The Journal of Experimental Biology 208, 603-610
Published by The Company of Biologists 2005
doi:10.1242/jeb.01422

Analysis of Na^+, Cl^-, K^+, H^+ and NH_4^+ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes

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Accepted 30 November 2004

Summary

Ion concentration gradients adjacent to the surface of the anal papillae of larvae of the mosquito *Aedes aegypti* were measured using self-referencing ion-selective microelectrodes. The gradients were used to calculate estimates of ion fluxes into and out of the papillae. There was a net influx of Na^+, Cl^- and K^+ from the bathing medium and a net efflux of acid and NH_4^+. No Ca^{2+} gradients were detectable. Na^+ and Cl^- influx occurred against a concentration gradient suggesting active transport. Although Na^+, Cl^- and NH_4^+ gradients were uniform along the length of the papillae, the proximal regions of the papillae *in vivo* revealed significantly higher H^+ and K^+ gradients compared with distal regions. The calculated ion fluxes at the papillae are sufficient for complete Na^+, K^+ and Cl^- haemolymph replacement in ~4 h with external ion concentrations of 5 mmol l^{-1}. Ion gradients were also detected adjacent to the surface of isolated papillae; however, Na^+ and H^+ gradients were higher, and Cl^- gradients were lower relative to papillae *in vivo*. The results support previous findings that the anal papillae of mosquito larvae are important structures for ion regulation, and suggest that these structures may be used for the excretion of nitrogenous waste.

Key words: anal papillae, mosquito, *Aedes aegypti*, ion transport, self-referencing ion selective microelectrode.

Introduction

The organs responsible for osmoregulation and ionoregulation in mosquito larvae are the midgut, Malpighian tubules, rectum and anal papillae (for an overview, see Bradley, 1987). The midgut is responsible for digestion and absorption of nutrients and ions. The anterior midgut lumen of mosquito larvae is maintained alkaline by carbonic anhydrase and secondary Cl^-/HCO_3^- exchange across the apical membrane that is energized by H^+ V-ATPase (Del Pilar Corena et al., 2004; Boudko et al., 2001a). The alkaline environment helps to dissociate tannin–protein complexes that occur in detritus, thereby permitting absorption of protein in the posterior midgut lumen, which has neutral pH (Boudko et al., 2001a). The Malpighian tubules produce the primary urine by actively secreting ions from the haemolymph into their lumen (Ramsay, 1950). The rectum is responsible for reabsorption of ions and some water from the primary urine into the haemolymph (Bradley and Phillips, 1977), a process that is particularly important to freshwater species, which face the dilution of haemolymph through passive loss of ions and entry of water across the body wall. The importance of the rectum in ionoregulation and osmoregulation is also evident in the salt-tolerant mosquito species, which possess a specialized rectal segment that secretes ions into the rectal lumen resulting in a hyperosmotic urine, thereby permitting these mosquitoes to survive in habitats containing high amounts of salt (Bradley and Phillips, 1975). The anal papillae of freshwater mosquito larvae are proposed to take up ions from the external medium (Stobbart, 1965, 1967, 1971), thereby contributing to the maintenance of proper haemolymph ion levels.

Mosquito larvae have four anal papillae of similar size and shape that arise from an extension of the terminal segment and project into the external medium. The lumen of the papillae is continuous with the haemolymph. The apical and basal plasma membranes of the cells of the papillae of *Aedes aegypti*, *Aedes togoi*, *Aedes campestris* and *Culiseta inornata* have many infoldings that are closely associated with mitochondria (Sohal and Copeland, 1966; Meredith and Phillips, 1973a,b; Garrett and Bradley, 1984). Such infoldings are characteristic of epithelial cells that transport water and ions, such as Malpighian tubules of insects (Bradley et al., 1982) and nasal salt glands of birds (Butt et al., 1985). The decrease in apical and basal membrane infoldings and the reduction in the number of mitochondria in the papillae of larval *Aedes aegypti* raised in saltwater are consistent with a role for the papillae in ion uptake from dilute media (Edwards and Harrison, 1983). These results are complemented by radioisotope uptake studies

that demonstrate uptake of Na^+ and Cl^- from the external medium by mosquito larvae (Stobbart, 1965, 1967, 1971). Measurements of base production by groups of larvae taking up Na^+ from Na_2SO_4 and Cl^- from KCl suggest that Na^+ and Cl^- influx is coupled to H^+ and HCO_3^- efflux, respectively (Stobbart, 1971). The anal papillae have been implicated as the site of Na^+ and Cl^- uptake on the assumption that there is negligible exchange of ions and water along the general body surface (Wigglesworth, 1933; Stobbart, 1965, 1967, 1971).

