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Sodium and chloride regulation in freshwater and osmoconforming larvae of *Culex* mosquitoes

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Summary

In this study, we examined aspects of Na^+ and Cl^- regulation in mosquito larvae of the genus *Culex*, a group that includes species that tolerate high salinity as well as other forms that are restricted to fresh water. When the euryhaline osmoconformer *C. tarsalis* was acutely transferred from 30 % to 50 % sea water, the patterns of hemolymph Na^+ and Cl^- regulation were similar. The underlying regulatory mechanisms for these two ions have very different characteristics. In *C. tarsalis*, Na^+ efflux was significantly elevated compared with the rates measured in the freshwater-restricted *C. quinquefasciatus*, while Cl^- influx was relatively lower. The modulation of Na^+ efflux and Cl^- influx allowed *C. tarsalis* to avoid a potential salt load and ionic disturbance in the hemolymph during an acute increase in salinity. The observed adjustment of NaCl regulation departs from that determined for other euryhaline organisms and is integral to the osmoconforming response. At the other extreme of the salinity spectrum, we observed that *C. tarsalis* faces difficulties in ion regulation in habitats with low NaCl levels because of its inability to reduce ion efflux and adjust ion absorption rates to maintain hemolymph ion balance. In contrast, *C. quinquefasciatus* exhibited a reduced ion efflux and the ability to upregulate Na^+ uptake, traits necessary to extend its lower salinity limit.

Key words: *Culex tarsalis*, *Culex quinquefasciatus*, sodium, chloride, ion regulation, uptake, efflux, mosquito, larva, osmoconformer.

Introduction

Over the past 70 years, an enormous body of work has been dedicated to describing ion regulation in freshwater and salt-water mosquito larvae (for a review, see Bradley, 1987). The focus, however, has principally fallen on one genus, *Aedes*. From these studies, the following picture has emerged. When held in fresh water, *Aedes* mosquito larvae osmoregulate by maintaining hemolymph [NaCl] at high levels relative to the external medium (i.e. they are hyper-regulators). To counteract the gradient for osmotic water gain and diffusive ion loss, larvae produce dilute urine at high rates, and Na^+ and Cl^- are actively absorbed from the environment across the anal papillae. The salt-tolerant mosquitoes of the genus *Aedes* are also osmoregulators. Their mechanism of ionic uptake in dilute media is identical to that of freshwater species. In saline media, the site of ion regulation is quite different. The larval forms of these salt-tolerant osmoregulating species (e.g. *A. taeniorhynchus*, *A. detritus*, *A. campestris*) drink the external medium to counter osmotic loss. Consequently, they ingest a large quantity of ions. To deal with this salt load, they have evolved an additional rectal segment where excess ions are actively secreted from the hemolymph into the rectal fluid, thereby maintaining hemolymph ion levels low relative to the saline environment (i.e. they are hypo-regulators).

While our understanding of ion regulation in *Aedes* is extensive, the eight other genera of mosquitoes that contain both freshwater and salt-tolerant species have been largely ignored. This present study is an attempt to fill that void by examining two species of the genus *Culex*. Species in this genus are of particular interest because recent studies show that they utilize a completely different strategy to tolerate saline environments, they osmoconform (Garrett and Bradley, 1987; Patrick and Bradley, 2000). *Culex tarsalis* can tolerate environmental salinities up to 70 % full-strength sea water. When held in 50 % sea water, *C. tarsalis* maintains hemolymph NaCl levels below that of the environment and accumulates high levels of two compatible solutes: trehalose, a disaccharide, and proline, an amino acid. This response allows hemolymph osmolality to conform to that of the environment, which eliminates the osmotic gradient for water loss and avoids the detrimental effects of high salt levels on protein function within the tissues. During an acute increase in external salinity, *C. tarsalis* larvae regulate their body volume by drinking the saline medium to compensate for water lost across the integument to the environment. A consequence of this drinking activity would be the incursion of a salt load into the hemolymph as ions and water are absorbed in the midgut of

mosquito larvae (Kiceniuk and Phillips, 1974). Despite this, *C. tarsalis* larvae are able to attenuate this potential rise in hemolymph Na^+ levels (Patrick and Bradley, 2000). From this work, it became apparent that osmoconforming larvae are able to regulate hemolymph NaCl levels during acute salinity changes and when acclimated to a range of salinities. We concluded that ion regulation is integral to the osmoconforming response, but little is known of the underlying mechanisms.

The goal of the present study was to compare the Na^+ and Cl^- regulatory responses of the osmoconformer *C. tarsalis* with those of *C. quinquefasciatus*, a species restricted to freshwater habitats. We measured whole-body Na^+ and Cl^- uptake and efflux to identify the key regulatory responses that have enabled *C. tarsalis* to extend its upper salinity limit and to tolerate acute salinity increases. In addition, we examined the patterns of Na^+ and Cl^- regulation of these two species at the other end of the salinity range, water with low levels of NaCl, to determine whether there is also a disparity in their lower salinity limits.

Materials and methods

Experimental animals and holding conditions

Colonies of *Culex tarsalis* (Coquillett) and *Culex quinquefasciatus* (Say) were established in the laboratory at University of California, Irvine, from colonies provided by Dr M. S. Mulla, Department of Entomology, University of California, Riverside. Mosquito larvae used in the propagation of the laboratory colonies were hatched and held in Irvine tapwater (4 mmol l^{-1} NaCl, 1 mmol l^{-1} Ca^{2+}, 0.1 mmol l^{-1} K^+) in large rectangular plastic trays (32 cm×18 cm×9 cm), and the water was changed each week. Larvae were fed ground rabbit chow pellets and dry yeast. Room temperature was 19–23 °C, and the light:dark cycle was set at 12 h:12 h. All experiments were conducted on fourth-instar larvae or large third-instar larvae.

Hemolymph Na^+ and Cl^- levels during long-term and acute salinity exposure

Hemolymph Na^+ and Cl^- concentrations were measured in both *C. quinquefasciatus* and *C. tarsalis* after 2 days in a low-NaCl medium [250 μmol l^{-1} NaCl, 1000 μmol l^{-1} $Ca(NO_3)_2$, 100 μmol l^{-1} KNO_3]. The low-NaCl medium was prepared from distilled water with the appropriate amount of each salt added. The 30 % seawater medium was made using Instant Ocean Salts (Aquarium Systems). Larvae were removed from the experimental medium, rinsed in distilled water and blotted dry on filterpaper disks. The larvae were placed on Parafilm and exsanguinated by making a small tear in the cuticle using fine forceps. The hemolymph was quickly collected using 1.0 μl microcapillary tubes (Drummond Microcaps). Hemolymph Cl^- concentrations were determined using a colorometric assay based on the liberation of thiocyanate from mercuric thiocyanate to form mercuric chloride (Patrick and Bradley, 2000). Hemolymph samples (1 μl) were diluted with 1 ml of distilled water, and 100 μl of a 1:1 solution of 13 mmol l^{-1} mercuric thiocyanate:0.5 mol l^{-1} ferric nitrate was added (Zall et al., 1956). Samples and Cl^- standards were read spectrophotometrically at an absorbance of 480 nm.

