The Characterization of Ion Regulation in Amazonian Mosquito Larvae: Evidence of Phenotypic Plasticity, Population-Based Disparity, and Novel Mechanisms of Ion Uptake

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ABSTRACT

This study is the first step in characterizing ion uptake mechanisms of mosquito larvae from the Amazon region of Brazil. Hemolymph NaCl levels and rates of unidirectional Na^+ and Cl^- uptake were measured in larvae of *Aedes aegypti* and *Culex quinquefasciatus* in a series of environmental manipulations that are known to challenge ion regulation in other aquatic animals. Despite being reared for numerous generations in dilute media (20 μmol L^{-1} NaCl), both species were able to maintain high hemolymph NaCl concentrations, a departure from previous studies. Exposure to distilled water or high-NaCl media did not affect hemolymph ion levels, but pH 3 caused significant decreases in hemolymph Na^+ and Cl^- levels in both species. Exposure to water from Rio Negro (pH 5.5), an organically rich but ion-poor body of water, did not disturb hemolymph Na^+ and Cl^- levels or the uptake of these ions. Acute exposure to control media or Rio Negro water titrated to pH 3.5 caused inhibition of Na^+ uptake and stimulation of Cl^- uptake in *C. quinquefasciatus*, but *A. aegypti* larvae experienced only a significant reduction of Na^+ uptake in Rio Negro/pH 3.5 treatment. The stimulation of Cl^- uptake at low pH has been documented only in aquatic insects and differs from all other invertebrate and vertebrate species. A similar pattern of Na^+ uptake inhibition and Cl^- uptake stimulation was observed in *A. aegypti* larvae exposed to bafilomycin A_1, a blocker of V-type H^+ ATPase. *Culex quinquefasciatus* larvae were unaffected by this drug. Both Na^+ and Cl^- uptake were reduced when *C. quinquefasciatus* larvae were exposed to acetazolamide, indicating that H^+ and HCO_3^-, derived from hydration of CO_2, are involved with Na^+ and Cl^- uptake. Kinetic analysis of Na^+ and Cl^- uptake in *C. quinquefasciatus*, *A. aegypti*, and *Anopheles nuneztovari* larvae indicate that these Amazonian species share similar high-capacity and high-affinity mechanisms. Comparison of the Amazonian *C. quinquefasciatus* with a Californian population provided evidence of both phenotypic plasticity and population disparity in Na^+ and Cl^- uptake, respectively. When the California population of *C. quinquefasciatus* was reared in a medium similar to that of the Amazonian group (60 μmol L^{-1} NaCl) instead of 4,000 μmol L^{-1} NaCl, larvae increased both Na^+ uptake capacity (J_{max}) and affinity (i.e., reduced K_m), yet Cl^- uptake did not change from its nonsaturating, low-capacity pattern. In the reverse experiment, Amazonian *C. quinquefasciatus* demonstrated plasticity in both Na^+ and Cl^- uptake by significantly reducing rates when held in 4,000 μmol L^{-1} NaCl for 3 d.

Introduction

Mosquito larvae, like aquatic vertebrates and other invertebrates that inhabit freshwater, maintain hemolymph Na^+ and Cl^- concentrations at levels much higher than those of the environment. Typical values of hemolymph Na^+ and Cl^- levels are 100 and 50 mmol L^{-1}, respectively (Clements 1992), whereas environmental NaCl concentrations can range from 0 to 8 mmol L^{-1}. Under these conditions, there is a diffusional gradient for ion loss to the environment. To counteract these losses, the animal must actively absorb Na^+ and Cl^- from the external medium back into the body. In mosquito larvae, Na^+

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and Cl^- absorption occurs across the anal papillae (Wigglesworth 1933*b*, 1938; Koch 1938; Ramsay 1953; Treherne 1954; Stobbart 1967).

Much of our understanding of freshwater ion regulation has been the result of intensive study of one particular species of freshwater mosquito larvae, *Aedes aegypti* (Stobbart 1960, 1965, 1967, 1971*a*, 1971*b*). Certain aspects of Na^+ and Cl^- uptake in larval *A. aegypti* were characterized in these studies. Na^+ and Cl^- uptake occurred through "pumps" that exhibited the classic enzyme-substrate kinetics (i.e., Michaelis-Menten) and were associated with the excretion of acidic and basic equivalents (i.e., H^+ and HCO_3^-, OH^-). These characteristics were similar to those described for freshwater fish (Kirschner 1997). It was thus assumed that all freshwater animals, including all mosquito species, might possess similar mechanisms of ion transport. However, until very recently, ion regulation in freshwater has been examined in only one species (*A. aegypti*) in any detail. Recently, our examination of two species of *Culex* mosquitoes, a freshwater-restricted species (*Culex quinquefasciatus*) and a euryhaline species (*Culex tarsalis*) revealed that despite being reared under identical freshwater conditions and sharing similar hemolymph ion composition, the dynamics of Na^+ and Cl^- uptake are very different (Patrick et al. 2001).

In both Stobbart's studies of *A. aegypti* larvae (1960, 1965, 1967, 1971*a*, 1971*b*) and our recent study of *Culex* species (Patrick et al. 2001), the colonies of mosquitoes were maintained in media with fairly high NaCl levels (>2,000 μmol L^{-1} NaCl; Stobbart 1959; Patrick et al. 2001). An exception was when Stobbart (1960) reared a batch of *A. aegypti* larvae in 2 μmol L^{-1} NaCl. This treatment challenged ion balance as it greatly compromised ion absorption mechanisms because of a paucity of ions available for transport. The larvae survived but experienced a dramatic drop in hemolymph Na^+ levels to 30 mmol L^{-1} (Stobbart 1960). Other studies have found that *A. aegypti* larvae are capable of surviving several days in distilled water, but hemolymph Na^+ and Cl^- levels drop significantly (Wigglesworth 1938; Ramsay 1953). There are a number of reports indicating that mosquito larvae are able to complete development in natural or artificial containers of rainwater that is essentially distilled water (reviewed by Clements [1992]). In our investigation, we posed the following question: Are mosquito larvae hatched and reared in ion-poor environments for many generations compromised in their hemolymph ion balance as described above?