Mosquito larval habitats are prone to sudden dilution by rain as well as increased salinity by evaporation and, in some cases, flooding of coastal marshes with seawater. There is evidence that mosquito larvae cope with these changes by regulating the physiological processes of their osmoregulatory organs. For example, the hormonal control of the Malpighian tubules is documented (Clark and Bradley, 1996, 1997), and hormones are found in neurosecretory cells of the midgut (Veenstra et al., 1995). In order to obtain an understanding of ionoregulation and osmoregulation in mosquito larvae, it is first necessary to identify specific ion-exchange pathways in all of the osmoregulatory organs. Recently the anal papillae have received little attention, and the current knowledge of ion-exchange processes is based on radioisotopic measurement of whole larval ion fluxes, which cannot entirely exclude ion exchange from the body surface or through drinking and excretion. Therefore, the present study utilized self-referencing ion-selective (SeRIS) microelectrodes to measure the concentration gradients of Na^+, Cl^-, H^+, K^+, Ca^{2+} and NH_4^+ adjacent to the surface of the anal papillae of larval *Aedes aegypti*. SeRIS microelectrodes have previously been used to record spatial and temporal variations in ion flux along the midgut of larval *Aedes aegypti* and Malpighian tubules of *Drosophila* (Boudko et al., 2001b; Rheault and O'Donnell, 2001). An estimate of net flux for each ion can be calculated from the measured gradients. Our results provide the first direct measurements of specific ion concentration gradients along the length of the anal papillae, suggesting that the papillae are indeed sites for ion exchange.

Materials and methods

Animals

Adult and larval mosquitoes of *Aedes aegypti* L. were raised at room temperature on a 12 h:12 h light:dark cycle. Eggs were hatched in plastic containers filled with distilled water. Larvae were maintained in these containers until they reached the fourth instar and were fed every second day with a solution of liver powder and yeast made up in distilled water. Prior to experiments, the Na^+, Cl^- and Ca^{2+} concentration and the pH of the rearing media were measured using ion-selective electrodes. At the time the larvae reached the fourth instar (8–10 days) the rearing media pH was 7.4 and it contained 1 mmol l^{-1} Na^+, 23 μmol l^{-1} Cl^- and 26 μmol l^{-1} Ca^{2+}. Fourth instar larvae were removed from the rearing containers and placed in distilled water without food for 1–3 days prior to each experiment so as to create conditions appropriate for active ion uptake (see Stobbart, 1965).

Construction of ion-selective microelectrodes

Construction of liquid–membrane ion-selective microelectrodes has been previously described in detail (Smith et al., 1999; Rheault and O'Donnell, 2001, 2004). The microelectrode tip diameters and ionophore cocktail column lengths were typically ~5 μm and 250–300 μm, respectively. The following ionophore cocktails (Fluka, Buchs, Switzerland) and back-fill solutions (in parentheses) were used: Na^+ Ionophore II Cocktail A (100 mmol l^{-1} NaCl); Cl^- Ionophore I Cocktail A (1 mol l^{-1} NaCl); K^+ Ionophore I Cocktail B, (100 mmol l^{-1} KCl); Ca^{2+} Ionophore I Cocktail A (100 mmol l^{-1} $CaCl_2$); H^+ Ionophore I Cocktail B (100 mmol l^{-1} NaCl + 100 mmol l^{-1} sodium citrate, pH 6.0) and NH_4^+ Ionophore I Cocktail A (100 mmol l^{-1} NH_4Cl). The tips of the ion-selective microelectrodes used to measure haemolymph ion concentrations were dipped in a solution of polyvinylchloride (PVC, Fluka) in tetrahydrofuran (Fluka), prior to use, as described by Rheault and O'Donnell (2004).

For measurement of haemolymph chloride concentration a solid state silver wire electrode was employed since the haemolymph contains other anions (i.e. HCO_3^-) that will be detected by microelectrodes based on a chloride ionophore cocktail. The solid state electrode was constructed by pulling a 2 mm diameter non-filamented glass capillary (A-M Systems Inc., Sequim, WA, USA) on a vertical micropipette puller. The tip was broken by touching it on tissue paper such that a fine silver wire (~ 10 μm diameter) could be inserted from the barrel through the tip of the micropipette. The silver wire was prepared by cutting a 0.005″ diameter wire obliquely with a razor blade so that the tip tapered to a fine point. A soldering iron was used to melt a glue stick in order to fill the gap between the wire and the glass tip of the micropipette, taking care not to cover the tip of the wire with melted glue. The same procedure was used to seal the gap between the end of the barrel and the wire, thereby securing the wire within the micropipette. The wire protruding from the barrel was soldered to the wire connected to the amplifier and the wire at the tip of the microelectrode was chlorided by immersion in a ferric chloride solution.

SeRIS microelectrode measurements of ion gradients adjacent the surface of the anal papillae in vivo *and* in vitro

An assay for the measurement of ion concentration gradients near the surface of the anal papillae using the SeRIS microelectrode technique was developed as follows. Vaseline mixed with charcoal was placed on the bottom of a 35 mm Petri dish and shaped into a rectangular block measuring approximately 8 mm in length, 6 mm in width and 5 mm in height. The entire body, with the exception of the anal papillae and siphon, of an intact, undissected larva was submerged in the vaseline to prevent movement. The siphon was then carefully pushed against the vaseline so that it was oriented perpendicular to the bottom of the dish and parallel to the

vertical side of the vaseline block. Initially, the anal papillae were in contact with the bottom of the dish. After addition of bathing solution all four papillae could be moved by the larva. Because these movements interfered with microelectrode measurements, some of the vaseline was carefully moved along one side of the length of one or two of the papillae, thereby limiting their movement and permitting subsequent measurement of ion concentration gradients near the opposite surface of the papillae. Papillae were continuously viewed on a video monitor and scans were discarded if the papillae moved. Care was taken to prevent submerging the siphon entirely so that the larvae had continuous access to air during the experiment.