Hemolymph Cl^- levels were also measured in both species following acute transfer from 30 % to 50 % sea water (250 mmol l^{-1} NaCl, 5 mmol l^{-1} Ca^{2+}, 5 mmol l^{-1} K^+). In our previous study (Patrick and Bradley, 2000), we had examined hemolymph Na^+ concentrations under identical conditions. Batches of larvae of both species were held for 2 days in 30 % sea water. Hemolymph samples were taken from both species held in 30 % sea water and following acute transfer to 50 % sea water. Hemolymph was sampled 1 h prior to transfer (N=5 samples) and at 0.5, 1, 2, 4, 6 and 24 h post-transfer (N=4–8 samples at each time).

Na^+ and Cl^- fluxes in different environmental salinities

Na^+ and Cl^- uptake

To quantify unidirectional Na^+ and Cl^- uptake, we used the radioisotopes ^{22}Na and ^{36}Cl. Preliminary tests determined the appropriate specific activity of the medium and flux time to ensure that a high activity of each isotope could be detected in the larvae. Na^+ and Cl^- uptake experiments were performed separately but followed similar protocols.

Rates of Na^+ and Cl^- uptake were measured in the two species in the following five treatments: tapwater, 2- and 7-day exposure to low-NaCl medium, 2-day exposure to 30 % sea water and 10 h post-transfer from 30 % to 50 % sea water.

Two hours prior to initiation of flux measurements, 6–8 larvae were transferred to a well of a cell culture plate (Costar, 24 wells). Holding medium (2 ml) was added to each well. To initiate the experimental flux period, the medium was removed, the larvae and well were rinsed twice with 2 ml of distilled water; 1.2 ml of appropriate experimental medium was then added to each well. Isotope (^{22}Na or ^{36}Cl) was either added to a batch of experimental medium prior to adding to the wells or a sample of the stock isotope solution was pipetted into the well immediately following addition of the medium. The specific activities of each isotope were as follows: 74 Bq μmol^{-1} for both the tapwater and low-NaCl treatment groups, 0.49 kBq μmol^{-1} for the 30 % seawater group and 0.30 kBq μmol^{-1} for the 50 % seawater groups. A 50 μl sample of the medium was taken after 5 min, initiating the flux period. Finally, a 50 μl sample of the medium was taken approximately 10 h later, at the end of the flux period. Larvae were then transferred, *via* a plastic Pasteur pipette, to a medium-sized weighing boat containing approximately 25 ml of experimental medium (without isotope). Larvae were rinsed in this medium for at least 30 s then blotted on a filterpaper disk. Individual larvae were weighed and transferred to a 6 ml plastic scintillation vial containing 200 μl of distilled water. Each larva was macerated, and 5 ml of scintillation cocktail (Ecolume, ACS) was added to each vial. Media and larvae samples were assayed for radioactivity using a liquid scintillation counter (Beckman). Experimental medium samples were diluted appropriately and assayed for Na^+ and Cl^- concentration.

Rates of Na^+ and Cl^- uptake (J_{in}; nmol mg^{-1} h^{-1}), as measured by the appearance of radioactivity in individual larvae, were calculated from the following equation:

$$J_{in} = cpm_{larva} \frac{1}{SA_{H_2O}} \frac{1}{m} \frac{1}{t}, \quad (1)$$

where cpm_{larva} is the whole-body activity of the isotope, m is the mass of the larva, t is time and SA_{H_2O} is the mean specific activity of the medium (cts min^{-1} nmol^{-1}) with regard to the isotope in question.

Na^+ and Cl^- efflux

Rates of Na^+ and Cl^- efflux were measured in the following five treatments: tapwater and 2-day exposure to low-NaCl medium (250 μmol l^{-1} NaCl, 1000 μmol l^{-1} Ca^{2+}, 100 μmol l^{-1} K^+), 2-day exposure to 30 % sea water, and 2 and 4 h post-transfer from 30 % to 50 % sea water.

The day prior to Na^+ and Cl^- efflux experiments, batches of larvae (N=8–16) were placed in 5 ml beakers with 2 ml of the appropriate holding medium. The activities of ^{22}Na or ^{36}Cl added to each container were as follows: 37 kBq for the 250 μmol l^{-1} NaCl acclimation groups, 74 kBq for the tapwater groups, 370 kBq for the 30 % sea water and 50 % sea water transfer groups. Larvae were held in the isotope loading baths for a minimum of 12 h. Two hours prior to experimentation, larvae were removed from the loading baths, rinsed twice in 50 ml of the appropriate holding medium and blotted on filterpaper disks. When individual larvae had been weighed, they were then transferred either to a 1.5 ml centrifuge tube containing 1 ml of the appropriate experimental medium (Na^+ efflux series) or to a well of a cell culture dish to which 2 ml of the experimental medium had been added (Cl^- efflux series). This initiated the flux period. When the flux period ended, the larvae were removed from the centrifuge tube or well, rinsed twice in distilled water and blotted on a filterpaper disk. Hemolymph samples were collected from each larva as before. Hemolymph samples were then diluted in 3 ml (Na^+ efflux) or 2 ml (Cl^- efflux) of distilled water. For Na^+ efflux analysis, the diluted hemolymph samples (3 ml) and medium samples (1 ml) were assayed for radioactivity of ^{22}Na using a γ counter. The hemolymph samples were then assayed for Na^+ concentrations using atomic absorption spectrophotometry. For Cl^- efflux experiments, 1 ml of the diluted hemolymph or undiluted medium sample was added to a scintillation vial containing 5 ml of scintillation cocktail. Samples were then assayed for β radioactivity. The remaining 1 ml of diluted hemolymph sample was assayed for Cl^- concentration, as described above.

The rates of efflux (J_{out}; nmol mg^{-1} h^{-1}) as measured by the appearance of radioactivity in the medium, were calculated by:

$$J_{out} = cpm_{H_2O} \frac{1}{SA_{larva}} \frac{1}{m} \frac{1}{t}, \quad (2)$$

where cpm_{H_2O} is the activity of the isotope in the medium and SA_{larva} is the initial specific activity of the hemolymph of the larva (cts min^{-1} nmol^{-1}).