To address this question, we examined ion regulatory properties, in vivo, of mosquito larvae that reside in the Amazon region of Brazil. The water chemistry of this region is typically described as ion poor, with Na^+ and Cl^- levels in the micromolar range (Furch 1984). We also wanted to determine whether the paucity of mosquitoes in the area around the Rio Negro, a major tributary of the Amazon river, is due to a disruption of ion balance of the aquatic larvae, as has been suggested (Janzen 1974). We examined the unidirectional Na^+ and Cl^- uptake in Amazonian populations of *C. quinquefasciatus*, *A. aegypti*, and *Anopheles nuneztovari* that had been maintained in an extremely dilute medium (20 μmol L^{-1} NaCl) for many generations. Our goal was to determine whether larvae are capable of maintaining high hemolymph NaCl levels and, if so, how the unidirectional Na^+ and Cl^- uptake mechanisms depart from those of populations reared in high NaCl media (Stobbart 1971*b*; Patrick et al. 2001). To characterize Na^+ and Cl^- uptake in the Amazonian population of mosquitoes, we employed various environmental manipulations (e.g., [NaCl], low pH) and pharmacological inhibitors of ion and acid-base regulation (amiloride, furosemide, bumetanide, bafilomycin A_1, acetazolamide) that have known disruptive effects on ion transport systems and have been useful in elucidating ion regulatory mechanisms in vivo in other freshwater organisms.

Material and Methods

Experimental Animals and Holding Conditions

Colonies of *Culex quinquefasciatus* (Say) and *Aedes aegypti* (Linnaeus) were established in a laboratory in the Department of Entomology at INPA (Instituto Nacional de Pesquisas da Amazonia) in Manaus, Brazil. Mosquito larvae used in the propagation of these lab colonies were hatched and held in INPA tap water (20 μmol L^{-1} Na^+, 20 μmol L^{-1} Cl, 9 μmol L^{-1} Ca^{2+}, 16 μmol L^{-1} K^+, pH 6.0–6.5) in large rectangular metal pans. Larvae of *Anopheles nuneztovari* (Gabaldon) were collected from sites around the city of Manaus, Brazil. The larvae were brought back to INPA, where they were held for 1 wk in INPA tap water. Ground fish food was added to the larval pans.

A colony of *C. quinquefasciatus* was established in the laboratory at the 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 lab colonies were hatched and held in Irvine tap water (4 mmol L^{-1} NaCl, 1 mmol L^{-1} Ca^{2+}, 0.1 mmol L^{-1} K^+) in large rectangular plastic trays (32 × 18 × 9 cm) with water changed each week. Larvae were fed ground rabbit chow pellets and dry yeast. For the Na^+ and Cl^- uptake kinetic analysis of *C. quinquefasciatus* reared in 60 μmol L^{-1} NaCl medium (series 4), egg rafts laid by adult female *C. quinquefasciatus* were transferred to a pan containing water with the following ionic composition: 60 μmol L^{-1} NaCl, 25 μmol L^{-1} KNO_3, 25 μmol L^{-1} $Ca(NO_3)_2$, pH 6–7. The larvae hatched in this medium. The water was changed every 2–3 d until larval development to the fourth instar. All experiments were conducted on fourth-instar larvae or large third-instar larvae.

Hemolymph Na^+ and Cl^- Levels

Hemolymph Na^+ and Cl^- concentrations were measured in both *C. quinquefasciatus* and *A. aegypti* held in the following

media for 2 d: control (rearing medium), 0 μmol L^{-1} NaCl, 8,000 μmol L^{-1} NaCl (5 d), pH 3, and Rio Negro water. For all but the last treatment, NaCl, KCl, and $Ca(NO_3)_2$ were added to distilled water to attain the appropriate concentrations of NaCl, K^+ (25 μmol L^{-1}), and Ca^{2+} (25 μmol L^{-1}). Because KCl was used to add K^+ to the media, the actual Cl^- concentrations were 25 μmol L^{-1} higher than the stated Na^+ values. The pH value was adjusted using H_2SO_4 or KOH. For all but the pH 3.0 and Rio Negro treatments, pH was adjusted to 6–7. Rio Negro water had the following chemical composition: 30 μmol L^{-1} Na^+, 30 μmol L^{-1} Cl^-, 18 μmol L^{-1} K^+, 10 μmol L^{-1} Ca^{2+}, pH = 5.5. Approximately 20 larvae were placed in small plastic containers with 50 mL of the appropriate experimental medium. For the 0 μmol L^{-1} NaCl treatment, the experimental medium was changed after 24 h. Ground fish pellet was added to each container. The addition of a small amount of ground fish pellet did not affect water Na^+ and Cl^- levels in the dilute treatments.

For sampling, larvae were removed from the experimental medium, rinsed in distilled water, and blotted dry on filter paper disks. The larvae were exsanguinated on Parafilm and the hemolymph was quickly collected using 0.5- or 1.0-μL microcapillary tubes (Drummond Microcaps). Hemolymph was then diluted in 0.5 or 1.0 mL distilled water for Na^+ and Cl^- analysis, respectively. Hemolymph and water Na^+ concentrations were determined using a flame photometer. Hemolymph and water Cl^- concentrations were determined using a modified version of a colorimetric assay (Zall et al. 1956).

Unidirectional Na^+ and Cl^- Uptake Rates

Experimental Protocol. Preliminary tests determined the appropriate specific activity of the medium and flux time to ensure that a high activity of isotope could be detected in the larvae. In all experiments, except for a part of series 4, as noted, unidirectional Na^+ and Cl^- uptake rates were measured simultaneously on the same group of larvae.

