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Changing salinity induces alterations in hemolymph ion concentrations and Na^+ and Cl^- transport kinetics of the anal papillae in the larval mosquito, *Aedes aegypti*

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Summary

Mosquito larvae are found in diverse aquatic habitats ranging from freshwater to hypersaline water and must often deal with rapid changes in habitat salinity. We transferred larvae of *Aedes aegypti* from freshwater to 30% seawater, or *vice versa*, and measured the time course of changes in their hemolymph ion concentrations, using ion-selective microelectrodes. We also reported the Michaelis–Menten kinetics of Na^+ and Cl^- transport by the anal papillae for the first time using the scanning ion-selective electrode technique (SIET). Hemolymph concentrations of Na^+, Cl^- and H^+ increased within 6 h, when larvae were transferred from freshwater to seawater and decreased within 6 h, when transferred from seawater to freshwater. Kinetic parameters for Na^+ and Cl^- transport by the anal papillae were altered after only 5 h following transfer between freshwater (FW) and 30% seawater (30%SW). The J_{max} (maximum transport rate) for both ions decreased when larvae were transferred to 30%SW, whereas the K_t (a measure of transporter affinity) increased for Na^+ transport but was unaltered for Cl^- transport, suggesting that Na^+ and Cl^- uptake are independent. Data reveal significant changes in ion transport by the anal papillae of mosquito larvae when they are faced with changes in external salinity such that Na^+ and Cl^- uptake decrease in higher salinity. The alterations in Na^+ and Cl^- uptake may be a consequence of changes in hemolymph ion levels when larvae encounter altered salinity. The rapid changes in ion transport described here compliment the previously observed long term alterations in the morphology and ultrastructure of the anal papillae.

Key words: anal papillae, mosquito, salinity, ion transport, ionoregulation.

Introduction

Larval mosquitoes live in a variety of aquatic habitats such as marshes, ditches, woodland pools and man-made containers that collect rainwater. One of the challenges they face in these habitats is the propensity for relatively rapid changes in salinity. For instance, heavy rainfall can quickly and significantly decrease salinity in these relatively low volume habitats creating a tendency for dilution of body fluids (Bradley, 1987). By contrast, evaporation of larval habitats can lead to increased salinity so that larvae tend to lose water to their habitat, resulting in the concentration of body fluids. The habitat of the salt marsh mosquito, *Ochlerotatus taeniorhynchus* experiences salinity fluctuations with freshwater influx from inland rivers and streams, and tidal influx of seawater. Survival of mosquito larvae depends on their ability to regulate the influx and efflux of ions under these dynamic conditions.

Mosquitoes that tolerate high salinity employ one of two strategies (Bradley, 1994). Osmoregulators have a specialized rectal segment that actively transports ions from the hemolymph into the rectal lumen, resulting in a concentrated urine (Bradley, 1994). Osmoconformers accumulate compatible solutes such as proline and trehalose in both intra- and extracellular compartments (Patrick and Bradley, 2000a).

Larval mosquitoes possess four anal papillae that extend from the terminal segment and project into the external medium. The walls of the papillae are composed of a one cell thick epithelial syncytium and the lumen is continuous with the hemolymph. Classical studies implicated the anal papillae of larval mosquitoes in the uptake of ions from dilute environments against large concentration gradients (Koch, 1938; Treherne, 1954; Ramsay, 1953). Martini-Hamburg (Martini-Hamburg, 1923) showed that anal papillae of mosquito larvae are shorter when larvae are reared in increased salinity. Some changes in the appearance of the epithelial cells, specifically cell shrinkage and a decrease in membrane infoldings, were also noted when larvae were acclimated to diluted seawater (Wigglesworth, 1933). These observations lead to the hypothesis that the anal papillae function only when larvae encounter dilute freshwater conditions. Subsequent

ultrastructural studies supported this hypothesis by demonstrating that epithelial cells in papillae of larvae reared in diluted seawater contain fewer mitochondria and reduced apical membrane folds relative to those of larvae reared in freshwater, suggesting a reduction in active ion transport (Sohal and Copeland, 1966). These profound morphological and ultrastructural changes are presumably adaptive for larvae exposed to chronic increases in ambient salinity; however, they raise the question of how larvae respond in the short term to variations in salinity.

The characteristics of Na^+ and Cl^- uptake by mosquito larvae have been determined in several species. Stobbart (Stobbart, 1965; Stobbart, 1967) measured radioisotopic ion fluxes in intact larvae of *Aedes aegypti* to demonstrate that both Na^+ and Cl^- uptake by the larvae were saturable and followed Michaelis–Menten kinetics, suggesting a carrier-mediated uptake mechanism. More recently the regulation of Na^+ and Cl^- levels in *Culex* mosquitoes subjected to both chronic and acute exposure to varying salinity has been studied (Patrick and Bradley, 2000a; Patrick et al., 2001). Interestingly, an increase in the affinity for Na^+ uptake (decrease in K_m) was observed in the obligate freshwater species *Culex quinquefasciatus* when acclimated to a low NaCl (0.25 mmol l^{-1}) medium, and this was accompanied by an increase in the maximum uptake capacity (J_{max}) for Na^+ (Patrick et al., 2001). Large influxes of both Na^+ and Cl^- were recently measured directly at the anal papillae using the scanning ion-selective electrode technique (SIET) (formerly known as the self-referencing ion-selective microelectrode technique, or SeRIS) such that the entire Na^+ and Cl^- content of the hemolymph could be replaced in just 3 h from the uptake occurring at the papillae, conclusively demonstrating that the anal papillae are the predominant sites for Na^+ and Cl^- uptake (Donini and O'Donnell, 2005). Taken together, these findings suggest a regulatory mechanism that stimulates the activity and/or the expression of Na^+ transporters in the anal papillae when larvae encounter dilute environments. Mechanisms regulating ion fluxes at other tissues such as the gut and Malpighian tubules may also exist.

The stimuli that trigger the regulatory mechanisms observed in *Culex* are not known. A probable and potential stimulus would be changes in the composition of the hemolymph. Efforts have been made to track hemolymph composition when larvae are either reared in or transferred to, higher salinity. The larvae of *Aedes aegypti* are capable of regulating their Na^+ and Cl^- hemolymph concentration to a higher but stable level up to an external rearing salinity of approximately 30% seawater (Edwards, 1982). These changes in hemolymph ion levels may lead to the activation of appropriate regulatory mechanisms. For instance, it has been shown that increases in Na^+ concentration of the hemolymph in *Culex tarsalis* is correlated with increased accumulation of the compatible solute proline (Patrick and Bradley, 2000b).