An *in vitro* preparation was also developed where a single papilla was removed from the larvae by pinching the papilla at the base using fine forceps. The papilla was placed in the vaseline so that one side was available for measurement of ion concentration gradients, and the base was sealed with vaseline to prevent the haemolymph from being washed out of the papilla when the bathing solution was added. For *in vivo* and *in vitro* measurements of Na^+, Cl^-, K^+, Ca^{2+}, H^+ and NH_4^+ the bathing solutions were; 5 mmol l^{-1} NaCl, 5 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} NaCl + 1 mmol l^{-1} Hepes (pH 8) and 1 mmol l^{-1} NH_4Cl, respectively.

The SeRIS microelectrode system and protocol employed in this study is described in detail in Rheault and O'Donnell (2001, 2004), with the following modifications. Excursion distances of 50 μm for Na^+, K^+, Ca^{2+}, Cl^- and NH_4^+, and 20 μm for H^+, were used. The 'wait' and 'sample' periods were 2 and 1 s, respectively, and fluxes were reported as an average of 3–5 repetitive measurements at each site.

SeRIS microelectrodes were calibrated in 0.1, 1 and 10 mmol l^{-1} solutions of NaCl for Na^+ and Cl^- electrodes, KCl for K^+ electrodes, $CaCl_2$ for Ca^{2+} electrodes and NH_4Cl for NH_4^+ electrodes. Microelectrode slopes (mV) for a tenfold change in ion concentration were [mean ± S.E.M. (*N*)]: 59.0±1.6 (4) for Na^+; 54.2±0.9 (5) for Cl^-; 54.4±2.1 (5) for K^+; 28.7±1.2 (3) for Ca^{2+}; and 55.3±1.1 (3) for NH_4^+. SeRIS pH microelectrodes were calibrated in solutions of 5 mmol l^{-1} NaCl containing 1 mmol l^{-1} Hepes and adjusted to pH 7, 8 or 9 with HCl or NaOH. The slope for a 1 pH unit change was 58.9 ± 2.4 (5). Selectivity coefficients of the Fluka ionophores (http://www.sigmaaldrich.com/Brands/Fluka___Riedel_Home/Analytical/Sensoric_Applications.html) used in construction of SeRIS microelectrodes are as follows (in brackets): H^+ ($K_{H,Na}$ $10^{10.4}$, $K_{H,K}$ $10^{9.8}$, $K_{H,Ca}$ $10^{11.1}$); Na^+ ($K_{Na,K}$ $10^{0.4}$, $K_{Na,Ca}$ $10^{1.3}$); K^+ ($K_{K,Na}$ $10^{3.9}$, $K_{K,Ca}$ $10^{4.9}$); Cl^- (K_{Cl,HCO_3} $10^{1.5}$, K_{Cl,SO_4} $10^{2.6}$); Ca^{2+} ($K_{Ca,Na}$ $10^{5.5}$, $K_{Ca,K}$ $10^{5.4}$); NH_4^+ ($K_{NH_4,H}$ $10^{2.2}$, $K_{NH_4,Na}$ $10^{2.9}$, $K_{NH_4,K}$ $10^{0.6}$). The Na^+ and NH_4^+ microelectrodes are only ~2.5 and 4 times more selective for their respective ions than for K^+. This relatively low selectivity did not compromise measurements of Na^+ and NH_4^+ concentration gradients since the bathing medium was nominally K^+-free (40–100 μmol l^{-1}) during such experiments.

Ion flux was calculated after subtracting the noise at a reference position 4 mm or more from the preparation from the differential signal measured at the site of interest near the preparation. Typical signals and noise (in parentheses) for each ion were as follows: Na^+ 450 μV (2 μV); K^+ 150 μV (3 μV); NH_4^+ 300 μV (3.5 μV); H^+ 1800 μV (13 μV); Cl^- 1100 μV (42 μV). The signal to noise ratios varied from a low of 25 for Cl^- to a high of 227 for Na^+.

The efficiency of the SeRIS microelectrode sampling protocol for Na^+, K^+, Ca^{2+} and Cl^- was tested using artificial gradients as described in Rheault and O'Donnell (2004). For Ca^{2+} and Cl^- there was no difference in the magnitude of the gradients determined using static and dynamic measurements, indicating that the efficiency of the SeRIS microelectrode measurements for each of these ions was 100%. For Na^+ and K^+ SeRIS microelectrodes the sampling protocols detected 90% and 95%, respectively, of the gradient measured using static microelectrodes, and flux calculations were adjusted accordingly to reflect the true gradient. The accuracy of the pH and NH_4^+ SeRIS microelectrode measurements was assessed by lengthening the 'wait' and 'sample' periods until the differential signal reached a plateau at a maximum value. The 'wait' and 'sample' periods used to measure the gradients from the papillae were chosen from within the plateau.