Freshwater Na^+ and Cl^- uptake kinetics

The rates of Na^+ and Cl^- uptake in larvae of *C. tarsalis* and *C. quinquefasciatus* raised and held in Irvine tapwater and exposed for 2 days to 250 μmol l^{-1} NaCl were measured at six different NaCl concentrations ranging from 250 to 8000 μmol l^{-1} (concentrations of other salts were held constant) to determine whether transport was carrier-mediated and saturable (i.e. conforming to Michaelis–Menten first-order kinetics). These kinetic experiments were performed in a defined freshwater medium [250, 500, 1000, 2000, 4000 or 8000 μmol l^{-1} NaCl, 1000 μmol l^{-1} $Ca(NO_3)_2$, 100 μmol l^{-1} KNO_3, pH 7.5]. Kinetic media were made up in batches with the appropriate NaCl concentration. For the Na^+ uptake measurements, a sample of the ^{22}Na stock solution was added to each well to reach the appropriate specific activity: 44.4 Bq μmol^{-1} for 250 μmol l^{-1} NaCl, 22.2 Bq μmol^{-1} for 500 and 1000 μmol l^{-1} NaCl, 11.1 Bq μmol^{-1} for 2000 μmol l^{-1} NaCl and 5.55 Bq μmol^{-1} for 4000 and 8000 μmol l^{-1} NaCl. For the Cl^- uptake studies, the experimental media were prepared using diluted ^{36}Cl stock at specific activities similar to those in Na^+ kinetic media.

The relationship between $[NaCl]_e$ and Na^+ uptake was examined using Michaelis–Menten analysis for first-order one-substrate kinetics. Values of J_{max} (the maximum uptake rate) and apparent K_m (the [ion] at which uptake is 50 % of J_{max}) were calculated using the following equation:

$$J_{in} = \frac{J_{max}[ion]_e}{K_m + [ion]_e}. \quad (3)$$

Michaelis–Menten analysis could not be performed on Cl^- uptake as it did not exhibit saturation kinetics over the NaCl concentration range tested.

Statistical analyses

All data are reported as means ± S.E.M. Comparisons among groups were performed using analysis of variance (ANOVA) (overall $P \leq 0.05$) with multiple comparisons (Scheffe's test) if ANOVA proved significant. The data for hemolymph Na^+ and Cl^- concentrations for the tapwater treatment groups of *C. quinquefasciatus* and *C. tarsalis* were taken from the results of Patrick and Bradley (Patrick and Bradley, 2000) and used to compare statistically with the values for the low-NaCl holding treatment. J_{max} and K_m values from the Michaelis–Menten kinetic analyses of Na^+ uptake were compared by inspection of 95 % confidence intervals. If they did not overlap, they were considered different.

Results

Hemolymph Na^+ and Cl^- concentrations

There were no significant differences in hemolymph Na^+ and Cl^- levels between *C. quinquefasciatus* and *C. tarsalis* held in low-NaCl medium (250 μmol l^{-1} NaCl), and the values were not significantly different from tapwater values. There were no significant differences between species (Table 1).

Table 1. *Hemolymph Na^+ and Cl^- concentrations of larval* Culex quinquefasciatus, *a freshwater obligate, and* C. tarsalis, *a euryhaline osmoconformer*

	C. quinquefasciatus		*C. tarsalis*	
	[Na^+] (mmol l^{-1})	[Cl^-] (mmol l^{-1})	[Na^+] (mmol l^{-1})	[Cl^-] (mmol l^{-1})
Tapwater	100.3±7.11	71.1±4.34	110.7±4.94	83.9±6.45
Low-NaCl medium	108.7±8.39	69.5±3.10	101.2±7.25	67.7±14.56

Larvae were held in tapwater (4 mmol l^{-1} NaCl), and low-NaCl (0.25 mmol l^{-1}) medium for a minimum of 2 days.

Hemolymph Na^+ and Cl^- concentrations for both species in tapwater, which were measured under the same conditions as the present study, were taken from Patrick and Bradley (Patrick and Bradley, 2000).

Values are means ± S.E.M., N=4–8. There were no statistically significant differences between treatments or between species.

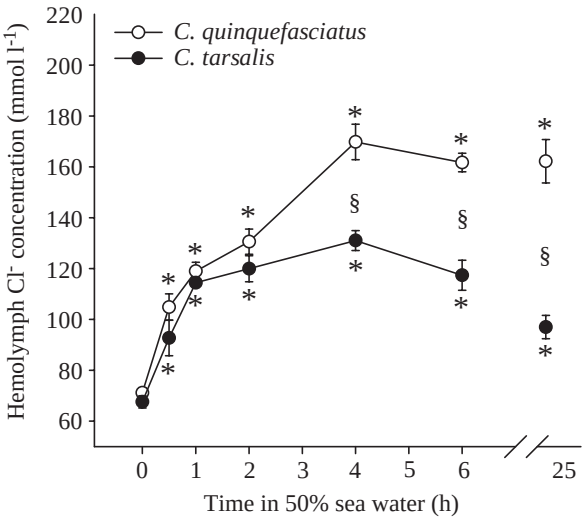

Fig. 1. Hemolymph Cl^- concentrations of larval *Culex quinquefasciatus*, a freshwater obligate, and *C. tarsalis*, a euryhaline osmoconformer, held in 30 % sea water and transferred to 50 % sea water. Values are means ± S.E.M., N=4–8. * denotes a significant difference from pre-transfer, 30 % seawater values (time 0 h) ($P \leqslant 0.05$). § denotes a significant difference between species at a given time point ($P \leqslant 0.05$).

Thirty minutes after transfer from 30 % sea water to 50 % sea water, both species experienced significant increases in hemolymph Cl^- levels (P<0.04; Fig. 1). In *C. quinquefasciatus*, Cl^- concentration continued to increase significantly throughout the first 6 h post-transfer and, at 24 h, remained at approximately 160 mmol l^{-1}. In contrast, hemolymph Cl^- levels in *C. tarsalis* plateaued at hour 4 at 120 mmol l^{-1}, but then decreased somewhat at 6 h and fell to 97 mmol l^{-1} by 24 h. Hemolymph Cl^- concentrations were significantly higher (P<0.001) in *C. quinquefasciatus* than in *C. tarsalis* by hour 4 post-transfer and remained significantly higher throughout the remainder of the experiment (P<0.001).