In the present study larvae of the yellow fever vector, *Aedes aegypti*, were exposed acutely to changes in external salinity from FW to 30%SW and *vice versa*, and the time courses of changes in hemolymph Na^+, K^+ and Cl^- concentrations were measured. In light of the documented changes in pH of hemolymph/blood associated with changing external salinity in crustaceans and fishes (Maxime et al., 1990; Nonnotte and Truchot, 1990; Truchot, 1981; Truchot, 1992), the time course of hemolymph pH was also assessed. In addition, the kinetic parameters of Na^+ and Cl^- fluxes were measured directly at the anal papillae from similarly treated larvae using SIET. Our findings reveal rapid changes in ion transport kinetics of the anal papillae that correspond well with the time courses of changes in hemolymph ion concentrations.

Materials and methods

Rearing of mosquitoes

A colony of *Aedes aegypti* (Linnaeus) was established at McMaster University with eggs obtained from Dr Marjorie Patrick at the University of San Diego, CA, USA. Adult mosquitoes had continuous access to a 5% aqueous sugar solution and females were fed once a week on warm sheep's blood in Alsever's solution (Cedarlane Laboratories, Hornby, ON, Canada) containing 0.1 mg ml^{-1} of ATP (Sigma, Oakville, ON, Canada). Females deposited eggs in freshwater (65 μmol l^{-1} Na^+)-filled Petri dishes. Eggs were transferred by pipette onto filter paper and stored in plastic bags until needed. Eggs were hatched in plastic containers filled with freshwater (0.06 mmol l^{-1} Cl^-, 0.1 mmol l^{-1} Na^+, 0.24 mmol l^{-1} K^+) and fed *ad libitum* with a solution of liver powder and yeast. Fourth instar larvae were used in all experiments.

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; Rheault and O'Donnell, 2004; Donini and O'Donnell, 2005). 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); and H^+ Ionophore I Cocktail B (100 mmol l^{-1} NaCl+100 mmol l^{-1} sodium citrate, pH 6.0). The tips of the ion-selective microelectrodes used to measure ion concentrations in hemolymph samples expelled under paraffin oil were dipped in a solution of polyvinylchloride (PVC, Fluka) in tetrahydrofuran (Fluka), prior to use, as described (Rheault and O'Donnell, 2004).

For measurement of hemolymph chloride concentration a solid-state silver wire electrode was employed. This type of electrode is unaffected by organic anions, and its construction and use has been described in detail (Donini and O'Donnell, 2005). In short, one end of a fine silver wire was shaped into a fine point by cutting the wire at oblique angles with a razor blade. The wire was subsequently inserted inside a micropipette and secured with melted glue such that the fine

point protruded from the tip of the micropipette. The fine point of the wire was coated with AgCl by dipping in a solution of ferrous chloride prior to use. The opposite end of the silver wire protruding from the barrel of the micropipette was then soldered to the wire connected to the amplifier.

Measurements of hemolymph ion concentrations

Concentrations of Na^+, Cl^-, H^+ and K^+ were measured in samples of hemolymph collected from larvae maintained in freshwater (FW: 0.06 mmol l^{-1} Cl^-, 0.1 mmol l^{-1} Na^+, 0.24 mmol l^{-1} K^+) and subsequently transferred to 30% seawater (30%SW: 100 mmol l^{-1} Cl^-, 97.6 mmol l^{-1} Na^+, 3.14 mmol l^{-1} K^+, Instant Ocean®, Aquarium Systems Inc., Mentor, OH, USA), or from larvae acutely exposed to 30%SW for a minimum of 24 h, and then transferred to FW. After transfer, larvae remained in either 30%SW or FW for periods ranging from 1 to 40 h prior to the collection of hemolymph. The procedure for collection of hemolymph has been described in detail (see Donini and O'Donnell, 2005). Ion concentrations were measured using ion-selective microelectrodes that were calibrated in the following solutions: Na^+ (25 mmol l^{-1} NaCl and/or 225 mmol l^{-1} LiCl and 250 mmol l^{-1} NaCl); K^+ (2.5 mmol l^{-1} KCl + 247.5 mmol l^{-1} NaCl; 25 mmol l^{-1} KCl + 235 mmol l^{-1} NaCl and 250 mmol l^{-1} KCl); Cl^- (25 mmol l^{-1} KCl + 225 mmol l^{-1} $KHCO_3$ and 250 mmol l^{-1} KCl); H^+ (larval saline solution titrated with NaOH to pH 7 or pH 8). The composition of the larval saline was modeled after that devised by Clark and Bradley (Clark and Bradley, 1996) and contained the following (in mmol l^{-1}): 64 NaCl; 3 KCl; 5 $NaHCO_3$; 0.6 $MgSO_4$; 5 $CaCl_2$; 25 Hepes; 5 L-proline; 9.1 L-glutamine; 8.74 L-histidine; 14.4 L-leucine; 3.37 L-arginine–HCl; 10 glucose; 5 succinic acid; 5 malic acid; 10 citric acid. Slopes of the electrodes [mV; mean ± s.e.m. (*N*)] for a tenfold change in ion concentration were 50.3±0.4 (11) for Na^+, 52.6±0.3 (11) for Cl^-, 55.8±0.9 (16) for K^+ and 59.1±1.0 (16) for H^+.

Calculations of ion concentrations were made using the following equation:

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

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

SIET measurements of ion gradients adjacent the surface of the anal papillae and calculation of ion fluxes across the anal papillae

The SIET system and protocol employed in this study are described in detail elsewhere (Rheault and O'Donnell, 2001; Rheault and O'Donnell, 2004) with the modifications described (Donini and O'Donnell, 2005). In short, microelectrode excursion distances of 50 μm were employed. The 'wait' and 'sample' periods were 3 s and 1 s, respectively, and fluxes were reported as an average of three repetitive measurements at each site. Microelectrodes used in SIET were calibrated in 0.1, 1 and 10 mmol l^{-1} solutions of NaCl. Slopes (mV) for a tenfold change in ion concentration were [mean ± s.e.m. (*N*)] 57.1±0.4, (40) for Na^+ and 59.3±1.1 (34) for Cl^-. Ion flux was calculated after subtracting the electrode noise, measured at a reference position 4–6 mm from the preparation, from the differential signal measured at the site of interest near the preparation. The efficiency of the Na^+ and Cl^- SIET protocol 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 were then chosen from within the plateau.