Calculation of ion fluxes

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

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

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

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

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

where J_I is the net flux of the ion in pmol cm^{-2} s^{-1}; D_I is the diffusion coefficient of the ion (1.55×10^{-5} cm^2 s^{-1} for Na^+ and Cl^-; 1.92×10^{-5} cm^2 s^{-1} for K^+; 1.19×10^{-5} cm^2 s^{-1} for Ca^{2+}; 9.4×10^{-5} cm^2 s^{-1} for H^+ and 2.09×10^{-5} cm^2 s^{-1} for NH_4^+); ΔC is the concentration gradient in pmol cm^{-3}; and Δx is the distance between the two points measured in cm. H^+ gradients were measured in Hepes-buffered solutions and the calculated flux values were therefore adjusted for the buffering capacity of the solution (see Somieski and Nagel, 2001; Smith and

Trimarchi, 2001; Kunkel et al., 2001) using the following equation:

$$J_I = [(D_H+D_I)B_H] \times (\Delta C/\Delta x) , \quad (3)$$

where D_H is the diffusion coefficient of Hepes (6.2×10^{-6} cm^2 s^{-1}); and B_H is the buffering capacity of Hepes, which is calculated from the following equation:

$$B_H = (C_H/C_B) \times [F/(1+F)^2] , \quad (4)$$

where C_H is the concentration of Hepes used in mol l^{-1} and F is calculated from the following equation:

$$F = \log_{10}(pK_{aH}) / C_B , \quad (5)$$

where $\log_{10}$ pK_{aH} is the dissociation constant of Hepes (pK_{aH}=7.55).

Measurement of haemolymph ion concentration

Fourth instar larvae were held in distilled water without food overnight. Larvae were dried on tissue paper and submerged in paraffin oil and the cuticle was torn with forceps, taking care not to rupture any internal organs. The resulting haemolymph droplet was held in the paraffin oil for no longer than 15 min before measurements were made. Ion concentration in the haemolymph was measured with ion-selective microelectrodes that were calibrated in the following solutions: Na^+ (15 mmol l^{-1} NaCl/135 mmol l^{-1} LiCl and 150 mmol l^{-1} NaCl); K^+ (1.5 mmol l^{-1} KCl/13.5 mmol l^{-1} NaCl and 15 mmol l^{-1} KCl); Cl^- (15 mmol l^{-1} KCl and 150 mmol l^{-1} KCl); H^+ (150 mmol l^{-1} NaCl containing 1 mmol l^{-1} Hepes at pH 7.5 and 8.5). Slopes of the electrodes (mV, mean ± S.E.M.) for a tenfold change in ion concentration were 52.3±0.4 for Na^+, 52.4±2.0 for Cl^-, 54.5±0.9 for K^+ and 53±1.2 for H^+ (N=4). Calculations of haemolymph ion concentration were made using the following equation:

$$a^h = a^c \times 10^{(\Delta V/S)} , \quad (6)$$

where a^h is the haemolymph ion concentration, a^c is the ion concentration in the calibration solution, ΔV is the difference in voltage between the haemolymph and the calibration solution and S is the slope of the electrode measured in response to a tenfold change in ion activity.

Calculation of papillae ion fluxes per larva and estimation of haemolymph ion content replacement time

Total ion fluxes across the papillae for a larvae were estimated by multiplying the calculated fluxes (pmol cm^{-2} s^{-1}) by the surface area of the papillae. The anal papillae of several fourth instar larvae were measured in length and width and the means were calculated. The surface area (SA) of the papilla was calculated assuming a generally cylindrical shape with only one closed end, using the formula: $SA=\pi r^2+2\pi rh$, where r is the radius or half the width of the papilla and h is the length of the papilla, both measured in cm. Total papillae ion fluxes (mol s^{-1}) were calculated by multiplying the measured average flux by the total surface area of the four papillae.

The time required for the papillae to transport a quantity of Na^+, K^+ or Cl^- equivalent to the total haemolymph content of each ion was calculated as follows. Fourth instar larvae were weighed after drying on tissue paper and larval haemolymph volume was calculated by assuming that the haemolymph comprised 62% of the body mass of the larvae (see Stobbart, 1965). The number of moles of each ion in the haemolymph was then calculated as the product of haemolymph ion concentration (mol l^{-1}) and haemolymph volume (l). The number of moles of each ion in the haemolymph was then divided by the calculated total ion flux at the papillae to yield the time required for complete replacement of haemolymph ion content by transport across the papillae.

Statistics

During the measurement of ion fluxes using the SeRIS microelectrode system the length of each papilla was arbitrarily divided into quarters designated 1 (proximal to anus) 2, 3 and 4 (distal to anus). The mean of 3–5 measurements at different sites within each quarter was calculated for each quarter, permitting statistical comparison of regional differences in ion transport. The data are expressed as the mean ± S.E.M. of ion fluxes from the quarters. The sample sizes represent the number of individual papillae sampled. The mean fluxes from the quarters were compared using parametric or non-parametric repeated-measures ANOVA with subsequent multiple comparison tests where appropriate.

Results

Ion fluxes across anal papilla in vivo

Flux estimates derived from SeRIS microelectrode measurements of ion concentration gradients indicated net fluxes of Na^+, Cl^-, K^+, H^+ and NH_4^+, but no net flux of Ca^{2+}. Although the magnitude of the flux of each ion varied between papillae in different larvae, the direction of net flux (influx or efflux) was the same for all larvae examined. The ranges of estimated net fluxes for each ion were as follows (pmol cm^{-2} s^{-1}, ion): 25–350, NH_4^+; 46–230, Cl^-; 122–664, Na^+; 26–144, K^+; 16–566, H^+.

The SeRIS microelectrode technique measured ion gradients at localized positions along the length of the papillae allowing for an assessment of any regional differences in net ion flux. Localized ion flux was illustrated by vectors superimposed on a digital image of the papilla (Fig. 1). The direction of the vector reflects the movement of ions into (influx) or out of (efflux) the papillae, whereas the length of the vector reflects the magnitude of the ion flux. Typical measurements of H^+ flux along the length of a papilla revealed a regional pattern of increased H^+ efflux in proximal regions of the papillae (compare quarter 1 with quarter 4 in Fig. 1A). In contrast to H^+ efflux, there was Cl^- influx along the length of the papillae (Fig. 1B).