Unidirectional Na^+ and Cl^- uptake rates

When both species were held in tapwater, *C. tarsalis* larvae had Na^+ uptake rates approximately twice as high as those of *C. quinquefasciatus* (Fig. 2A, P<0.0001), but there were no

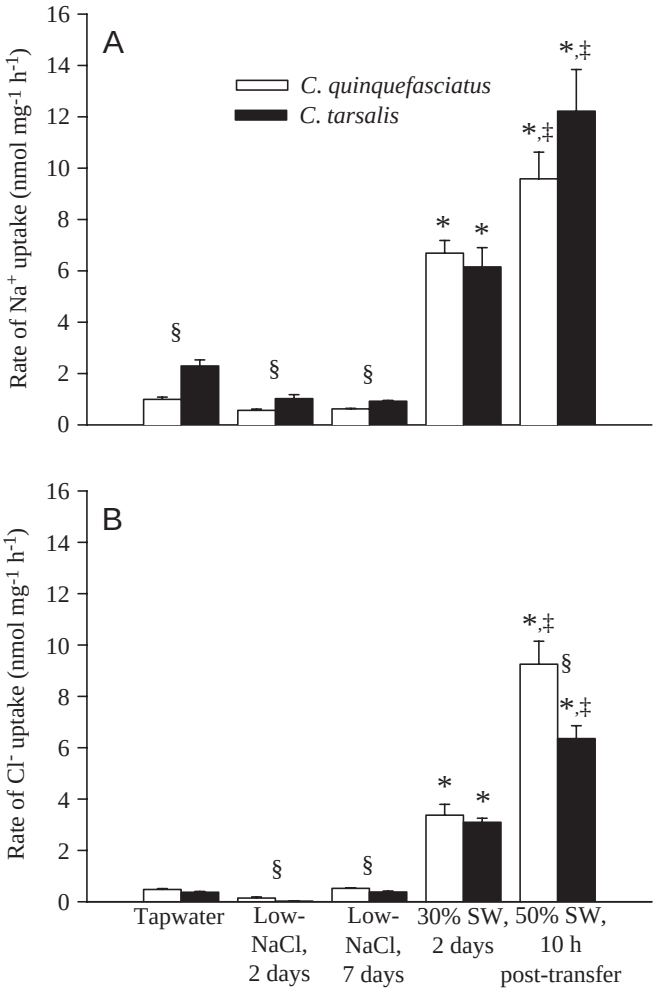

Fig. 2. Unidirectional, whole-body Na^+ (A) and Cl^- (B) uptake rates of *Culex quinquefasciatus*, a freshwater-obligate (open columns), and *C. tarsalis*, a euryhaline osmoconformer (filled columns), held in Irvine tapwater, low-NaCl medium for 2 or 7 days, 30 % sea water (SW) for 2 days and acutely transferred to 50 % sea water at 10 h post-transfer. Values are means + S.E.M.; N=5–12 for Na^+ uptake, N=6–7 for Cl^- uptake. * denotes a significant difference from the tapwater value ($P \leqslant 0.05$). ‡ denotes a significant difference from the 30 % seawater value ($P \leqslant 0.05$). § denotes a significant difference between species for a given treatment ($P \leqslant 0.05$).

differences in Cl^- uptake rates (Fig. 2B). In low-NaCl water (2 and 7 days of holding), Na^+ and Cl^- uptake rates were not significantly different from those in tapwater, but species differences persisted in both Na^+ and Cl^- uptake rates (P<0.001). Also, Na^+ uptake rates for *C. quinquefasciatus* and *C. tarsalis* were approximately two and six times those of Cl^- uptake rates under all freshwater treatments. After 2 days in 30 % sea water, *C. quinquefasciatus* and *C. tarsalis* experienced a 6.7-fold (P<0.0001) and 2.7-fold (P<0.038) increase, respectively, in Na^+ uptake rates relative to tapwater values. Cl^- uptake rates increased 8.4-fold in *C. quinquefasciatus* (P<0.0001) and sevenfold in *C. tarsalis* (P<0.0082). Na^+ uptake rates were double the rates for Cl^- uptake in both species held in 30 % seawater medium. When larvae were transferred to 50 % sea water from 30 % sea water, Na^+ and Cl^- uptake rates increased even further in *C. quinquefasciatus* (Na^+ P<0.0081, Cl^- P<0.0001) and *C. tarsalis* (Na^+ P<0.001, Cl^- P<0.0001). Uptake rates of Cl^-, but not of Na^+, were significantly higher in *C. quinquefasciatus* (P<0.021) than in *C. tarsalis.* Na^+ uptake rates approximated Cl^- uptake rates in *C. quinquefasciatus* held in 50 % sea water, whereas Na^+ uptake was double that of Cl^- uptake in *C. tarsalis*.

Unidirectional Na^+ and Cl^- efflux rates

While being held in tapwater medium, Na^+ efflux rates were similar in both species (Fig. 3A); however, *C. tarsalis* had a Cl^- efflux rate that was 50 % higher than that of *C. quinquefasciatus* (Fig. 3B; P<0.0034). Rates of Na^+ and Cl^- efflux were similar in the two species during freshwater holding. In contrast, when larvae were held for 2 days in the low-NaCl medium, *C. quinquefasciatus* had a Na^+ efflux rate that was significantly lower (P<0.0027) than that of *C. tarsalis*, but this rate was not significantly different from the tapwater value. There were no significant differences between Cl^- efflux rates of larvae held in low-NaCl water *versus* tapwater and also no significant difference between species held in low-NaCl water. In 30 % seawater, Na^+ efflux increased approximately 4.2-fold (P<0.0003) in *C. quinquefasciatus* and 3.3-fold (P<0.0003) in *C. tarsalis*, but Cl^- efflux rates did not change significantly in either species. Consequently, Na^+ efflux rates were 3.2- and 2.6-fold higher than Cl^- efflux rates in *C. quinquefasciatus* and *C. tarsalis*, respectively. When the larvae in 30 % sea water were acutely transferred to 50 % sea water, *C. tarsalis* experienced a further 1.9-fold increase (P<0.0001) in the Na^+ efflux rate after 2 h, whereas the Na^+ efflux rate for *C. quinquefasciatus* did not change. The difference between the species was significant (P<0.014). Cl^- effluxes increased significantly and to approximately the same rates in *C. quinquefasciatus* (P<0.0001) and *C. tarsalis* (P<0.0071) during the first 2 h in 50 % sea water. After hour 4, however, Na^+ effluxes had returned to 30 % seawater levels in both *C. quinquefasciatus* (P<0.0045) and *C. tarsalis* (P<0.0001) and were not significantly different between species. Similar trends were observed in the Cl^- effluxes, with both species experiencing significant decreases (*C. quinquefasciatus* 38 %, P<0.0021; *C. tarsalis* 43 %, P<0.002). Throughout the 4 h of holding in 50 % sea water, Na^+ efflux rates were 2–3 times greater than the corresponding Cl^- efflux rates in both species.