Calculated Na^+ and Cl^- fluxes at a single point along a papilla were obtained for each of several bath concentrations of NaCl. The bath initially consisted of 0.1 mmol l^{-1} NaCl and progressively higher concentrations were added to a maximum of 10 mmol l^{-1}. SIET measurements of Na^+ and Cl^- concentration gradients adjacent the surface of the anal papillae at a single point along the papilla were taken at each concentration of NaCl. The background voltage signal of the SIET microelectrode was used to calculate the actual NaCl concentration of each treatment using the following equation:

$$C_B = 1 \times 10^{(Vb - V_1/S)} \times 1000 ,$$

where C_B is the background ion concentration in μmol l^{-1}; V_b is the average voltage (mV) recorded at each sampling time; V_1 is the voltage (mV) recorded when the electrode is placed in 1 mmol l^{-1} NaCl; and S is the slope of the electrode. Ion fluxes were then calculated by first obtaining the concentration gradient and subsequently converting this gradient into a flux measurement using Fick's Law of diffusion. A detailed description of the calculations including all relevant equations has been published (Donini and O'Donnell, 2005).

The *in vivo* preparation employed in this study was developed and described previously (Donini and O'Donnell, 2005). An *in vitro* preparation was also previously developed and how a single papilla can be removed from the larvae by pinching the papilla at the base using fine forceps was described (see Donini and O'Donnell, 2005). This *in vitro* preparation was modified such that a small binder clip was used to keep the forceps closed once the papilla was pinched. In this manner, the base of the papilla remained sealed, thereby preventing an exchange of fluids between the hemocoel and the external solution. The forceps were subsequently fastened to a micromanipulator, which was used to submerge the papilla in the bath and aid in positioning the papilla relative to the SIET microelectrode. The *in vivo* preparation allowed for the measurement of ion fluxes at the papillae of intact, undissected larvae in near real-time. The *in vitro* preparation greatly facilitated the derivation of kinetic parameters for Na^+ and Cl^- influx because it eliminated the papillae movements normally associated with the *in vivo* preparation, which made it difficult to measure repeatedly from a single site along the length of the papillae. The validity of this modified *in vitro* preparation was tested by recording ion concentration gradients at a single point along the papilla

for at least 45 min in a bath containing 1 mmol l^{-1} NaCl. In addition a limited number of *in vivo* preparations of FW larvae were used to derive the kinetics of Na^+ and Cl^- influx and these results were compared to similar results obtained using the *in vitro* preparation with FW larvae. The metabolic dependency of the concentration gradients was assessed by the addition of 30 μmol l^{-1} KCN to the bath. The *in vitro* preparation was utilized to assess the effects of external salinity on the ion concentration gradients adjacent to the papillae. For these studies, larvae held in FW were transferred to 30%SW for 5 h or were initially transferred to 30%SW for 5 h and then transferred back to FW for an additional 5 or 20 h prior to experimentation. To assess whether the anal papillae play a role in the elimination of excess ions, some larvae were transferred to 30%SW for between 15 and 24 h and ion fluxes at the anal papillae were measured using the *in vivo* preparation in a bath NaCl concentration of 0.1 mmol l^{-1}.

Treatment of data and statistical analyses

All statistical analyses used a 5% level of significance. Data collected for hemolymph ion concentrations were analyzed using a single factor non-parametric ANOVA (Kruskal–Wallis test) for each ion with the length of time the larvae were held in the 30%SW (FW to 30%SW) or FW (30%SW to FW) as the independent variable. A significant ANOVA was followed by a Dunn's multiple comparison test to determine in which groups hemolymph ion concentrations differed from those measured immediately prior to transferring larvae (time=0). ANOVA was also employed to validate the use of the modified *in vitro* preparation and the effect of cyanide on ion fluxes at the anal papillae.

The effects of the concentration of Na^+ and Cl^- in the bath on the respective ion fluxes across individual papillae were measured. Data collected for each papilla were fitted with a nonlinear regression, one-site binding model (Graphpad Prism 3.0, Graphpad Software Inc., San Diego, CA, USA), which was used to determine the kinetic parameters of Na^+ and Cl^- transport. The equation used was $J=J_{max}[ion]/(K_t+[ion])$ where J is the rate of ion uptake into the papilla from an external concentration of the ion, J_{max} is the maximum rate of ion uptake that is achievable by the papilla, K_t is the external concentration of the ion that results in a rate of uptake that is half of the maximal rate of uptake and [ion] is the external concentration of the ion. Only papillae that yielded curves with R^2 values $\geqslant 0.9$ were used in subsequent analyses. Generally, the papillae that yielded curves with lower R^2 values also exhibited relatively lower overall fluxes and were thus considered to have sustained damage during dissection. In addition, a small number of papillae sampled from larvae that were subjected to 30%SW showed an unsaturable Cl^- uptake. Data for these papillae could be more accurately fitted with a linear regression and were therefore excluded from subsequent analyses. Mean values of kinetic parameters for different treatments were compared using an ANOVA followed by Bonferroni multiple comparison tests.

Results

Hemolymph ion concentrations changed significantly within 6 h of transferring larvae of *Aedes aegypti* between FW and 30%SW (in either direction). Na^+, Cl^- and H^+ concentrations increased when larvae were transferred to 30%SW. Over the initial 5 h after transfer of larvae from FW to 30%SW, the Na^+ concentration increased significantly from 82.9±3.0 (16) to 103.8±3.6 mmol l^{-1} (10) (ANOVA, Fig. 1A) and the Cl^- concentration significantly increased from 50.3±1.5 (16) to 76.8±2.0 mmol l^{-1} (10) in just 3 h after transfer (ANOVA, Fig. 1B). By 16 h after transfer of larvae from FW to 30%SW the Na^+ and Cl^- concentrations in hemolymph remained relatively unchanged at approximately 130 mmol l^{-1}. There was no change in K^+ concentration in the hemolymph (Fig. 1C), whereas H^+ concentration increased significantly from 19.5±1.2 (16) to 49.7±4.7 nmol l^{-1} (10) over 40 h (Fig. 1D).

When larvae were transferred from 30%SW to FW the hemolymph Na^+ and Cl^- concentrations significantly decreased from 130.9±3.2 (7) to 103.5±1.1 (7) and 126.6±2.0 (7) to 69.8±6.9 mmol l^{-1} (7), respectively in 6 h (ANOVA, Fig. 2A,B). The Na^+ and Cl^- concentrations in the hemolymph of larvae held in 30%SW for a minimum of 24 h (Fig. 2A,B) were similar to those of the FW-reared larvae that had been transferred from FW to 30%SW for 40 h (Fig. 1A,B). There was no change in hemolymph K^+ concentration when larvae were transferred from 30%SW to FW (Fig. 2C). The H^+ concentration decreased significantly from 28.6±2.9 (8) to 8.9±0.7 nmol l^{-1} (5) in 25 h (Fig. 2D), a change of 9.7 nmol l^{-1} which is similar to the magnitude increase in H^+ concentration of 12.3 nmol l^{-1} over the same time period when larvae were transferred from FW to 30%SW (see Fig. 1D).