In addition to the influx of Cl^- and the efflux of H^+, there was a net influx of Na^+ and K^+ and a net efflux of NH_4^+ (Fig. 2). The influx of Na^+ was greater than either influx or efflux of any of the other ions studied. Mean fluxes of Na^+ from

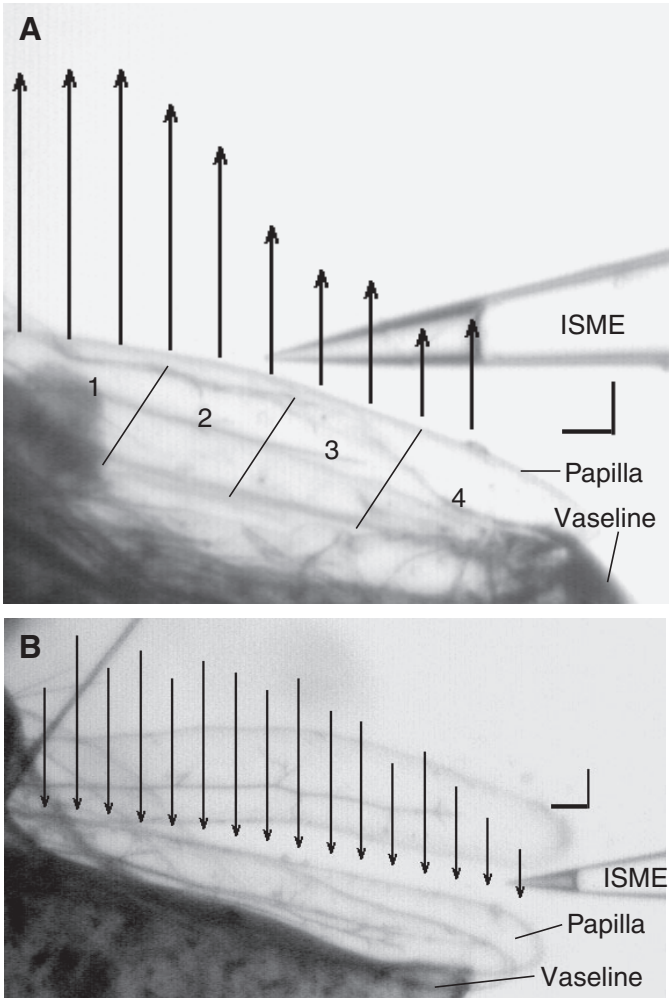

Fig. 1. Representative example of SeRIS microelectrode measurements showing calculated ion fluxes along the surface of the anal papillae *in vivo*. The length of the arrows and the arrowheads indicate the magnitude and direction of flux, respectively. The papillae were arbitrarily divided into quarters such that regions 1 and 2 comprised the proximal half of the papillae and regions 3 and 4 the distal portion of the papillae. (A) H^+ flux. H^+ is released by the papillae into the bathing medium. Note that H^+ flux is higher in the proximal regions of the papillae. Vertical scale bar, 6.5 pmol cm^{-2} s^{-1}; horizontal scale bar, 90 μm. (B) Cl^- flux. Cl^- is taken up by the papillae from the bathing medium. The papillae that measurements were taken from was positioned approximately 500 μm below the other papilla that is seen in the field of view. Vertical scale bar, 42 pmol cm^{-2} s^{-1}, horizontal scale bar, 54 μm.

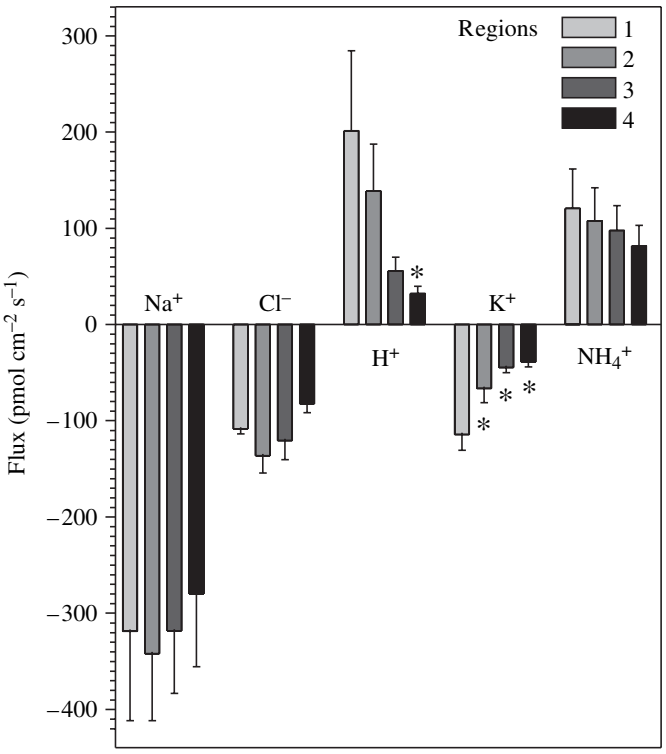

Fig. 2. Summary of ion fluxes in the four regions of the anal papilla *in vivo*. Negative values denote influx of ions from the bathing medium into papilla, and positive values denote efflux of ions from the papilla into the bathing medium. H^+ and K^+ fluxes were significantly higher in region 1 (asterisks; K^+ flux: ANOVA $P<0.01$; Tukey–Kramer $P<0.05$; H^+ flux: ANOVA $P<0.05$; Dunn's test $P<0.05$). There were no regional differences in Na^+, Cl^- and NH_4^+ fluxes. Values are mean + S.E.M. (Na^+, N=5; Cl^-, N=7; H^+, N=6; K^+, N=5; NH_4^+, N=7).