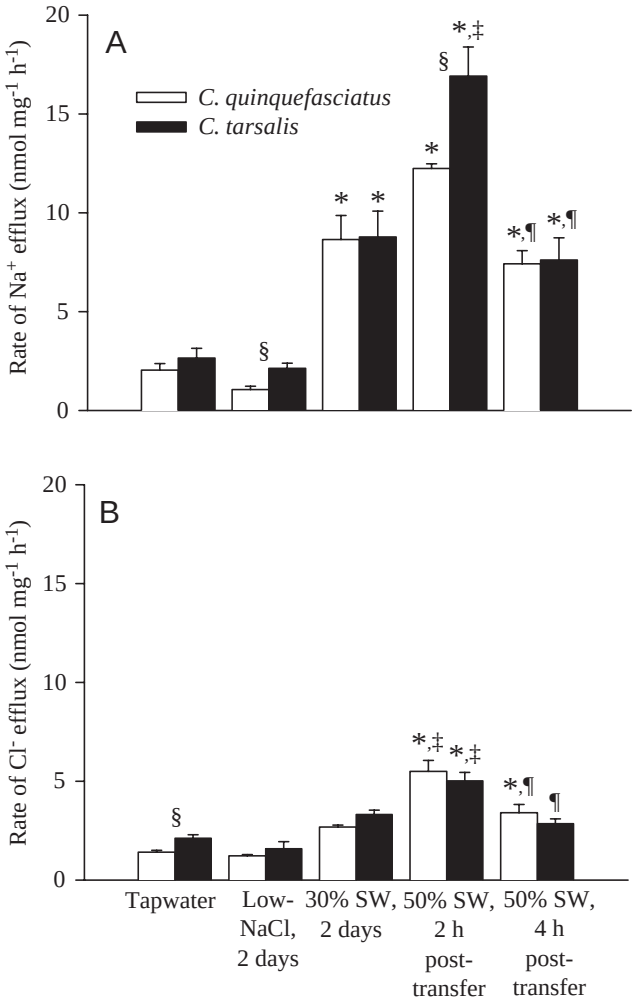

Fig. 3. Unidirectional, whole-body Na^+ (A) and Cl^- (B) efflux rates of *Culex quinquefasciatus*, a freshwater-obligate (open columns), and *C. tarsalis*, a euryhaline osmoconformer (filled columns), held in Irvine tapwater, low-NaCl medium for 2 days, 30 % sea water (SW) for 2 days, and transferred to 50 % sea water at 2 and 4 h post-transfer. Values are means + S.E.M.; N=5–14 for Na^+ efflux, N=5–8 for Cl^- efflux. * denotes a significant difference from the tapwater value ($P \leqslant 0.05$). ‡ denotes a significant difference from 30 % seawater value ($P \leqslant 0.05$). § denotes a significant difference between species for a given treatment ($P \leqslant 0.05$). ¶ denotes a significant difference from the hour 2 post-transfer, 50 % seawater value ($P \leqslant 0.05$).

Freshwater Na^+ and Cl^- uptake kinetic analysis

The relationship between Na^+ and Cl^- uptake rates and external NaCl concentrations were examined in both species during holding in tapwater and low-NaCl medium. In tapwater and low-Na^+ medium, Na^+ uptake of both *C. quinquefasciatus* and *C. tarsalis* exhibited typical saturation kinetics (Fig. 4A,C) when external NaCl concentration was increased from 0.25 to

Table 2. *The affinity constant (K_m), maximum uptake capacity (J_{max}) and r^2 value of non-linear regression of Na^+ uptake rates in* Culex quinquefasciatus *and* C. tarsalis *larvae acclimated to Irvine tapwater and low-NaCl medium*

		Na^+ uptake		
		K_m (mmol l^{-1})	J_{max} (nmol mg^{-1} h^{-1})	r^2
C. quinquefasciatus	Tapwater (4 mmol l^{-1} NaCl)	1.06±0.11	1.08±0.03	0.99
	Low-NaCl (0.25 mmol l^{-1}) medium	0.65±0.14	1.64±0.10	0.98
C. tarsalis	Tapwater (4 mmol l^{-1} NaCl)	0.75±0.20	2.05±0.15	0.94
	Low-NaCl (0.25 mmol l^{-1}) medium	0.51±0.21	2.24±0.23	0.91

Values are means ± S.E.M. (N=4–6).
Note that K_m and J_{max} values could not be estimated for Cl^- uptake because it did not exhibit saturation kinetics (see Fig. 4).

8 mmol l^{-1} NaCl. Michaelis–Menten kinetic analysis of the tapwater acclimation groups determined that the Na^+ uptake system of *C. tarsalis* had a maximum capacity (J_{max}) that was almost double that of *C. quinquefasciatus* (confidence intervals did not overlap), but affinity values (K_m) did not differ (Table 2). In contrast, the increase in Cl^- uptake over the concentration range was linear in both species (Fig. 4B,D). When held in low-NaCl water, Na^+ uptake kinetics did not change in *C. tarsalis* larvae, whereas *C. quinquefasciatus* larvae exhibited a 54 % increase in J_{max} (confidence intervals did not overlap), but no change in affinity (Table 2). Acclimation to low-NaCl medium did not affect the linear increase in Cl^- uptake rates in either *C. quinquefasciatus* or *C. tarsalis* (Fig. 4B,D).

Discussion

Na^+ and Cl^- regulation in high-salinity media

Until this study, ion regulation in osmoconforming larvae of mosquitoes had not been characterized. By manipulating external salt concentration and observing the patterns of hemolymph ion levels and whole-body influxes and effluxes of Na^+ and Cl^- in the larvae of the euryhaline *C. tarsalis* and the freshwater obligate *C. quinquefasciatus*, we were able to gain insight into how the former species has extended its upper salinity limit. These experiments indicate that there are significant differences in underlying transport mechanisms of Na^+ and Cl^- that may explain this.

Exposure to high salinity revealed that *C. tarsalis* was able to regulate hemolymph Na^+ and Cl^- levels while *C. quinquefasciatus* was not. When the external salinity was raised from 30 % to 50 % sea water, an increase that reverses the diffusion and osmotic gradients for the larvae, *C. tarsalis* experienced a smaller increase in hemolymph Cl^- concentration relative to the considerable rise in *C. quinquefasciatus* (Fig. 1). This pattern in the regulation of hemolymph Cl^- parallels previous observations for hemolymph Na^+ levels during an identical acute salinity increase (Patrick and Bradley, 2000). These results suggest that *C. tarsalis*, although an osmoconformer in higher salinity, regulates hemolymph NaCl at levels well below that of the environment (i.e. hyporegulates). In both studies, the potential rise in hemolymph Na^+ and Cl^- concentrations was attenuated in *C.*