Utilizing the *in vitro* preparation, sustained Na^+ concentration gradients, indicating Na^+ influx (from bath to lumen), were recorded from a single point along the surface of individual papillae for at least 45 min. There was no evidence of run down over the course of the experiment (see Fig. 3) indicating that the *in vitro* preparation was suitable for the experiments. The Na^+ influx was reduced within 5 min of the addition of the metabolic inhibitor KCN (30 μmol l^{-1}).

This study is the first to calculate the kinetic parameters of Na^+ and Cl^- uptake by anal papillae. The Na^+ and Cl^- uptake recorded at the papillae utilizing the *in vitro* preparation was saturable and conformed to Michaelis–Menten kinetics in both FW and in 30%SW (Fig. 4A,B). Since mosquito larvae are capable of moving the anal papillae, it was difficult to measure repeatedly from a single point along the length of the papillae using the *in vivo* preparation, while changing the NaCl bath concentration. Therefore, data based on *in vivo* preparations were only collected for larvae held in freshwater and these data were used as a secondary validation of the *in vitro* preparation. These data revealed that the kinetic parameters for Na^+ and Cl^- uptake calculated from data collected with the *in vitro* preparation did not differ from the data collected with the *in vivo* preparation (see Fig. 4C,D: FW *in vivo vs* FW *in vitro*). The papillae showed a greater affinity for Na^+ uptake relative

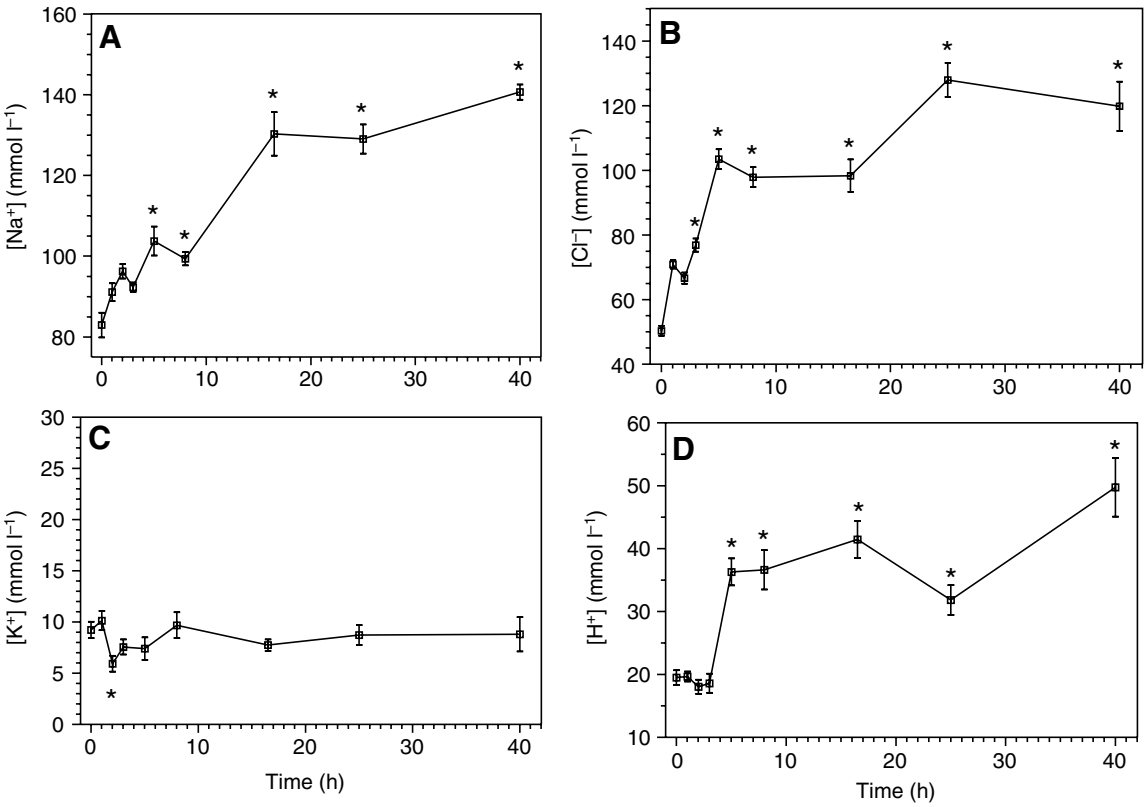

Fig. 1. Transfer of mosquito larvae from freshwater (FW) to 30%SW (seawater) leads to significant increases in Na^+, Cl^- and H^+ concentrations in the hemolymph within 5 h of transfer. Groups of mosquito larvae were transferred from FW to 30%SW at time=0, where they were held for varying periods of time prior to the collection of hemolymph. Ion-selective microelectrodes were used to measure the Na^+ (A), Cl^- (B), K^+ (C) and H^+ (D) ion concentrations of the hemolymph. Values are means ± s.e.m., N=16 for time=0 and N=10 for all other groups. An asterisk denotes a significant difference from value at time=0.

to Cl^- uptake when larvae were held in FW, as shown by the significantly lower K_t values for Na^+ uptake (see Fig. 4C, unpaired *t*-test). Conversely, the papillae had an overall greater capacity for Cl^- uptake relative to Na^+, as indicated by the relatively higher J_{max} values when larvae were held in FW (see Fig. 4D, unpaired *t*-test). The relative efficiencies of the papillae to transport both Na^+ and Cl^- can be assessed by calculating the ratio of J_{max}/K_t from the individual papillae, for each ion [see Bresler et al. (Bresler et al., 1990) and Murata et al. (Murata et al., 1999) for the use of the J_{max}/K_t ratio]. Under freshwater conditions the transport efficiencies of 0.51±0.1 for Na^+ and 0.44±0.1 for Cl^- do not differ significantly.