Two hours before the initiation of flux measurements, six to eight larvae (mean weight 1.7 mg) were transferred to a well of a cell culture plate (Costar; 24 wells). Two milliliters of holding medium was added to each well. To initiate the experimental flux period, water was removed, larvae and well were rinsed twice with 2 mL distilled water, and 1.5 mL of appropriate experimental medium was added to each well. Isotope (1.85 kBq 22NaCl + 3.7 kBq $Na^{36}Cl$) was added to each well immediately following addition of the medium. A 50-μL water sample was taken after 5 min, initiating the flux period. A final 50-μL water sample was taken approximately 4 h later at the end of the flux period. Larvae were then transferred, via a plastic pasteur pipette, to a weigh boat containing approximately 25 mL of experimental medium (without isotope). Larvae were rinsed in this medium for at least 30 s and then blotted on a filter paper disk. Individual larvae were weighed and transferred to a 0.5-mL microcentrifuge tube. Water samples and larvae were assayed for gamma radiation using a gamma counter. Next, 50 μL of distilled water was added to each microcentrifuge bullet tube containing larvae. The larvae were macerated, and 500 μL of scintillation cocktail (Ecolite, ACS) was added to each tube, including the tubes containing 50-μL water samples. Water and larvae samples were assayed for beta radioactivity using a scintillation counter. Experimental water samples were assayed for Na^+ and Cl^- concentration as described for hemolymph samples above.

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

$$J_{in} = \text{cpm}_{larva} \times \frac{1}{SA_{H_2O}} \times \frac{1}{\text{mass}_{larva}} \times \frac{1}{\text{time}}.$$

When Na^+ and Cl^- uptake rates were measured separately, cpm_{larva} designated whole-body activity and SA_{H_2O} was the mean specific activity of the water (cpm $nmol^{-1}$) with regards to the isotope used. ^{22}Na is a dual gamma and beta radioactivity emitter. ^{36}Cl is a pure beta radioactivity emitter. Scintillation counting detects both types of radiative emission. When the ^{22}Na and ^{36}Cl isotopes were used simultaneously, cpm_{larva} and SA_{H_2O} for Na^+ were determined from the measurements of gamma radioactivity only of ^{22}Na. For Cl^- uptake, cpm_{larva} and SA_{H_2O} refer to the ^{36}Cl beta radioactivity. We determined the ^{36}Cl beta radioactivity by scintillation counting, subtracting the total radioactivity of ^{22}Na (gamma radioactivity measurements corrected for the counting efficiency differences between the gamma and liquid scintillation counters) from the total scintillation radioactivity count of the larvae and water samples. Gamma counting was performed using a Picker-Cliniscaler counter attached to a 4-inch NaI Crystal with a well, and scintillation counting was performed using a Triathler portable scintillation counter. Estimates of whole-body specific activity of Na^+ and Cl^- at the end of the experiment were consistently less than 5% of the water specific activity, the upper limit for radioisotopic measurement of unidirectional ion flux (Wood 1988).

Series 1. The first series of experiments examined patterns in larval ion regulation under environmental conditions typical of the Amazon region (ion-poor conditions, low-pH conditions, or a combination thereof). An additional experiment was performed to compare ion uptake rates in the Amazonian population of *C. quinquefasciatus* with those of a California population maintained in high-NaCl water (4 mmol L^{-1} NaCl) at the University of California, Irvine (Patrick et al. 2001). The rates of Na^+ and Cl^- uptake in larvae of *C. quinquefasciatus* and *A. aegypti* were measured in the following six treatments: (1) control; (2) acute exposure to control media adjusted to pH 3.5 (20 μmol L^{-1} Na^+, 25 μmol L^{-1} K^+, 25 μmol L^{-1} Ca^{2+}, 45 μmol L^{-1} Cl^-); (3) acute exposure to Rio Negro water (30

μmol L^{-1} Na^+, 30 μmol L^{-1} Cl^-, 18 μmol L^{-1} K^+, 9 μmol L^{-1} Ca^{2+}, pH 5.5); (4) acute exposure to Rio Negro water with a pH of 3.5; (5) 3-d exposure to 4 mmol L^{-1} NaCl medium (25 μmol L^{-1} K^+, 25 μmol L^{-1} Ca^{2+}, pH 6.5); and (6) 3-d exposure to Rio Negro water. Larvae for the control, acute pH 3.5, acute Rio Negro, and acute Rio Negro/pH 3.5 exposures were held initially in plastic trays containing INPA tap water (ion composition; see above). For the 3-d treatments, approximately 20 larvae were placed in small plastic containers with 50 mL of the appropriate medium. A small amount of ground fish pellet was added to each container. The addition of food did not significantly alter the ionic composition of the media. Uptake rates for the control, 3-d 4 mmol L^{-1} NaCl, and 3-d Rio Negro treatment groups were all determined in the same conditions as the control medium (20 μmol L^{-1} Na^+, 25 μmol L^{-1} K^+, 25 μmol L^{-1} Ca^{2+}, 45 μmol L^{-1} Cl^-, pH 6). For the other treatments, the initiation of the acute exposures (to pH 3.5, Rio Negro, and Rio Negro/pH 3.5) was the start of the flux period and the fluxes were determined in the treatment water.

Series 2. This series investigated the transporters responsible for larval Na^+ and Cl^- uptake in the two species of mosquitoes. We employed three known pharmacological inhibitors of ion transport: amiloride (Sigma), a general blocker of Na^+ transport (Kleyman and Cragoe 1990), and bumetanide (Sigma) and furosemide (Sigma), known blockers of Na^+/Cl^-, $Na^+/K^+/2Cl^-$, and K^+/Cl^- cotransporters (Schlatter et al. 1983). Stock solutions (100 mmol L^{-1}) of each blocker were made up in dimethlysulfoxide (DMSO; Sigma). At the beginning of the flux period, an aliquot of the appropriate inhibitor was added to individual wells to reach a final concentration of 100 μmol L^{-1}. A DMSO control flux was run to determine whether the 1 μL of DMSO added to each well (0.07%), in addition to the inhibitor, had any effect on Na^+ and Cl^- uptake rates.

Series 3. This set of experiments examined the relationship between ion regulation and acid-base balance using acetazolamide (ACZ), a pharmacological inhibitor of carbonic anhydrase, a catalyst for the hydration reaction of CO_2, and bafilomycin A1 (Biomol, Plymouth Meeting, Pa.), a vacuolar-type H^+-ATPase blocker (Bowman et al. 1988). The 100 mmol L^{-1} stock solution of acetazolamide (Sigma) was made up by first dissolving ACZ in 5% KOH and then titrating this solution to pH 7 with HNO_3 (concentrated). A 400 μmol L^{-1} stock solution of bafilomycin was made with DMSO as the solvent. Aliquots of ACZ and bafilomycin stock solution were added to the appropriate wells to reach a final concentration of 100 and 2 μmol L^{-1}, respectively. A DMSO control flux was run to determine whether the 8.24 μL of DMSO added to each well (0.5%) had any effect on Na^+ and Cl^- uptake rates.