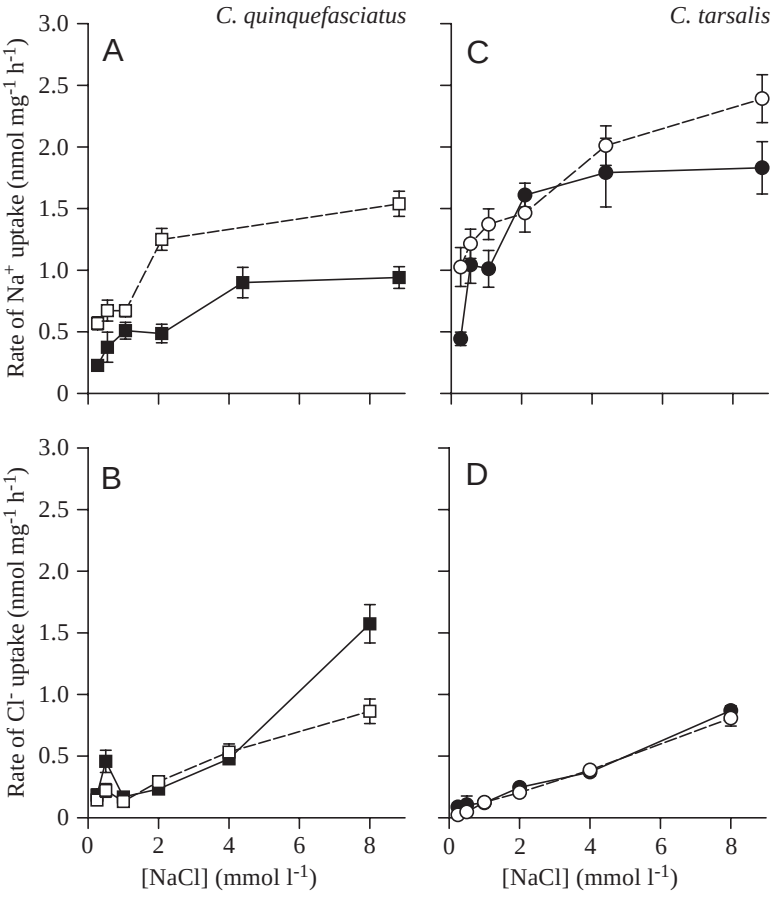

Fig. 4. Effect of water NaCl concentration on whole-body Na^+ and Cl^- uptake rates of *Culex quinquefasciatus*, a freshwater-obligate (A,B), and *C. tarsalis*, a euryhaline osmoconformer (C,D), held in Irvine tapwater (filled symbols) and low-NaCl medium for 2 days (open symbols). Values are means ± S.E.M.; N=4–6 for Na^+ uptake, N=6–7 for Cl^- uptake.

tarsalis and, in fact, a slight decrease occurred after 4 h in 50 % sea water. In contrast, *C. quinquefasciatus* experienced substantial increases in concentrations of both ions, probably contributing to their death at this salinity.

During the initial few hours following transfer to 50 % sea water, *C. tarsalis* briefly stimulate their drinking rate to maintain a constant body volume in the face of osmotic water loss and, consequently, experience a salt load (Patrick and Bradley, 2000). The patterns in hemolymph ion levels indicate that the mechanisms of dealing with this salt load were effective by hour 4 and that, by 24 h, new and slightly higher [Na^+] and [Cl^-] equilibria had been reached within the hemolymph. On the basis of these observations, it might be presumed that the regulatory mechanisms for Na^+ and Cl^- in *C. tarsalis* larvae are identical. However, examination of unidirectional uptake and efflux indicates otherwise.

Our results show that, upon transfer to 50 % sea water, *C. tarsalis* modulated its Na^+ efflux (Fig. 3A) and Cl^- uptake (Fig. 2B) components in a manner that attenuated the rise in hemolymph Na^+ (Patrick and Bradley, 2000) and Cl^- concentrations (Fig. 1). We observed that *C. tarsalis* larvae had a significantly higher Na^+ efflux rate (Fig. 3A) and lower Cl^- uptake rate (Fig. 2B) than *C. quinquefasciatus* larvae during the initial 2 h and 10 h post-transfer, respectively. Thus, despite the similar time course and pattern of changes in levels of the two major hemolymph ions, two separate mechanisms are functioning to control Na^+ and Cl^- concentrations.

The greater rate of Na^+ efflux observed in *C. tarsalis* (Fig. 3A) would function to eliminate excess Na^+ that had entered the larvae as a result of the greater influx rate of this ion (Fig. 2A). The trend in Na^+ efflux reflects the rapidity of the compensatory response to external salinity changes and that the modulation of efflux must be separate from the Na^+ uptake mechanism. An increase in the number of Na^+ transporters and/or an increase in the activity of the existing transporters must occur to increase the rate of Na^+ elimination, although the latter mechanism is more plausible given the rapidity of the response (2 h).

The transport of Na^+ out of the larvae is against a concentration gradient. The electrical gradient existing across the body wall of the mosquito larvae is not known. Given the hemolymph and water Na^+ concentrations, an electrical gradient of greater than +16 mV (hemolymph-positive) would be required for Na^+ to be passively distributed when *C. tarsalis* larvae are held in 50 % sea water. To date, transepithelial potential (TEP) has not been measured in mosquito larvae held in media of varying salinity, but previous studies on hyporegulating, euryhaline crustaceans and fish held in 100 % seawater medium report TEP values ranging between +10 to +25 mV (Kirschner, 1997; O'Donnell, 1997). Hypo-osmoregulating brine shrimp (*Artemia salina*) held in 50 % sea water drink the external medium, secrete excess salts and exhibit a TEP of +10 mV (Holliday et al., 1990). On the basis of these data, we cannot rule out the possibility that Na^+ transport could be passive under these conditions. It would be interesting to examine the molecular moieties responsible for Na^+ transport in this species and to determine whether Na^+ transport is active or passive in this brackish salinity.

Following transfer to 50 % sea water, the lower rate of Cl^- uptake observed in *C. tarsalis* relative to *C. quinquefasciatus* (Fig. 2B) suggests that the former species has a lower Cl^- permeability at higher external salinities. In 50 % sea water, Cl^- uptake is presumed to be passive because the concentration gradient and likely electrical gradient would favor inward diffusion (inside-positive). The lower rate of influx of Cl^- in *C. tarsalis* relative to *C. quinquefasciatus* (Fig. 2B) could be interpreted as a reduced number of sites for Cl^- entry. This strategy would reduce not only the potential Cl^- load during exposure to high salinity but also the need to expend additional energy to transport Cl^- out of the hemolymph against the electrochemical gradient. This lower Cl^- permeability contrasts with that of Na^+ in the two species, with *C. tarsalis* exhibiting a slightly, but not significantly, greater Na^+ uptake rate than *C. quinquefasciatus* (Fig. 2A). Overall, the unidirectional fluxes of Na^+ were at least twice as great as the Cl^- fluxes, indicating a greater Na^+ permeability, a trend consistent among seawater vertebrates (Kirschner, 1997) and invertebrates (Smith, 1969; O'Donnell, 1997).

