those in the bathing saline over the range 0.1–10 mmol l^{-1}. Given that the transepithelial potential of the Malpighian tubules of the Oregon R strain of *D. melanogaster* is lumen positive by ~30 mV (O'Donnell et al., 1996), NH_4^+ is transported against an electrochemical gradient, indicating that some form of active transport is present. Our results suggest that competition between K^+ and NH_4^+ is not simple. Secreted fluid ammonium concentrations equal those in the bath at ammonium concentrations approximately in the physiological range, then trend towards concentrations slightly below those in the bath above 20 mmol l^{-1} ammonium. This pattern may indicate more than one mechanism for ammonium secretion. For example, amiloride-sensitive cation:proton exchangers that are energized by the V-ATPase mediate ammonia transport across the apical membrane in the midgut of larval *M. sexta* (Blaesse et al., 2010). It will thus be of interest in future studies to examine whether cation:proton exchangers mediate transport of ammonium as well as K^+ and Na^+ into the lumen of Malpighian tubules, or whether there is a separate transport pathway selective for NH_4^+ that is most effective over a specific range of bath ammonium concentrations.

Our measurements can also be used to estimate the contribution of the Malpighian tubules to clearance of NH_4^+ from the haemolymph. Haemolymph contains ~550 pmol of NH_4^+ based on the concentration of ~1 mmol l^{-1} NH_4^+ (Fig. 5) and the volume of haemolymph in third instar larvae of ~0.55 µl (Naikkhwah and O'Donnell, 2011). Malpighian tubules bathed in saline whose ionic composition is based on the larval haemolymph secrete NH_4^+ at the rate of 0.65 pmol min^{-1} in the presence of 1 mmol l^{-1} NH_4^+ (Fig. 6). The four tubules thus secrete at the combined rate of 156 pmol h^{-1}. The rates of NH_4^+ transport by the Malpighian tubules are thus sufficient to clear the haemolymph content of NH_4^+ in ~3.5 h. These estimates suggest that the Malpighian tubules play an important role in clearance of ammonia from the haemolymph, although other epithelia such as the hindgut may also contribute.

Although we have examined ammonium secretion by Malpighian tubules of *D. melanogaster*, in part because the larvae may ingest high concentrations (>10 mmol l^{-1}) of ammonia in the diet (Borash et al., 1998), mosquitoes also excrete ammonia following ingestion of ammonia-rich fluids or blood (Scaraffia et al., 2005). The techniques developed in this paper will be useful for assessing the role of the Malpighian tubules in ammonia excretion by mosquitoes and other blood-feeding insects.