Table 1. *Haemolymph ion concentrations and pH*

Haemolymph constituent	Concentration (mmol l^{-1}) or pH
Cl^-	65.8±5.2 (14)
Na^+	85.1±2.2 (10)
K^+	3.7±0.2 (10)
pH	7.9±0.02 (10)

Ion-selective micrelectrodes were used to measure ion concentrations and pH in samples of haemolymph collected from larvae held in distilled water for 14–16 h.

Values are mean ± S.E.M. (N).

the four quarters of the papillae were ~300 pmol cm^{-2} s^{-1} (see Fig. 2). The corresponding calculated influx of Na^+ across all four papillae of a single larvae was 16.1 pmol s^{-1} (Table 1). This was 2.1 and 4.8 times greater than the Cl^- and K^+ influx, as well as 3.1 times greater than the H^+ and NH_4^+ efflux, measured under similar conditions.

The proximal regions of the papillae revealed significantly higher H^+ and K^+ net fluxes than distal regions (Fig. 2). The mean H^+ efflux and K^+ influx for the first quarter were 6.3 and 2.9 times greater, respectively, than for the fourth quarter. Although some preparations indicated lower Cl^- influx near the tip of the papilla (Fig. 1B), mean values showed no statistically significant regional differences in Na^+ and Cl^- influx or NH_4^+ efflux along the length of the papillae (Fig. 2).

Ion fluxes across anal papilla in vitro

All calculated ion fluxes measured in the *in vitro* preparation were in the same direction as in the *in vivo* preparation. The

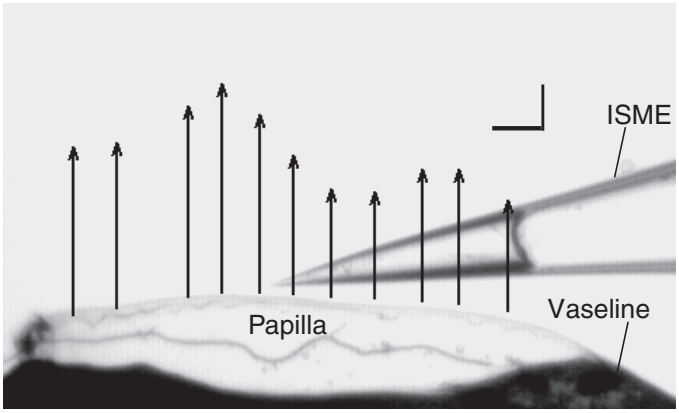

Fig. 3. H^+ flux along the length of a papilla *in vitro*. The papilla was removed from the larva and sealed at the open end with Vaseline. Note that in comparison with Fig. 2A there were no regional differences in H^+ flux. Vertical scale bar, 7 pmol cm^{-2} s^{-1}; horizontal scale bar, 115 μm.

range of estimated net fluxes for each ion were as follows (pmol cm^{-2} s^{-1}, ion): 26–474, Cl^-; 85–295, Na^+; 29–287, K^+; 9–67, H^+.

Although the magnitude of K^+ flux was similar *in vitro* and *in vivo*, fluxes of other ions increased or decreased *in vitro* and the regional differences in flux were abolished (Figs 3, 4). The influx of Na^+ and efflux of H^+ *in vitro* were reduced approximately twofold and fourfold, respectively, relative to the corresponding fluxes *in vivo*. In contrast, Cl^- influx was approximately twofold greater *in vitro* than *in vivo*. Only four of nine *in vitro* preparations showed the same regional difference in H^+ efflux that was seen in all six *in vivo* preparations, and only five of nine *in vitro* preparations showed the same regional difference in K^+ influx seen in all five intact preparations. There were no significant differences in the mean values of H^+ and K^+ fluxes across the four regions of the papilla *in vitro* (Fig. 4).

Haemolymph ion composition

The Na^+ and Cl^- concentrations in haemolymph were 17 and 13 times greater than those in the experimental external medium used for the SeRIS microelectrode measurements (5 mmol l^{-1}) (see Table 1). In contrast, the K^+ concentration of the external medium of 5 mmol l^{-1} was greater than the haemolymph K^+ concentration of 3.7 mmol l^{-1}. Although the pH of the external medium was 8, the efflux of H^+ from the papillae decreased the pH of the unstirred layer to 7.4, ~0.5 pH units acid to the haemolymph (pH 7.9).