On the basis of our current findings and previous studies, we can propose possible mechanisms for the patterns of Na^+ and Cl^- regulation in brackish water. When *C. tarsalis* larvae are acutely transferred to 50 % sea water, a salt load is incurred from drinking and/or entry across the anal papillae, but with a reduced influx of Cl^-. This ion load is rapidly counteracted (i.e. within 2 h) by the significant increase in Na^+ and Cl^- extrusion at the anal papillae against their respective concentration, and most probably electrical, gradients.

In a recent study (Patrick and Bradley, 2000), we reported that *C. tarsalis* larvae rapidly increase their rate of drinking to regulate body volume during an acute increase in salinity. The midgut has been indicated as the site of water absorption and transport of most of the ions in the ingested medium (Kiceniuk and Phillips, 1974). With regard to the external body surface, it has been suggested that the anal papillae are the region most permeable to water (Wigglesworth, 1933b) and ions (Wigglesworth, 1933a; Koch, 1938; Stobbart, 1967). On the basis of these studies, the increase in Na^+ and Cl^- uptake observed when both species are held in 50 % sea water (Fig. 2) could occur at one or both of these two sites, with *C. tarsalis* larvae possessing a reduced Cl^- permeability (relative to *C. quinquefasciatus*), indicated by a lower influx rate, in the midgut and/or anal papillae. This is particularly intriguing since *C. tarsalis* must experience a greater Cl^- load in the midgut because of the significantly greater intake of the external medium (Patrick and Bradley, 2000).

Possible sites for the excretion of the salt load from the hemolymph are the hindgut and anal papillae. Previously, Garrett and Bradley (Garrett and Bradley, 1984b) examined the morphology of key osmoregulating tissues in the larvae of *Culiseta inornata*, an osmoconformer and ion regulator like *C. tarsalis*. These authors examined the ultrastructure of the Malpighian tubules, the hindgut and the anal papillae of larvae

and did not report any morphological changes associated with varying environmental salinity. The authors did suggest that the anal papillae could be the site of ion transport in osmoconforming larvae. Several morphological and radioisotopic studies have provided evidence that the anal papillae are the site for ion regulation in freshwater mosquito larvae (Wigglesworth, 1938; Ramsay, 1953; Sohal and Copeland, 1966; Stobbart, 1967). Treherne (Treherne, 1954) determined that in freshwater *Aedes aegypti* most Na^+ exchange occurs through the anal papillae, with a very small percentage occurring across the gut. In addition, Phillips and Meredith (Phillips and Meredith, 1969) showed that the anal papillae function to regulate hemolymph Na^+ and Cl^- to lower levels in *Aedes campestri*s that had been acclimated to Ringer's solution and transferred acutely to 430 mmol l^{-1} NaCl. Garrett and Bradley (Garrett and Bradley, 1984a) ruled out the hindgut as the site of ion regulation in osmoconforming larvae (*Culiseta inornata*) held in saline media because neither *in vivo* nor *in vitro* preparations of the rectum produced hyperosmotic urine.

Na^+ and Cl^- regulation in dilute media

The present study is the first to characterize freshwater Na^+ and Cl^- regulation in two species belonging to the same genus (*Culex*) but possessing very different osmoregulatory abilities (Patrick and Bradley, 2000). The similarity in the hemolymph NaCl concentrations in the two species held in tapwater (4 mmol l^{-1} NaCl) and for 2 days at 0.25 mmol l^{-1} NaCl (Table 1) suggests that Na^+ and Cl^- regulation might be similar in these two species. However, the unidirectional Na^+ and Cl^- fluxes (Fig. 2, Fig. 3) and kinetic analysis (Fig. 4; Table 2) indicate that this is not the case. Acclimation to a medium with one-sixteenth of the NaCl levels found in tapwater did not alter rates of ion efflux in either species, but *C. tarsalis* exhibited a significantly higher rate of Na^+ loss than *C. quinquefasciatus* (Fig. 3). A higher Na^+ permeability across the body at higher salinity, as indicated by a greater Na^+ loss, is common among euryhaline organisms (Croghan and Lockwood, 1968; Taylor and Harris, 1986; Patrick et al., 1997). Despite the fact that this laboratory colony of *C. tarsalis* has been maintained in fresh water for several years, it does not exhibit a reduced Na^+ permeability similar to that of *C. quinquefasciatus*. Because of the higher rates of Na^+ loss, *C. tarsalis* larvae require a greater rate of transport of Na^+ from the environment to maintain hemolymph NaCl balance. Michaelis–Menten kinetic analyses of Na^+ uptake (Fig. 4C; Table 2) showed that *C. tarsalis* had a J_{max} double that of the freshwater-restricted *C. quinquefasciatus* (Fig. 4C; Table 2). In addition, *C. tarsalis* did not upregulate Na^+ uptake (Fig. 4C) following 2 days in low-NaCl medium. Consequently, this species did not maintain Na^+ uptake at a rate comparable with the value measured in tapwater (Fig. 2A, Fig. 4C). If *C. tarsalis* larvae were held for a longer period in low-NaCl water, hemolymph Na^+ balance could not be maintained unless the efflux component was reduced to a level comparable with the decrease in Na^+ uptake.

Other studies of the ion-regulatory abilities of euryhaline and freshwater animals have yielded similar results. For example, Taylor and Harris determined that the euryhaline amphipod *Corophium curvispinum*, found in brackish water, is excluded from invading freshwater habitats with NaCl levels lower than 0.5 mmol l^{-1} because it cannot maintain ion balance (Taylor and Harris, 1986). Sutcliffe reported that the brackish-water amphipod *Gammarus duebeni* has a high Na^+ permeability and could maintain ion balance only in water with a NaCl concentration greater than 1 mmol l^{-1} (Sutcliffe, 1967). In contrast, other closely related amphipods successfully inhabit low-NaCl waters by reducing their ion permeability and demand for active uptake of Na^+. Similar patterns of high Na^+ turnover rates have been found in the euryhaline fish *Fundulus heteroclitus*, a species that is able to tolerate freshwater medium but can achieve a balance only at 1 mmol l^{-1} Na^+ and 1.7 mmol l^{-1} Cl^- (Patrick et al., 1997). As a consequence, it is unable to invade ion-poor bodies of water that other freshwater species of *Fundulus* can inhabit (such as *Fundulus diaphanus*; Griffith, 1972).