Series 4. The rates of Na^+ and Cl^- uptake in larvae of *C. quinquefasciatus* and *A. aegypti* were measured in 10 different NaCl concentrations ranging from 10 to 8,000 μmol L^{-1} (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 (10, 20, 40, 80, 160, 320, 1,000, 2,000, 4,000, or 8,000 μmol L^{-1} NaCl; 25 μmol L^{-1} $Ca(NO_3)_2$, 25 μmol L^{-1} KCl, pH 6.5). Because of the limited number of larvae, only Na^+ uptake was measured in *A. nuneztovari* in the range of 10–320 μmol L^{-1} Na^+. As mentioned above, KCl salt was used in making the experimental media; therefore, the actual water Cl^- concentrations were 25 μmol L^{-1} higher than the reported Na^+ concentration. For the Californian population of *C. quinquefasciatus* reared in 60 μmol L^{-1} NaCl medium, Na^+ and Cl^- uptake rates were measured in six different NaCl concentrations, as described in our previous study (Patrick et al. 2001).

Kinetic media were made up in batches with the appropriate pH and ion concentrations. Na^+ and Cl^- uptake measurements in the 10–320 μmol L^{-1} range were performed separately. For the 10, 20, 40, and 80 μmol L^{-1} NaCl treatment, 0.05 μCi of either ^{22}Na or ^{36}Cl was added to each well, and for the 160 and 320 μmol L^{-1} NaCl treatments, 0.1 μCi of either ^{22}Na or ^{36}Cl was added to each well. For the 1,000–8,000 μmol L^{-1} NaCl range, Na^+ and Cl^- uptake were measured simultaneously. For the 1,000 and 2,000 μmol L^{-1} NaCl treatments, 0.1 μCi of ^{22}Na and 0.2 μCi of ^{36}Cl were added to each well, and for the 4,000 and 8,000 μmol L^{-1} NaCl treatments, 0.2 μCi of ^{22}Na and 0.4 μCi of ^{36}Cl were added to individual wells.

The relationship between $[NaCl]_{ext}$ and Na^+ and Cl^- uptake was examined using Michaelis-Menten analysis for first-order one-substrate kinetics. FigP curve-fitting software was used to determine the values of J_{max} (the maximum uptake rate) and apparent K_m (the [ion] at which uptake is 50% of J_{max}) using the following equation:

$$J_{in} = \frac{J_{max} \times [ion]_{ext}}{K_m + [ion]_{ext}}.$$

Statistical Analyses. All data are reported as means ± SEM. Comparisons between groups were performed using ANOVA (overall $P \leq 0.05$) with multiple comparisons (Scheffé's test) if ANOVA proved significant. For the comparison of the kinetic parameters of ion uptake, K_m and J_{max}, if the 95% confidence intervals of each treatment did not overlap, then those parameters were considered different.

Results

Hemolymph Na^+ and Cl^- Levels

Hemolymph Na^+ and Cl^- levels in larval *Culex quinquefasciatus* (Fig. 1) were not significantly different from those of *Aedes aegypti* (Fig. 1) when maintained in the rearing medium of INPA tap water (i.e., 20 μmol L^{-1} NaCl). There were no significant differences in hemolymph Na^+ and Cl^- concentrations of *C. quinquefasciatus* larvae held in the control medium versus

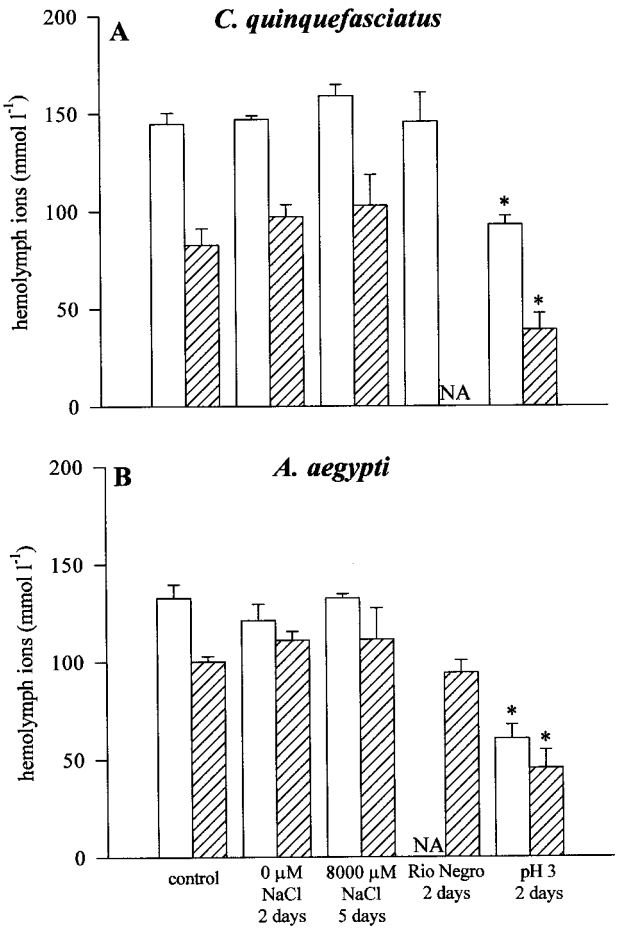

Figure 1. Hemolymph Na^+ (*open bars*) and Cl^- (*crosshatched bars*) concentrations of larval *Culex quinquefasciatus* (*A*) and *Aedes aegypti* (*B*) held in control, 0 and 8,000 μmol L^{-1} NaCl, Rio Negro, and pH 3 media for 2–5 d. $N = 3$–5. An asterisk denotes a significant difference from 20 μmol L^{-1} NaCl treatment value ($P = 0.05$). NA indicates that hemolymph Cl^- and Na^+ were not measured in *C. quinquefasciatus* and *A. aegypti*, respectively, when held in Rio Negro water.

the 0 and 8,000 μmol L^{-1} NaCl and Rio Negro treatments. However, both Na^+ and Cl^- levels dropped by 35.7% and 52.7%, respectively, in *C. quinquefasciatus* held at pH 3 for 2 d (Na^+, $P < 0.0001$; Cl^-, $P < 0.0107$). This trend was also found in *A. aegypti* larvae held in pH 3, and the decrease was greater (Na^+, 54.4%, $P < 0.0001$; Cl^-, 54.6%, $P < 0.0003$).