The time required for replacement of the total haemolymph content of each ion by the papillae was estimated from measurements of the surface area of the papillae, the concentrations of the ions in the haemolymph and the calculated fluxes of these ions across the papillae. A papilla from a fourth instar larva is approximately 0.16 cm in length and 0.02 cm in width. The surface area of one papilla is therefore 1.27×10^{-2} cm^2, thus a fourth instar larva has

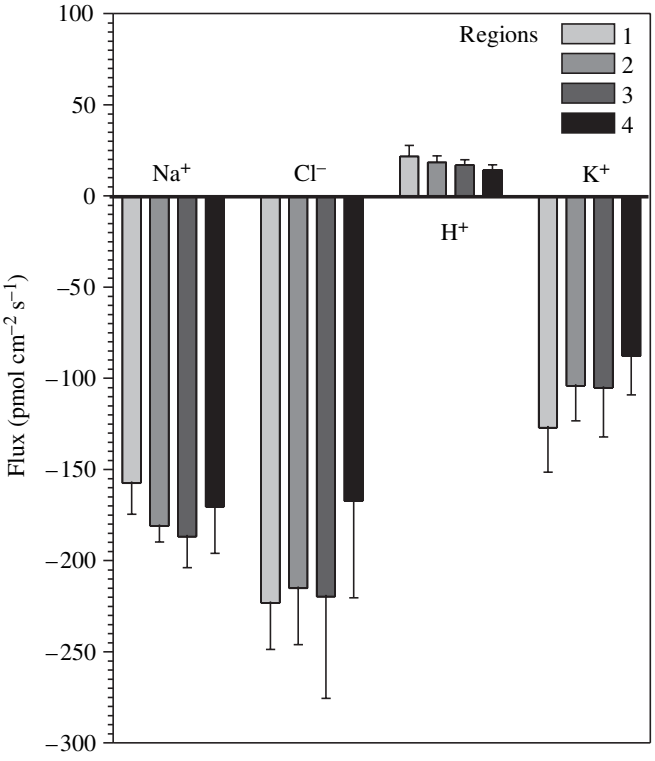

Fig. 4. Summary of ion fluxes in the four regions of the anal papilla *in vitro*. There were no regional differences in ion fluxes. Values are mean + S.E.M. (Na^+, *N*=6; Cl^-, *N*=7; H^+, *N*=9; K^+, *N*=9).

5.08×10^{-2} cm^2 of papillae surface area across which to exchange ions with the external medium. With an external concentration of 5 mmol l^{-1} of each ion, the complete replacement of Cl^- haemolymph levels may be accomplished in approximately 4 h compared with K^+ and Na^+, which require 3.3 and 2.4 h, respectively (Table 2).

Discussion

Previous whole animal studies have indirectly implicated the anal papillae of mosquito larvae as sites of Na^+ and Cl^- uptake (Stobbart, 1965, 1967, 1971; Phillips and Meredith, 1969). The present study is the first to localize ion fluxes specifically to the papillae through direct measurement of ion concentration gradients along their length. Flux estimates based on our measurements indicate net influx of Na^+, Cl^- and K^+ and net efflux of acid and NH_4^+. These findings strengthen the hypothesis that the anal papillae are osmoregulatory/ionoregulatory organs. Our data indicate that the papillae can replace haemolymph Na^+ and Cl^- content in ~4 h when the external medium contains 5 mmol l^{-1} NaCl. These high rates of transport suggest that the papillae may play an important role in compensating for osmotic and ionic perturbations associated with changes in the external medium.

Our data are in agreement with previous findings that studied the uptake of radioisotopes of Na^+ and Cl^- from dilute solutions of NaCl (see Stobbart, 1965, 1967, 1971). The net Na^+ and Cl^-

Table 2. *Estimates of time for replacement of haemolymph ion content by transport across the anal papillae*

Ion transported	Papillae ion flux per larva (pmol larva^{-1} s^{-1})	Papillae ion flux per larva (nmol mg^{-1} h^{-1})	Time to complete ion haemolymph replacement (h)
Influx			
Na^+	16.1	22.3	2.4
Cl^-	7.6	10.5	4.0
K^+	3.4	4.6	3.3
Efflux			
NH_4^+	5.1	7.2	NA
H^+	5.2	7.2	NA

The surface area of the papilla (1.27×10^{-2} cm^2) and the mean ion fluxes across all four regions of the papilla (Fig. 3) were used to estimate the ion flux resulting from all four papillae in a single larva. For comparisons with Stobbart (1965, 1967) flux was converted to nmol mg^{-1} h^{-1}, based on a larval mass of 2.66 mg. The values were subsequently used to estimate the time to replace the total ion content of the haemolymph, based on a mean larval haemolymph volume of 1.65 µl. These values are single calculations using an average papilla surface area, therefore no variation is associated with the values.

NA, not applicable.

influx occurs against a concentration gradient since haemolymph Na^+ and Cl^- concentrations are over tenfold greater in the haemolymph than the external medium, suggesting that these ions may enter the papillae by a form of active transport. Our results also confirm previous suggestions that Ca^{2+} exchange does not occur at the anal papillae since there was no Ca^{2+} concentration gradient adjacent to the surface of the papillae (see Barkai and Williams, 1983).