Transferring larvae from FW to 30%SW resulted in significant changes in the Michaelis–Menten kinetic parameters for Na^+ uptake by the papillae, and these changes occurred within 5 h. The J_{max} for Na^+ uptake significantly decreased from 364.3±49 to 211.2±49 pmol cm^{-2} s^{-1}, whereas the K_t increased from 0.8±0.1 to 2.4±0.7 mmol l^{-1} (Fig. 4C,D). As a result, the papillae transport efficiency for Na^+ significantly decreased from 0.51±0.1 to 0.12±0.02. Four out of a total of 21 papillae that were sampled from larvae exposed to 30%SW for 5 h revealed an unsaturable Cl^- uptake. These papillae were not included in the calculation of mean kinetic parameters since they did not conform to Michaelis–Menten kinetics. The mean K_t for Cl^- uptake from the remaining papillae was no different than that for FW larvae; however, the J_{max} for Cl^- uptake decreased from 674.4±99 to 286.6±38 pmol cm^{-2} s^{-1}, resulting in a significant decrease in transport efficiency for Cl^- from 0.44±0.1 to 0.27±0.03.

The observed decrease in the J_{max} for Cl^- uptake was reversible within 5 h of transferring larvae back to FW (see Fig. 4D). By contrast, the decreased J_{max} for Na^+ uptake was irreversible up to 20 h after transferring larvae back to FW, but the increased K_t was reversible (Fig. 4C,D). As a result the Na^+ transport efficiency of 0.26±0.05 remained significantly lower than in papillae from freshwater-held larvae.

We exposed larvae to 30%SW for a minimum of 15 h and then measured Na^+ and Cl^- fluxes from the anal papillae using the *in vivo* preparation in 0.1 mmol l^{-1} NaCl. Mean Na^+ and Cl^- efflux (from lumen to bath) of 16.2±3.3 pmol cm^{-2} s^{-1} (8) and 84.3±37.5 pmol cm^{-2} s^{-1} (6) respectively, were measured at the anal papillae within 0.5 h of transfer from 30%SW to 0.1 mmol l^{-1} NaCl. These larvae were treated similarly to the larvae used to measure the time course of hemolymph ion changes when transferred from 30%SW to FW. As discussed

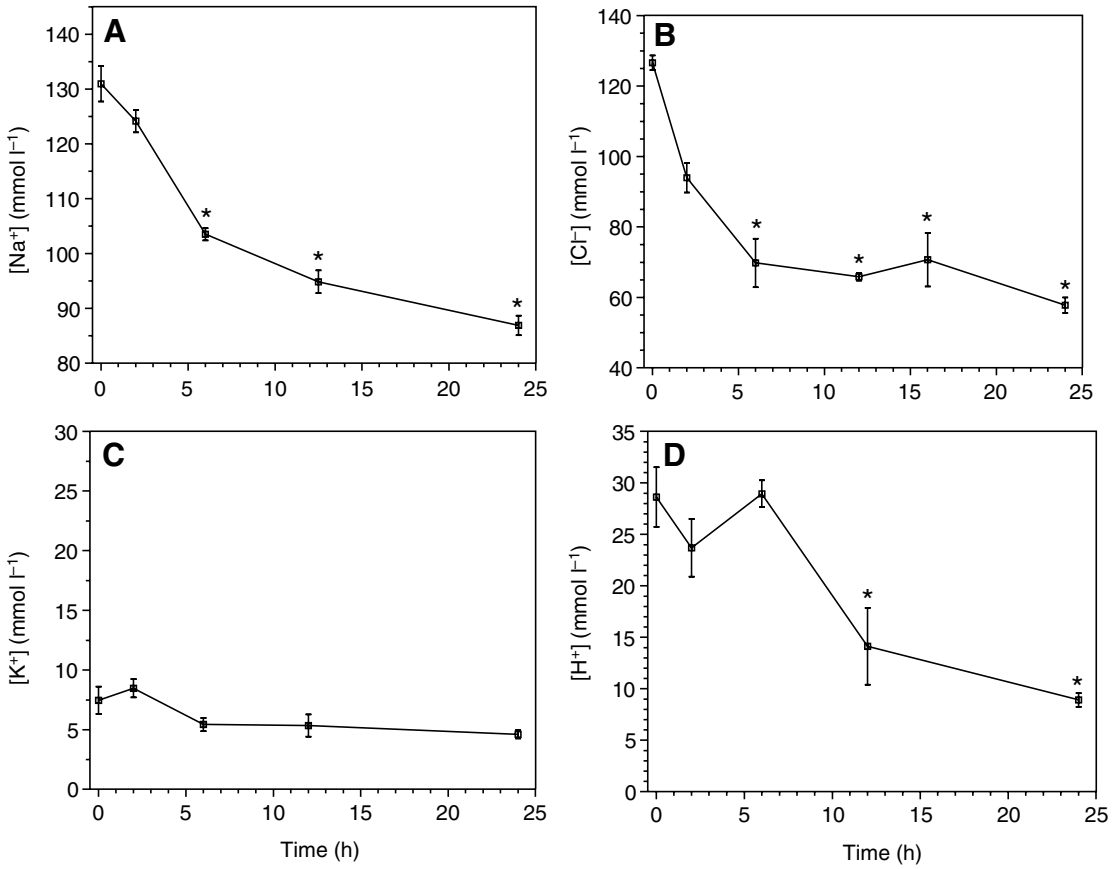

Fig. 2. Transfer of mosquito larvae from 30%SW (seawater) to FW (freshwater) results in significant decreases of Na^+, Cl^- and H^+ concentrations in the hemolymph within 6 h of transfer. Groups of mosquito larvae that were reared in FW were transferred and held in 30%SW for at least 24 h. Larvae were subsequently transferred back to FW at time=0, where they were held for varying periods of time prior to the collection of hemolymph. Ion-selective microelectrodes were used to measure the Na^+ (A), Cl^- (B), K^+ (C) and H^+ (D) ion concentrations of the hemolymph. Values are means ± s.e.m., N=6–14. An asterisk denotes a significant difference from value at time=0.

below, the magnitude of Na^+ and Cl^- efflux from the papillae can be utilized to calculate the contribution of the papillae towards the observed decrease in Na^+ and Cl^- concentration of the hemolymph shown in Fig. 2A,B.

Discussion

Significant differences in the kinetic parameters for Na^+ and Cl^- uptake at the anal papillae develop within 5–6 h when larvae are transferred between freshwater and 30% seawater. The changes in the maximum rate of transport for Na^+ and Cl^- uptake (lower J_{max} in 30%SW) as well as the change in the affinity of the Na^+ uptake mechanism (higher K_t in 30%SW) are consistent with a decrease in both Na^+ and Cl^- uptake when larvae encounter higher salinity. This is emphasized by the comparatively lower Na^+ and Cl^- transport efficiencies obtained from the papillae of larvae exposed to 30%SW. The timing of changes in the kinetic parameters for Na^+ and Cl^- uptake by the papillae coincide with the time of significant changes in Na^+ and Cl^- concentrations in the hemolymph, suggesting that concentrations of hemolymph ions may trigger the changes in ion transport observed at the anal papillae.