Na^+ and Cl^- Uptake Rates

Series 1. The two species had similar rates of Na^+ uptake, but the Cl^- uptake rate in *C. quinquefasciatus* was greater than the rate in *A. aegypti* ($P < 0.0007$; Fig. 2). When acutely exposed to a medium of pH 3.5, *C. quinquefasciatus* larvae experienced a 74.5% decrease in Na^+ uptake ($P < 0.0001$) and a 52.7% increase in Cl^- uptake ($P < 0.0134$; Fig. 2*A*). Both the acute and 3-d exposure to Rio Negro water did not affect uptake rates; however, when *C. quinquefasciatus* larvae were acutely transferred to Rio Negro water with a pH of 3.5, Na^+ uptake decreased by 80.9% ($P < 0.0001$) and Cl^- uptake was stimulated by 97.5% ($P < 0.0003$), a response greater than the pH 3.5 treatment ($P < 0.037$) and significantly different from the uptake rates for larvae held in Rio Negro water (Na^+, $P < 0.0001$; Cl^-, $P < 0.0001$). When *C. quinquefasciatus* larvae were held in 4 mmol L^{-1} NaCl medium for 3 d, Na^+ and Cl^- uptake were reduced by 84.3% ($P < 0.0001$) and 59.2% ($P < 0.0029$), respectively.

In contrast to *C. quinquefasciatus*, *A. aegypti* larvae did not

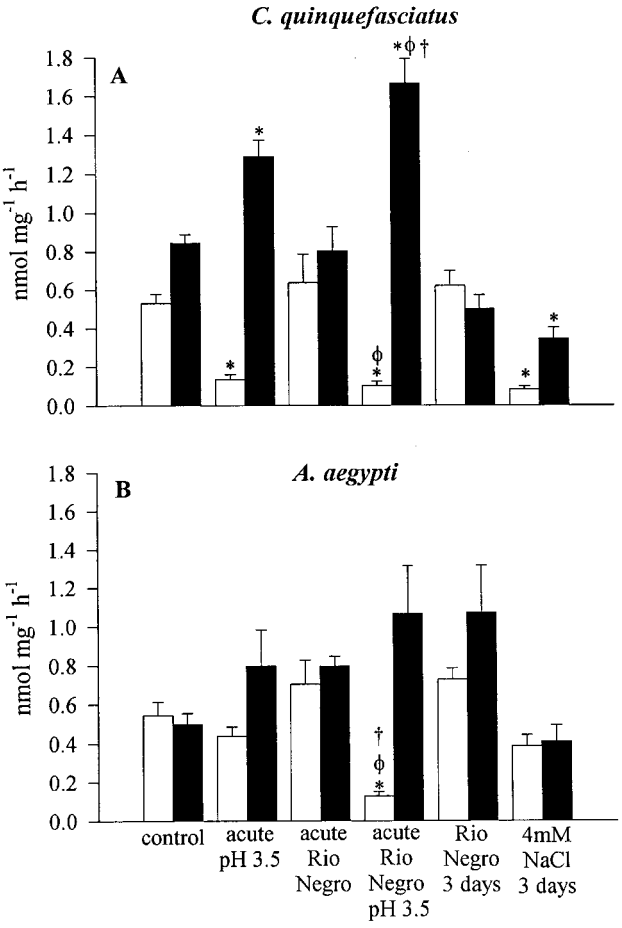

Figure 2. Unidirectional, whole-body Na^+ (*open bars*) and Cl^- (*solid bars*) uptake rates of *Culex quinquefasciatus* (*A*) and *Aedes aegypti* (*B*) measured in control medium, acute pH 3.5, Rio Negro, Rio Negro/pH 3.5, 3-d holding in Rio Negro, and 4 mmol L^{-1} NaCl media. Values are means ± SEM. $N = 5$–6. An asterisk denotes a significant difference from control value ($P = 0.05$). A lowercase phi denotes a significant difference from the acute Rio Negro value ($P = 0.05$). A dagger denotes a significant difference from acute pH 3.5 value ($P = 0.05$).

experience changes in rates of Na^+ and Cl^- uptake when exposed to pH 3.5 or Rio Negro water and, surprisingly, 4 mmol L^{-1} NaCl (Fig. 2*B*). The only significant change was the 76.6% drop in Na^+ uptake when *A. aegypti* were placed in Rio Negro water with a pH of 3.5 ($P < 0.0003$), whereas Cl^- uptake did not vary from the control rate. This reduced Na^+ uptake rate was also significantly different from the acute Rio Negro ($P < 0.001$) and pH 3.5 ($P < 0.009$) treatments.

Series 2. There were no significant changes in rates of Na^+ and Cl^- uptake in either *C. quinquefasciatus* (Fig. 3*A*) or *A. aegypti* (Fig. 3*B*) when exposed to DMSO, 100 μmol L^{-1} amiloride, 100 μmol L^{-1} bumetanide, or 100 μmol L^{-1} furosemide.

Series 3. When *C. quinquefasciatus* larvae were exposed to 100 μmol L^{-1} ACZ, Na^+ and Cl^- uptake rates were reduced by 17% ($P < 0.039$) and 41.3% ($P < 0.043$), respectively (Fig. 4*A*). The addition of 8.2 μL of DMSO (solvent for bafilomycin A_1) to the 1.5 mL flux medium did not alter Na^+ uptake, but Cl^- uptake was reduced by 66.6% ($P < 0.0008$). In *C. quinquefasciatus* larvae exposed to 2 μmol L^{-1} bafilomycin A_1 (in DMSO), Na^+ uptake rate again did not vary, but Cl^- uptake was 73% less than the control rate ($P < 0.0003$), similar to the response caused by DMSO alone. Neither Na^+ nor Cl^- uptake rates during the bafilomycin exposure were significantly different from the DMSO control values.