The estimated net Na^+ influx of 22.3 nmol mg^{-1} h^{-1} for larvae bathed in 5 mmol l^{-1} NaCl is greater than the value of 9–10 nmol mg^{-1} h^{-1} derived by Stobbart (1965, 1967) for uptake of [^{22}Na] by whole larvae in the same concentration of NaCl. Similarly, the estimated net Cl^- influx of 10.5 nmol mg^{-1} h^{-1} for larvae bathed in 5 mmol l^{-1} NaCl, is greater than the values of 6–7 nmol mg^{-1} h^{-1} for [$^{36}Cl^-$] uptake by whole larvae (Stobbart, 1965, 1967). Values obtained from the current study were derived from direct measurements of ion concentration gradients adjacent to the surface of the papillae, whereas the values of Stobbart (1965, 1967) were obtained for whole larvae in which the effects of drinking rate and loss of ions through excretion may have affected the calculated flux values. Our results indicate that Na^+ influx is approximately double that of Cl^-, in contrast to previous reports of near equivalence under similar conditions (Stobbart, 1967). Taking into account the net movements of all the positive ions (Na^+, K^+, H^+ and NH_4^+) across the papillae, there was a net cation influx of 9.2 pmol s^{-1}. This was accompanied by net anion influx (Cl^-) of 7.6 pmol s^{-1}, leaving 1.6 pmol s^{-1} of cation efflux or anion influx unaccounted for by any of the ions measured. This discrepancy is in part a consequence of summing the mean values of five ion fluxes, each with its associated standard error. Fluxes of other ions, such as an efflux of Mg^{2+} or influx of HCO_3^-, for example, could also contribute to the discrepancy. For the *in vitro* papillae (Fig. 4), the sum of Na^+ and K^+ influx, minus the H^+ efflux, is within 27% of the magnitude of the Cl^- influx, suggesting that fluxes of other unidentified ions are relatively small.

Our data are of use in considering which ion transporters are present in the anal papillae. Na^+/H^+ exchange across the papillae has been suggested by the change in pH of the external medium during Na^+ uptake by whole larvae (Stobbart, 1971). Our estimates of H^+ efflux from the papillae of larvae held in dilute NaCl support this hypothesis and indicate that H^+ efflux can account for 32% of Na^+ influx. Given the saturable nature of Na^+ influx (see Stobbart, 1965), these results may indicate the presence of a specific Na^+/H^+ transporter in the anal papillae. Increased H^+ efflux and K^+ influx in proximal regions of the papillae suggest that these regions, relative to those more distal, are specialized for H^+ efflux and K^+ influx. Na^+/H^+ exchange cannot explain the regional difference in H^+ efflux since there is no complimentary increase in Na^+ influx at the proximal regions of the papillae. The presence of a specific K^+/H^+ exchanger is a possibility given the regional difference in K^+ influx, which is complimentary to H^+ efflux. A K^+/H^+ exchanger is believed to regulate alkalinization of the midgut lumen in *Manduca sexta* (Lepier et al., 1994). Although the mechanism providing the driving force for ion movements across the anal papillae is unknown, the exchange of H^+ for Na^+ and/or K^+ occurs as secondary transport that is energized by a vacuolar type H^+-ATPase in other osmoregulatory organs such as the midgut and Malpighian tubules (Wieczorek et al., 2003; Weng et al., 2003).

There was no evidence for regionalization of H^+ efflux and K^+ influx across papillae *in vitro*. The possibility of tissue damage contributing to the differences in magnitudes of estimated fluxes *in vitro* relative to those *in vivo* cannot be ruled out; however, physiological factors may also contribute. For example, levels of CO_2 in the tracheal system within the papillae may be higher *in vivo*, particularly in the proximal regions closest to the metabolically active tissues in the rest of the body. Corresponding increases in H^+ production by carbonic anhydrase could sustain higher H^+ efflux in the proximal regions of the papillae *in vivo*. Irrespective of the quantitative differences in the fluxes across the papillae *in vitro* relative to those *in vivo*, the *in vitro* preparation developed in this study will aid subsequent identification of ion transporters involved. Importantly, the *in vitro* preparation facilitates application of drugs, putative hormonal factors or second messengers to basolateral surfaces of the epithelial cells of the papilla, while providing an immobile preparation that facilitates repeated SeRIS microelectrode measurements at the same point over an extended period of time.

The mechanism of ammonia excretion by the anal papillae remains unclear. Previous results demonstrated very little effect of NH_4^+ on Na^+ flux in the anal papillae, suggesting that Na^+/NH_4^+ exchange is not involved in ammonia excretion (see Stobbart, 1967). Our data reveal that the unstirred layer (as

measured with pH-selective microelectrodes), adjacent to the papillae is acidic to the haemolymph, thus favouring formation of NH_4^+ from NH_3. The calculated flux values (~100 pmol cm^{-2} s^{-1}) are equivalent to 360 nmol cm^{-2} h^{-1}. This value is similar to those previously reported for isolated locust hindgut (580 nmol cm^{-2} h^{-1}; Thomson et al., 1988) and those calculated from whole animal data for the cockroach *Periplaneta americana* (220 nmol cm^{-2} h^{-1}, Mullins, 1974). This suggests that the papillae may play a prominent role in the excretion of nitrogenous waste.

In summary, this study provides the first direct measurements of ionic concentration gradients along the anal papillae of mosquito larvae, which are likely to be indicative of ion transport. Furthermore, our data suggest that the papillae are not only sites of ion uptake, as previously reported, but can be used to eliminate acid equivalents and nitrogenous waste. The development of both *in vivo* and *in vitro* assays for direct measurement of ion transport by the papillae provides a basis for further studies characterizing the ion transport mechanisms involved.

The authors would like to thank Dr Marjorie Patrick for supplying *Aedes aegypti* eggs that enabled the establishment of a colony. Funding was provided by the Natural Sciences and Engineering Research Council of Canada in the form of a Discovery Grant to M.J.O'D. and a Postdoctoral Fellowship to A.D.

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