The underlying mechanisms responsible for these changes remain unclear. There is a paucity of information regarding the identity of the molecular mechanisms of ion transport by the anal papillae. The abolishment of ion uptake at the anal papillae by cyanide supports the apparent dependence of ion uptake on oxidative metabolism. Recently, the presence of V-type-H^+-ATPase in the apical (exterior facing) membrane was demonstrated (Patrick et al., 2006), and Na^+/K^+-ATPase in the basolateral (lumen facing) membrane of the syncytial epithelium of the anal papillae. The localization of the V-type-H^+-ATPase is consistent with the recorded acidification of the unstirred layer adjacent the anal papillae (Donini and O'Donnell, 2005). The V-type-H^+-ATPase may serve to set up a negative potential across the apical membrane through the pumping of H^+ into the external medium, which would create conditions favorable for the electrodiffusive entry of Na^+ through channels across the apical membrane, similar to the scenario found in frog skin (Ehrenfeld et al., 1985) and freshwater fish gills (Lin and Randall, 1991). Indeed,

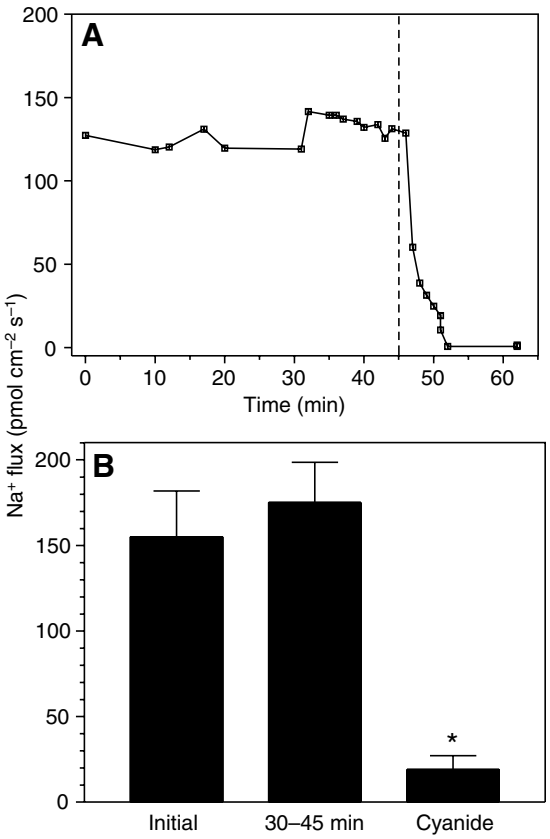

Fig. 3. Potassium cyanide significantly reduces Na^+ uptake by anal papillae of mosquito larvae. Na^+ concentration gradients at a single point adjacent the surface of isolated (*in vitro*) anal papillae were measured using the SIET system in a 1 mmol l^{-1} Na^+ bath. After a minimum of 40 min, 30 μmol l^{-1} KCN was added to the bath. Results are shown as Na^+ flux. (A) A representative preparation illustrates the time course of KCN effects on Na^+ influx. Influx remained consistent for 45 min. KCN was added at the vertical broken line and Na^+ influx immediately decreased and was abolished in 6 min. (B) Na^+ influx measured immediately after the preparation was set up (initial), 1 min prior to the addition of KCN (30–45 min) and 5 min after the addition of KCN. Values are mean ± s.e.m. of seven individual papillae. The asterisk denotes a statistically significant difference from the initial measurements.

Edwards (Edwards, 1983) reported negative apical membrane potentials for the anal papillae of *A. aegypti*. The movement of Na^+ across the basolateral membrane might occur through the actions of the Na^+/K^+-ATPase, which can overcome the positive basolateral membrane potential reported by Edwards (Edwards, 1983). The decrease in J_{max} for Na^+ uptake at the papillae may be caused by the observed increases in Na^+ hemolymph levels, which would act to lower the electrochemical driving force for Na^+ entry. It is also possible that hormonal factors are released which may lead to the modulation of the V-type H^+-ATPase and/or other transporters involved in Na^+ and Cl^- uptake. Hormonal regulation may cause differential expression in the number and/or subtypes of transporters involved in Na^+ and Cl^- uptake and these changes would be manifested by changes in the J_{max} and K_t, respectively.

Irrespective of the underlying mechanisms involved in changes of the kinetic parameters of Na^+ and Cl^- uptake, it is clear that Na^+ and Cl^- uptake processes can be independent of one another. This is evident in the differential changes in ion transport kinetics when larvae are transferred between FW and 30%SW. For instance, the K_t for Na^+ uptake is altered, but the K_t for Cl^- uptake remains unaltered, when larvae are transferred from FW to 30%SW. Furthermore, the seawater-induced change in the J_{max} for Na^+ uptake remains after at least 20 h of re-transferring larvae to FW whereas the changes in the J_{max} for Cl^- transport are reversible within 5 h.

The kinetic parameters reported here are the first measured exclusively from the anal papillae, although, similar parameters have been previously derived for whole larvae. For the purposes of making comparisons between the kinetic parameters that we calculated and those previously reported from whole larval studies we expressed our J_{max} values in units of nmol mg^{-1} h^{-1} in the following section [see Donini and O'Donnell (Donini and O'Donnell, 2005) for detailed description of calculation]. K_t for whole larval Na^+ uptake between 0.5 and 0.6 mmol l^{-1} was reported for two laboratory strains of *A. aegypti* that were kept in an external NaCl concentration of 5 μmol l^{-1} (Stobbart, 1967), whereas a much greater affinity (K_t=0.08 mmol l^{-1}) was found for an Amazonian population of the same species reared in 50 μmol l^{-1} NaCl (Patrick et al., 2002). Our K_t of 0.8 mmol l^{-1} for Na^+ uptake by the anal papillae of larvae held in FW (100 μmol l^{-1} Na^+, 60 μmol l^{-1} Cl^-) is similar to that reported by Stobbart for whole larval Na^+ uptake. By contrast, our K_t of 1.5 mmol l^{-1} for Cl^- uptake by the anal papillae is greater than any previously reported value for whole larval Cl^- uptake, which range from 0.09 to 0.5 mmol l^{-1} (Stobbart, 1967; Patrick et al., 2002). The discrepancies in the K_t for Cl^- uptake may reflect population-based differences, which have been shown in the larvae of the species *Culex quinquefasciatus*, in which the K_t for an Amazonian population is considerably lower than that of a Californian population reared under similar conditions (see Patrick et al., 2002). Our J_{max} values of 25.1 and 44.7 nmol mg^{-1} h^{-1} for Na^+ and Cl^- uptake, respectively, were also higher than previously reported values from whole larval studies. Stobbart reported J_{max} values of 6–7 and 12 nmol mg^{-1} h^{-1} for Cl^- and Na^+, respectively (Stobbart, 1967), whereas Patrick et al. found values of around 3 nmol mg^{-1} h^{-1} for an Amazonian population of *A. aegypti* (Patrick et al., 2002). The much greater J_{max} values obtained directly from the anal papillae did not come as a surprise since it was previously noted that influx of Na^+ and Cl^- measured with the SIET (SeRIS) at the anal papillae were higher than those reported from whole larval studies (see Donini and O'Donnell, 2005). It has been suggested that values obtained from whole larval studies cannot distinguish between ion movements at the papillae and those occurring at other sites such as the gut and body wall (Donini and O'Donnell, 2005). The larger fluxes recorded at the anal papillae with the SIET