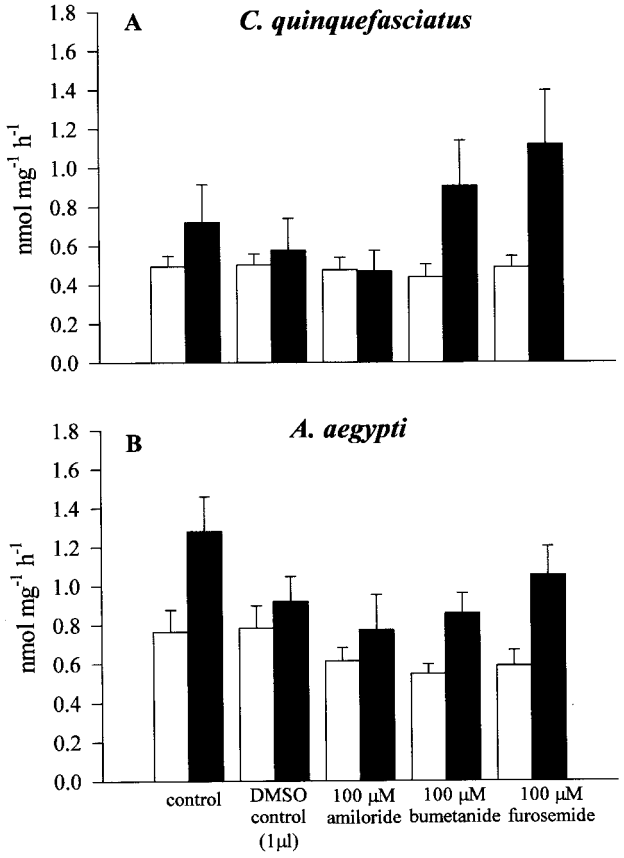

Figure 3. Unidirectional, whole-body Na^+ (*open bars*) and Cl^- (*solid bars*) uptake rates of *Culex quinquefasciatus* (*A*) and *Aedes aegypti* (*B*), measured in control medium, DMSO control, 100 μM amiloride, 100 μM bumetanide, and 100 μM furosemide media. Values are means ± SEM. $N = 4$–6.

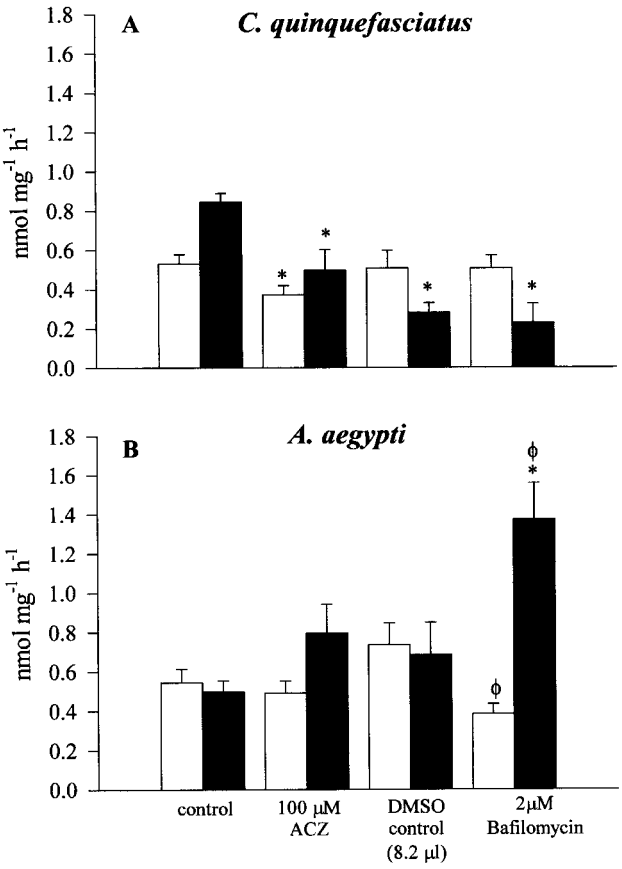

Figure 4. Unidirectional, whole-body Na^+ (*open bars*) and Cl^- (*solid bars*) uptake rates of *Culex quinquefasciatus* (*A*) and *Aedes aegypti* (*B*), measured in control medium, 100 μM acetazolamide, DMSO control, and 2 μM bafilomycin media. Values are means ± SEM. $N = 5$–6. An asterisk denotes a significant difference from control value ($P = 0.05$). A lowercase phi denotes a significant difference from the DMSO control value ($P = 0.05$).

The larvae of *A. aegypti* did not show changes in Na^+ and Cl^- uptake rates when exposed to 100 μmol L^{-1} ACZ or DMSO (Fig. 4*B*). However, when larvae were exposed to bafilomycin A_1, Na^+ uptake was reduced by 47.9% ($P < 0.034$), and Cl^- uptake rate doubled ($P < 0.034$) relative to the DMSO control rates.

Series 4. From Figure 5, it is apparent that Na^+ uptake did not

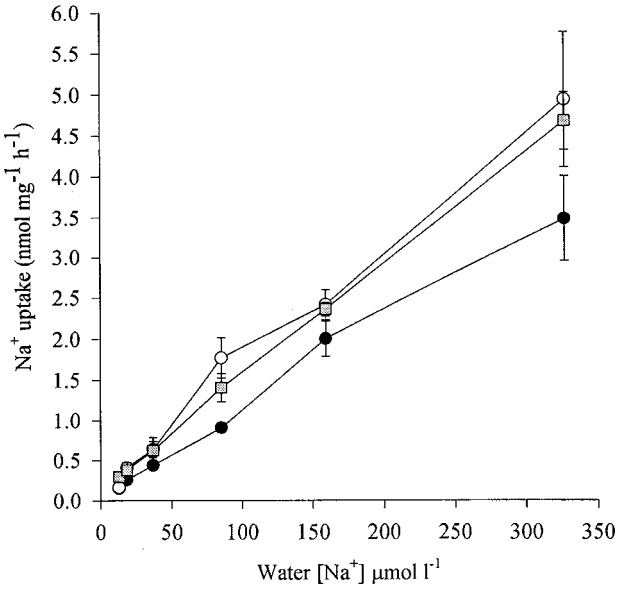

Figure 5. Effect of water Na^+ concentration on whole-body Na^+ uptake rates of *Culex quinquefasciatus* (*solid circles*), *Aedes aegypti* (*open circles*), and *Anopheles nuneztovari* (*squares*). Values are means ± SEM. N = 5–6.