While *C. tarsalis* has trouble maintaining ion balance in low-NaCl water, *C. quinquefasciatus* is equipped to survive water of lower ion concentrations by being able to stimulate Na^+ absorption (Fig. 4A). By substantially increasing maximum transport capacity (J_{max}) (Fig. 4A; Table 2), the freshwater obligate species maintained Na^+ uptake at a rate comparable with the tapwater value, despite the large reduction in concentration of external ions available for transport. The upregulation of J_{max} of *C. quinquefasciatus* (Fig. 4A; Table 2) could be attributed to the expression of more Na^+ transporters. The time frame in which these changes took place (2–4 h *versus* more than 12 h) would provide insight into how the changes in Na^+ absorption in *C. quinquefasciatus* are regulated (i.e. at the genetic or transporter level).

Although J_{max} values were quite different in *C. tarsalis* and *C. quinquefasciatus*, affinity values were similar (Table 2). Previously, it has been argued that truly freshwater animals reduce the energy required for maintaining ion balance by first reducing integumental permeability to ions and then reducing both the K_m and J_{max} of the ion-uptake mechanism (Shaw, 1961). Indeed, *C. quinquefasciatus* has a lower Na^+ efflux and J_{max} for Na^+ uptake relative to *C. tarsalis*; however, these two species share a similar K_m for Na^+ uptake (Table 2), a finding that contrasts with studies of freshwater and euryhaline amphipods (Sutcliffe, 1967; Taylor and Harris, 1986), chironomids (Wright, 1975), other crustaceans (Shaw, 1961) and teleosts (Patrick et al., 1997). The K_m values determined in the present study for *Culex* mosquito larvae and also by Stobbart (Stobbart, 1965) for the freshwater obligate mosquito *A. aegypti* (K_m=0.55 mmol l^{-1} NaCl) are higher than the range typically reported for truly freshwater animals (typically <0.4 mmol l^{-1} NaCl) (Shaw, 1961; Wright, 1975; Taylor and Harris, 1986; Patrick et al., 1997). This suggests that mosquitoes cannot increase the affinity of Na^+ transport to survive lower-NaCl water despite the fact that mosquito larvae have been found to complete their development in distilled water (Wigglesworth, 1938; Ramsay, 1953). Stobbart (Stobbart, 1965) argued that an enhanced Na^+ uptake

mechanism could be achieved without an increase in affinity *via* the recruitment of more transporters, a pattern similar to that observed in *C. quinquefasciatus* held in 0.25 mmol l^{-1} NaCl water after only 2 days (Fig. 4A; Table 2). Perhaps the K_m of Na^+ uptake is plastic, but changes require long-term exposure (over several generations) to low-ion medium.

Unlike Na^+ uptake, the Cl^- uptake mechanism of the two species appears to be similar. In both cases, we see a lack of saturable uptake kinetics and a lack of response to low-NaCl water (Fig. 4B,D). These results signify completely independent mechanisms of Na^+ and Cl^- transport in both *C. tarsalis* and *C. quinquefasciatus*. In addition, Cl^- uptake rates were quite low relative to Na^+ uptake (Fig. 2A,B, Fig. 4), a trait matched by the low Cl^- efflux (Fig. 3). This situation differs from that in *A. aegypti* larvae, in which Na^+ and Cl^- uptake are saturable processes with similar kinetic properties (Stobbart, 1971a; Stobbart, 1971b). Is the unsaturable pattern of Cl^- uptake in *C. tarsalis* and *C. quinquefasciatus* characteristic of all *Culex* species? These disparities in Cl^- uptake patterns among the mosquito genera go beyond the differences described for Na^+ uptake mechanisms (i.e. J_{max}) and suggest that there must be completely different moieties capable of active transport.

The underlying mechanisms by which Na^+ and Cl^- are transported have been examined, using radioisotopic and electrophysiological methods, in freshwater and salt-tolerant mosquitoes belonging to the osmoregulating genus *Aedes*. Stobbart (Stobbart, 1959; Stobbart, 1960; Stobbart, 1965; Stobbart, 1967; Stobbart, 1971a; Stobbart, 1971b) thoroughly examined the freshwater-restricted *A. aegypti* and reported that Na^+ and Cl^- uptake from the external medium *via* the anal papillae is independent, active and involves an intimate coupling to the excretion of acidic and basic equivalents, respectively (i.e. H^+ and HCO_3^-). For larvae in sea water, the nature of ion excretion by the additional rectal segment found in several salt-tolerant *Aedes* species (*A. campestris*, *A. detritus*, *A. taeniorhychus* and *A. dorsalis*) involves the active transport of Na^+, Mg^{2+} and Cl^- (for a review, see Bradley, 1987). However, in both the freshwater and seawater models, the primary source of energy driving these processes has yet to be identified.

In the freshwater, seawater and euryhaline vertebrates and invertebrates studied to date, Na^+/K^+-ATPase plays a role in the models proposed for ion transport (Gilles and Delpire, 1997; Kirschner, 1997; O'Donnell, 1997). In both freshwater and seawater organisms, numerous studies have reported correlations between Na^+/K^+-ATPase activity and ion-transport rates when environmental salinity is varied, thus supporting the role of Na^+/K^+-ATPase in ion regulation (Holliday et al., 1990; McCormick, 1995; Piermarini and Evans, 2000; Postel et al., 2000). Both models are far from complete, and the freshwater model has recently been challenged by the identification of H^+-ATPase (V-type) in osmoregulating tissues, including those of insects. The V-ATPase is central to the alkalization of the insect midgut (Klein, 1992; Zhuang et al., 1999) and the secretion of KCl-rich fluid by the Malpighian tubules of insects (Maddrell and O'Donnell, 1992; Beyenbach et al., 2000). Filippova et al. (Filippova et al., 1998), using an antibody for the β-subunit of the V-ATPase, examined freshwater-acclimated *C. quinquefasciatus* and reported that the H^+-ATPase was located throughout the gut and the Malpighian tubules of the larvae. They did not examine the anal papillae, a key site for ion regulation in mosquito larvae. Zhuang et al. (Zhuang et al., 1999) found that antibodies for the E-subunit of V-ATPases co-localized with portasomes, which are small particles found on ion-transporting tissue and believed to be the V_1 part of the H^+ V-ATPase. Garrett and Bradley (Garrett and Bradley, 1984b), in examining the ultrastructure of osmoregulating tissues of the euryhaline osmoconformer *Culiseta inornata*, reported the presence of portasomes lining the epithelium of the anal papillae. Taken together, the above findings suggest that H^+-ATPase may be present on the anal papillae of osmoconforming larvae. Whether an H^+-ATPase or a Na^+/K^+-ATPase is driving Na^+ and Cl^- transport in either the rectum of osmoregulating larvae (e.g. *A. taeniorhynchus*) or the anal papillae of osmoconforming species (e.g. *C. tarsalis*) during a salinity challenge has not been addressed. We are currently examining this question in the hope of advancing the understanding of ion regulation in osmoconforming mosquito larvae which was, until this present study, a mystery.

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