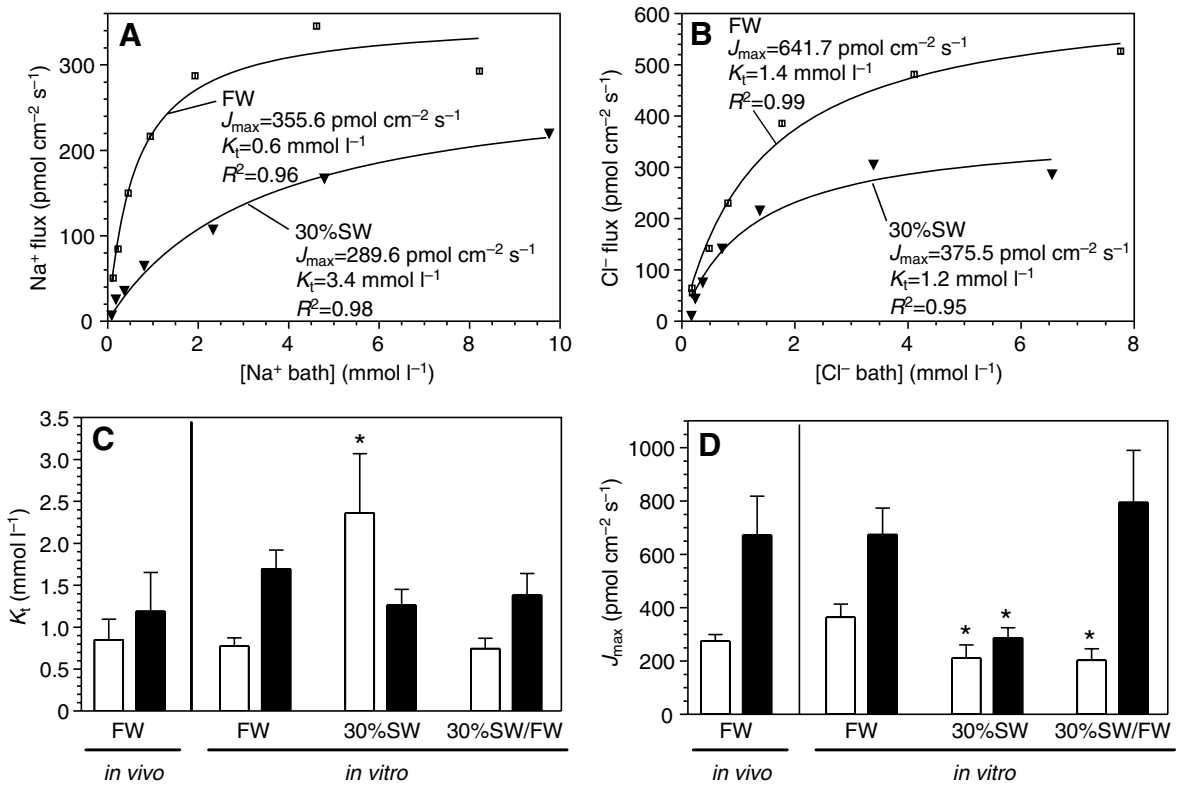

Fig. 4. Exposure to 30%SW significantly changes the Michealis–Menten kinetics of Na^+ and Cl^- influx at the papillae. Na^+ or Cl^- influx was calculated using the measurements obtained from the SIET system from a single point on the surface of the anal papillae while changing the bath NaCl concentration. The results obtained from each individual papilla were fitted to the Michaelis–Menten equation using non-linear regression analysis. J_{max}, maximum rate of transport; K_t, Na^+ or Cl^- concentration that yields a flux of 50% J_{max}. (A) Na^+ and (B) Cl^- influx from single papillae obtained from larvae maintained in FW (freshwater; squares) or in 30%SW (seawater) for 5 h (triangles). (C) K_t and (D) J_{max} (means ± s.e.m.) for groups of papillae obtained from larvae that were either held in freshwater (FW), had been transferred from FW to 30%SW for 5 h (30%SW), or were held in 30%SW for 5 h and subsequently transferred to FW (30%SW/FW) for 20 h (Na^+, white bars, N=6–13) or 5 h (Cl^-, black bars, N=8–13). An asterisk denotes a statistically significant difference from the FW group. Kinetic parameters for Na^+ (N=6) and Cl^- (N=5) uptake by the papillae of intact larvae (*in vivo*) held in FW are also shown (no difference from FW, t-test, P<0.05).

suggest a site such as the gut, where substantial amounts of ions are lost to the environment therefore diminishing the recorded ion uptake in whole larval studies.

The aforementioned Californian population of *Culex quinquefasciatus* responded to changes in the concentration of external NaCl by increasing and decreasing the K_t and J_{max} for Na^+ uptake, respectively (Patrick et al., 2002). This response is consistent with the results presented here for Na^+ and Cl^- uptake by the anal papillae of *A. aegypti*. Together these results suggest that alterations of ion transport by the anal papillae may be a common trait in mosquito larvae. It remains to be seen whether the larvae of other aquatic dwelling insects such as midges and black flies, which also possess anal papillae, have similar compensatory mechanisms.