exhibit saturation within the low range of water Na^+ concentration of 10–320 μmol L^{-1} Na^+ in larvae of Amazonian *C. quinquefasciatus*, *A. aegypti*, and *Anopheles nuneztovari*. All three species exhibited linear and large increases in Na^+ uptake and shared similar rates in the lower NaCl concentrations tested (≥320 μmol L^{-1} Na^+). However, both Na^+ and Cl^- uptake exhibited saturation in the Amazonian populations of *C. quinquefasciatus* and *A. aegypti* when the range of water NaCl concentrations was extended up to approximately 8,000 μmol L^{-1} (Fig. 6). Both species had similar K_m and J_{max} values for Na^+ uptake (Table 1), but *C. quinquefasciatus* larvae had a J_{max} for Cl^- uptake that was approximately 30% greater than *A. aegypti* (no overlap in confidence intervals; data not shown). The K_m value for Cl^- uptake in *C. quinquefasciatus* was approximately threefold higher than *A. aegypti*, but there was overlap in the confidence intervals.

There were many differences in the kinetic parameters of ion uptake between the Amazonian and Californian populations of *C. quinquefasciatus* (Fig. 7; Table 1). The data for the Californian *C. quinquefasciatus* that were reared in 4,000 μmol L^{-1} NaCl or held in 250 μmol L^{-1} NaCl media were taken from Patrick et al. (2001). The Amazonian population had the highest Na^+ uptake capacity (J_{max}), and the Californian population reared in 60 μmol L^{-1} NaCl had a J_{max} that was 60% that of the Amazonian group. The K_m values of the Amazonian and Californian populations reared in similar media were not different, as indicated by the overlapping of confidence intervals.

Both the Amazonian and the Californian population of *C. quinquefasciatus* that were hatched and reared in low NaCl exhibited saturable Cl^- uptake (Fig. 7*B*). However, their kinetic parameters where quite different, with the Amazonian group exhibiting much higher affinity (i.e., low K_m) and transport capacity for Cl^-, whereas the Californian population had a K_m value that was 6.2 times higher and a J_{max} that was one-fourth the value for the Amazonian population. The other two treatment groups of the Californian population did not exhibit clear saturation of the Cl^- uptake system (Fig. 7*B*). Regardless of treatment, the Californian population had very low rates of Cl^- uptake and did not increase much when external Cl^- levels were raised. In contrast, *C. quinquefasciatus* from the Amazon region exhibited very high rates of Cl^- uptake that increased rapidly within the lower NaCl concentrations tested (i.e., <320 μmol L^{-1} Cl^-) and started to plateau by 4,000 μmol L^{-1} Cl^-.

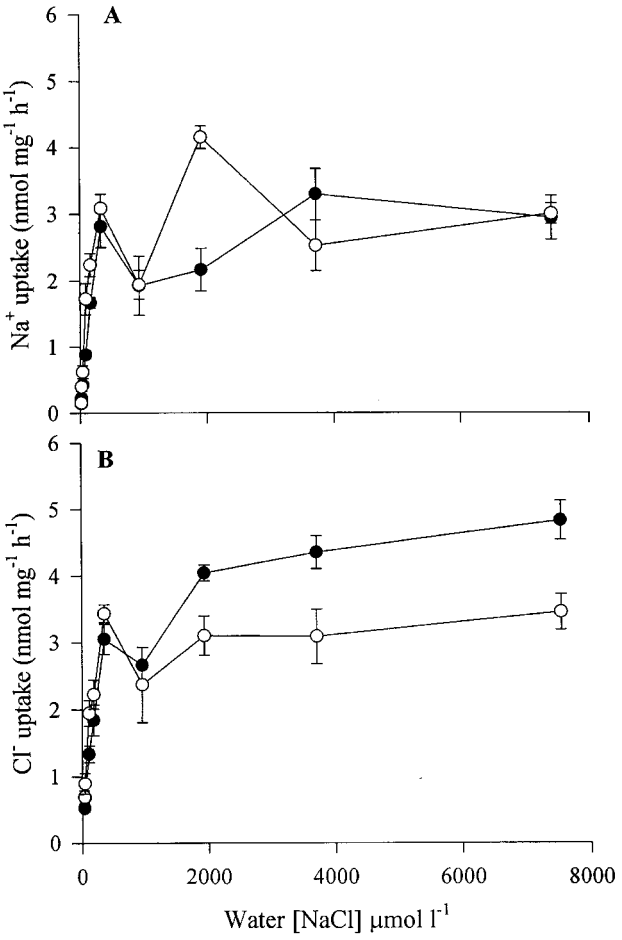

Figure 6. Effect of water NaCl concentration on whole-body Na^+ (*A*) and Cl^- (*B*) uptake rates of *Culex quinquefasciatus* (*solid circles*) and *Aedes aegypti* (*open circles*). Values are means ± SEM. N = 5–6.

Table 1: The affinity constant, K_m (μmol L^{-1}), maximum uptake capacity, J_{max} (nmol mg^{-1} h^{-1}), and r^2 value of nonlinear regression of Na^+ and Cl^- uptake rates in *Culex quinquefasciatus* and *Aedes aegypti*

	Na^+			Cl^-		
	K_m	J_{max}	r^2	K_m	J_{max}	r^2
Culex quinquefasciatus:						
Amazon population, 50 μM NaCl reared	134.00 ± 49.55	3.04 ± .70	.92	286.81 ± 73.88	4.64 ± .30	.96
California population:						
60 μM NaCl reared	144.00 ± 40.48	1.82 ± .07	.98	1,792.5 ± 1,134.30	1.16 ± .27	.80
250 μM NaCl 2 d[a]	650.00 ± 140.00	1.64 ± .10	.98	…	…	
4,000 μM NaCl reared[a]	1,060.00 ± 110.00	1.08 ± .03	.99	…	…	
Aedes aegypti:						
Amazon population, 50 μM NaCl reared	81.51 ± 42.04	3.16 ± .34	.84	88.08 ± 29.81	3.29 ± .24	.91

Note. The Amazonian population of *C. quinquefasciatus* reared in INPA tap water (20 μmol L^{-1} NaCl) and the Californian population of *C. quinquefasciatus* reared in Irvine tap water (4,000 μmol L^{-1} NaCl), 60 μmol L^{-1} NaCl, and held for 2 d in 250 μmol L^{-1} NaCl (but reared in Irvine tap water). Values are means ± SEM. Note that K_m and J_{max} values of Cl^- uptake could not be estimated for the Californian population held in 250 and 4,000 μmol L^{-1} NaCl because of the lack of saturation kinetics (see Fig. 7).