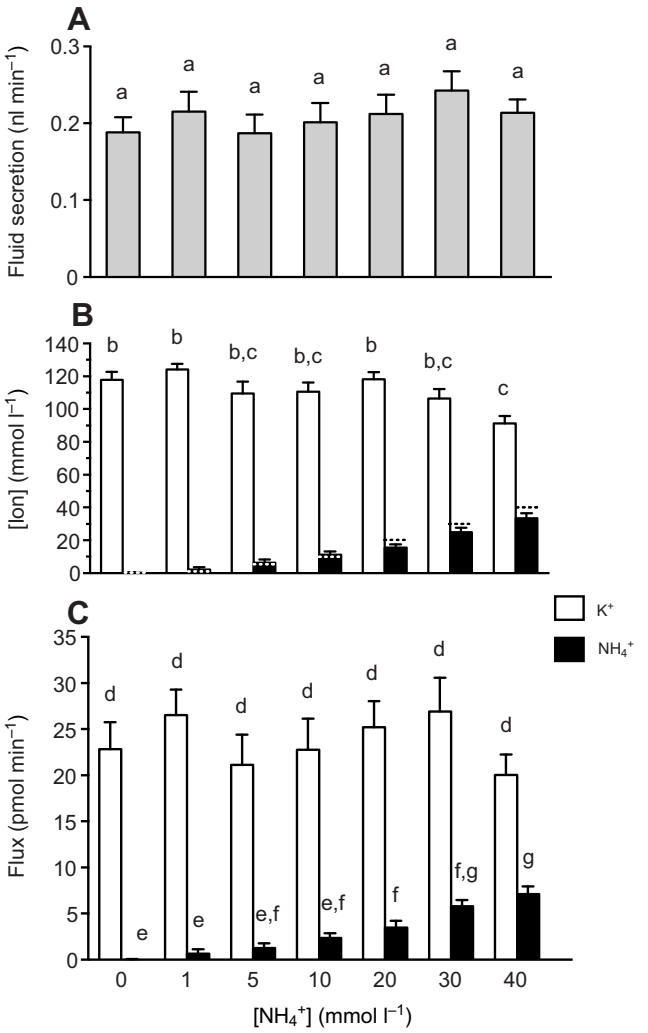

Fig. 6. Effects of bathing saline $[NH_4^+]$ on (A) fluid secretion rate (nl min^{-1}), (B) K^+ and NH_4^+ concentrations in secreted fluid (mmol l^{-1}) and (C) K^+ and NH_4^+ fluxes (pmol min^{-1}) of tubules bathed in *Drosophila* saline containing 25 mmol l^{-1} KCl and 40 mmol l^{-1} NaCl. Flux was calculated as the product of fluid secretion rate and ion concentration ([Ion]). Dashed lines indicate the known NH_4^+ concentration of the saline for the corresponding bar. Ionic strength of all solutions was maintained by equimolar substitution of NH_4^+ for NMDG. Potassium selectivity coefficients used to correct for potassium interference were obtained from the equation: $y=-0.0027x-0.994$, where x is the uncorrected ammonium concentration. Data are expressed as means ± s.e.m. Bars labelled with different letters are significantly different (one-way ANOVA, $P<0.05$, $N=7–11$).

APPENDIX

Using the Nicolsky–Eisenman equation to correct measured concentrations for interference

The response of an ion-selective electrode to interfering ions can be corrected using the Nicolsky–Eisenman equation:

$$\mathrm{EMF} = E_0 + S\log(c_i + K_{ij}c_j), \quad \text{(A1)}$$

where EMF is the electromotive force (mV), E_0 is the reference potential (mV), S is the slope, c_i is the primary ion concentration, c_j is the interfering ion concentration and K_{ij} is the selectivity coefficient for an i-selective electrode towards the interfering ion j (Ammann, 1986).

Using the example of K^+ interference on NH_4^+ microelectrodes, where all measurements are referenced to a calibration solution containing 15 mmol l^{-1} NH_4Cl, 135 mmol l^{-1} NaCl and 0 mmol l^{-1} KCl, we can calculate the corrected NH_4^+ concentration in the droplet ($[NH_4^+]_{corrected}$) as follows:

$$EMF_1 = E_0 + S\log\left([NH_4^+]_{corrected} + (K_{NH4,K})[K^+]\right), \quad (A2)$$

$$EMF_2 = E_0 + S\log\left(15 + (K_{NH4,K})(0)\right), \quad (A3)$$

$$\Delta V = EMF_1 - EMF_2 = S\log\left([NH_4^+]_{corrected} + (K_{NH4,K})[K^+]\right) - S\log\left(15 + (K_{NH4,K})(0)\right) = S\log\left(\left([NH_4^+]_{corrected} + (K_{NH4,K})[K^+]\right)/15\right), \quad (A4)$$

where ΔV is the change in voltage recorded by the electrode as it is moved from the solution containing both NH_4^+ and K^+ to the K^+-free calibration solution.

By re-arrangement and solving for $[NH_4^+]_{corrected}$:

$$\Delta V/S = \log\left([NH_4^+]_{corrected} + (K_{NH4,K})[K^+]\right)/15, \quad (A5)$$

$$10^{(\Delta V/S)} = \left([NH_4^+]_{corrected} + (K_{NH4,K})[K^+]\right)/15, \quad (A6)$$

$$(15)[10^{(\Delta V/S)}] = [NH_4^+]_{corrected} + (K_{NH4,K})[K^+], \quad (A7)$$

$$[NH_4^+]_{corrected} = (15)[10^{(\Delta V/S)}] - (K_{NH4,K})[K^+]. \quad (A8)$$

In other words, the corrected ammonium concentration is calculated as the difference between the uncorrected ammonium concentration and the product of the selectivity coefficient for potassium and the potassium concentration:

$$[NH_4^+]_{corrected} = [NH_4^+]_{uncorrected} - (K_{NH4,K})[K^+]. \quad (A9)$$

For example, if ΔV=–0.2 mV relative to the calibration solution containing 15 NH_4^+, S=54.9 mV decade^{-1}, $[K^+]$=120 mmol l^{-1} and $\log K_{NH4,K}$=–0.94 (i.e. $K_{NH4,K}$=0.115), then:

$$-0.2 = 54.9\log\left(\left([NH_4^+]_{corrected} + (0.115)(120)\right)/15\right) = 54.9\log\left(([NH_4^+]_{corrected} + 13.8)/15\right), \quad (A10)$$

$$[NH_4^+]_{corrected} = (15)[10^{(-0.2/54.9)}] - 13.8 = 14.9 - 13.8 = 1.1\ \text{mmol}\,l^{-1}. \quad (A11)$$

Selectivity coefficients are usually reported as $\log K_{NH4,K}$, so it is necessary to convert them as in $10^{\log K_{NH4,K}}$ and use the arithmetic value of the selectivity coefficient in the equations above.

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AUTHOR CONTRIBUTIONS

This work was conceived from initial observations by M.J.O. The experiments were conducted and data were collected by A.B. Experimental design and data interpretation resulted from continuing interactions between M.J.O and A.B., who were also both closely involved in drafting and revising the article.

COMPETING INTERESTS

No competing interests declared.

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