At first glance it appears that the anal papillae of *A. aegypti* have a much greater capacity for Cl^- transport relative to Na^+, considering that the J_{max} for Cl^- is double that for Na^+. However, the overall greater capacity for Cl^- uptake is offset by the relatively lower affinity of the Cl^- uptake mechanism compared with that for Na^+. As a result, at a given external concentration of NaCl below 2 mmol l^{-1}, the magnitude of Na^+ and Cl^- uptake are not dissimilar (see Fig. 4). Thus under freshwater conditions the anal papillae are likely to take up equivalent amounts of Na^+ and Cl^-. Above external NaCl concentrations of 2 mmol l^{-1}, the Cl^- uptake at the anal papillae begins to exceed the Na^+ uptake and this is likely to be occurring in larvae that were just transferred from freshwater to 30%SW. This may explain the relatively higher rates of accumulation of Cl^- compared with Na^+ in the hemolymph that we observed over the initial 5 h of transfer. Over this time period, Cl^- was accumulated in the hemolymph at twice the rate of Na^+ (see Fig. 1A,B), and since the transport mechanisms are saturated in 30%SW, Cl^- influx would be roughly twice that of Na^+ at the anal papillae. By our calculations the uptake of Na^+ and Cl^- at the anal papillae

Table 1. *Estimated rates of Na^+ and Cl^- accumulation and loss attributed to the anal papillae in comparison with the timing of observed increases and decreases in hemolymph Na^+ and Cl^- levels upon transfer between freshwater and 30% seawater*

	Na^+	Cl^-
Transfer from FW to 30%SW		
Observed initial rate of increase in hemolymph (pmol s^{-1})	2.20	2.86
Estimated rate of uptake attributed to all four papillae (pmol s^{-1})	4.51	8.14
Observed time required to achieve increase (h)	5	3
Estimated time required to achieve observed increase (h)	2.4	1.4
Transfer from 30%SW to FW		
Observed initial rate of decrease in hemolymph (pmol s^{-1})	1.91	4.13
Estimated rate of loss attributed to all four papillae (pmol s^{-1})	0.21	1.07
Observed time to achieve decrease (h)	6	6
Estimated time required to achieve observed decrease (h)	55.6	23.1

FW, freshwater; SW, seawater

The J_{max} values from freshwater-held larvae were used to estimate rates of ion uptake upon transfer from FW to 30%SW. The mean effluxes measured from papillae of individual larvae held in 30%SW for between 15 and 24 h (Na^+ N=8; Cl^- N=6) were used to estimate rates of ion loss attributed to the anal papillae upon transfer from 30%SW to FW. Total ion fluxes across all four papillae were estimated by multiplying the calculated fluxes by the surface area of the papillae (see Donini and O'Donnell, 2005). The time required for ion fluxes at the anal papillae to achieve the observed changes in hemolymph ion levels was estimated by first determining the magnitude of the ion change in mmol l^{-1} and multiplying by 1.65 μl (total hemolymph volume). The magnitude of the ion change in moles was divided by the total ion flux to obtain an estimate of the time required for changes to occur. Observed rates of increase and decrease in hemolymph ion levels were calculated from the results presented in Figs 1 and 2 using the time at which changes were first found to be significant.

alone is sufficient to explain the observed increases of both ions in the hemolymph (see Table 1). The estimated rates of Na^+ and Cl^- uptake by the papillae were 2.1 and 2.8 times higher, respectively, than the observed initial rates (see Table 1), resulting in shorter estimated times to achieve the observed changes. This might be explained by concomitant losses of NaCl at other sites of ion exchange such as the gut.

Our results indicate that under certain circumstances the anal papillae may contribute to the elimination of excess ions. The relative contribution of the anal papillae to the overall elimination of ions appears to be minor as witnessed by the relatively low efflux of Na^+ and Cl^- measured from papillae of larvae that had been exposed to 30%SW for between 15 and 24 h and subsequently assayed under freshwater conditions. The estimated rates of Na^+ and Cl^- loss from the anal papillae were only one tenth and one quarter of the observed rates of decrease in hemolymph Na^+ and Cl^- concentrations, respectively, when larvae were transferred from 30%SW to FW (see Table 1). Therefore, our results suggest that other sites such as the Malpighian tubules and gut are likely to play a major role in the elimination of excess ions and that the anal papillae of *A. aegypti* only play a minor role. It was recently shown that ion transport by the Malpighian tubules of larval *A. aegypti* is altered such that there is an increase in Na^+ secretion at the expense of K^+ when larvae are raised in high salinity (Donini et al., 2006). This alteration occurs within 12 h of transferring larvae from freshwater to high salinity (A.D., unpublished data) and is probably the result of changes in the ion transport machinery in the membranes of the tubule epithelial cells (Donini et al., 2006). In the larvae of *Ochlerotatus taeniorhynchus* a specialized posterior rectal segment is important in the elimination of excess ions from the hemolymph. Ion secretion in the posterior rectum occurs when Na^+ and Cl^- hemolymph levels rise (Bradley and Phillips, 1977).

The transfer of larvae between FW and 30%SW was associated with changes in hemolymph pH such that there was an inverse relationship between salinity and hemolymph pH. This inverse relationship is common in many osmoregulating aquatic animals (Henry and Cameron, 1982; Truchot, 1981; Nonnotte and Truchot, 1990; Maxime et al., 1990) and is thought to be a consequence of regulating hemolymph and/or blood ion levels since ion transport is often associated with H^+ and/or HCO_3^- exchange but, may also involve adjustments that regulate cell volume (Truchot, 1992; Whiteley et al., 2001). It is possible that similar processes are occurring in these larval mosquitoes particularly in light of evidence for the association of Na^+ transport with H^+ movements (see Stobbart, 1967; Patrick et al., 2002). In this regard, the observed decrease in Na^+ uptake may be coupled to a decrease in H^+ efflux at the papillae, which would affect hemolymph pH levels. By contrast, the K^+ concentration of the hemolymph was unaffected by changes in external salinity which is likely a reflection of the low amount of K^+ in seawater (3.14 mmol l^{-1} in 30%SW) relative to the Na^+ (97.6 mmol l^{-1} in 30%SW) and Cl^- (100 mmol l^{-1} in 30%SW) levels of seawater.

In summary, our data reveal significant changes in ion transport by the anal papillae of mosquito larvae when they are faced with changes in external salinity such that Na^+ and Cl^- uptake decrease in higher salinity. The alterations in the transport kinetics for Na^+ and Cl^- uptake may be a consequence of the increase in hemolymph ion levels when larvae encounter higher salinity. These changes in ion transport, which occur within 5 h, compliment the long-term alterations in the morphology and ultrastructure of the papillae.

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