[a] Data from Patrick et al. 2001.

Discussion

This first look at the ion regulatory properties of mosquito larvae inhabiting the Amazon region has revealed differences among the species studied, as well as distinctions in the mechanisms of unidirectional Na^+ and Cl^- uptake from that of other aquatic organisms, including vertebrate and invertebrate species. Our results also provide evidence of both phenotypic plasticity and population differences in ion uptake within a single species. This study takes the initial step in characterizing the underlying mechanisms of ion uptake in the Amazonian mosquitoes and sets the foundation for elucidating the species and population distinctions that are presented below.

The Amazonian populations of *Aedes aegypti* and *Culex quinquefasciatus* have been maintained in water containing micromolar levels of NaCl for generations. Their ability to maintain high hemolymph NaCl levels under these conditions, as well as the lack of ion disruption when held in distilled water, indicates a departure from those populations examined in the previous studies. Hemolymph NaCl levels, however, were approximately 30–40 mmol L^{-1} higher than previously reported for both freshwater *C. quinquefasciatus* and *A. aegypti* (Na^+, 100 mmol L^{-1}; Cl^-, 50–70 mmol L^{-1}; Stobbart 1960, 1967; Patrick and Bradley 2000), even though in these previous studies the larvae were reared in media with much higher NaCl concentrations (>2 mmol L^{-1} Na^+). The distilled water treatment did not disturb hemolymph NaCl levels in either species (Fig. 1), which contrasts with the more than 30% lower levels when *A. aegypti* larvae were exposed to distilled water or when *A. aegypti* were reared in 2 μmol L^{-1} NaCl (Ramsay 1953; Stobbart 1960, 1967).

Although these two species maintained hemolymph NaCl levels similarly during exposure to different environmental treatments, our examination of Na^+ and Cl^- uptake revealed fundamental differences in the underlying mechanisms of ion regulation. *Culex quinquefasciatus* had significantly reduced rates of ion uptake relative to control values after 3 d in 4 mmol L^{-1} NaCl water, while *A. aegypti* was unaffected (Fig. 2), suggesting that the former species downregulated ion uptake in response to high concentration of NaCl in the water. This plasticity in ion uptake was also evident by the upregulation of Na^+ uptake in the Californian population of *C. quinquefasciatus* held in low-NaCl medium (Patrick et al. 2001). This modulation of uptake capacity could be realized by regulating the number of transport sites, allowing *C. quinquefasciatus* to maintain the same rate of uptake across a range of water NaCl levels. Presumably, the rate of diffusive ion loss was also reduced in *C. quinquefasciatus* in order to maintain ion balance (Fig. 1*A*). Similar plasticity in ion uptake has been reported in fish that withstand environmental challenges such as low pH (Gonzalez et al. 1997) or ion-poor media (McDonald and Rogano 1986; Perry and Laurent 1989). The lack of response in Na^+ and Cl^- uptake by *A. aegypti* (Fig. 2*B*), relative to its control group and to *C. quinquefasciatus* in the same high NaCl medium, means that this species must have high turnover rates (i.e., influx and efflux) when held in high-NaCl water (Fig. 2). This inflexibility would be costly as the uptake of Na^+ and Cl^- from the medium is against their respective electrochemical gradients, thus requiring the input of energy (Stobbart 1965).

The acute and 3-d holding in Rio Negro water (pH 5.5) did not disturb either hemolymph NaCl levels (see Fig. 1) or ion uptake rates (Fig. 2). However, when Rio Negro water was titrated to a pH of 3.5, we did observe an inhibitory effect on

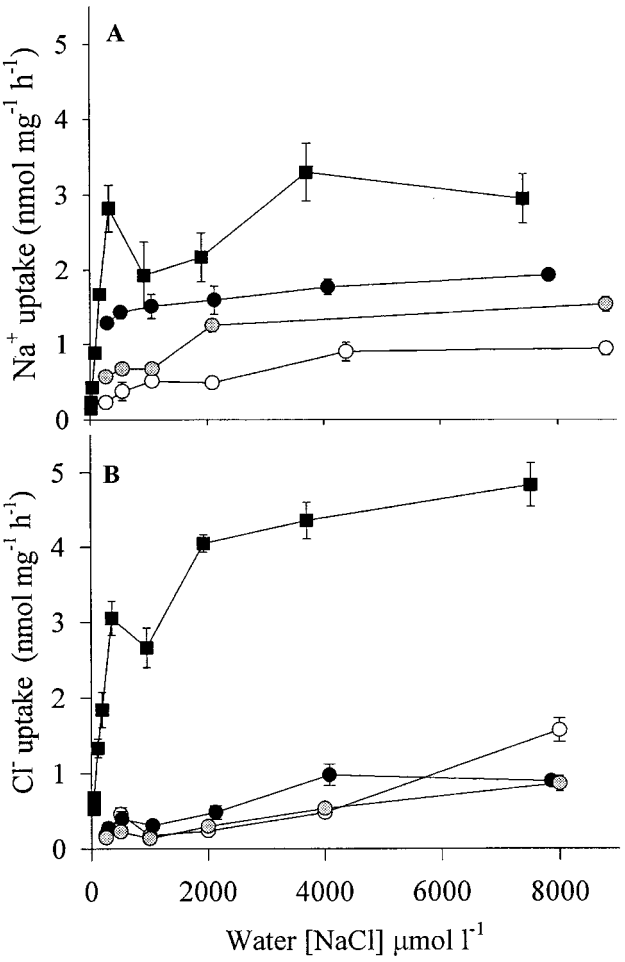

Figure 7. Effect of water NaCl concentration on whole-body Na^+ (*A*) and Cl^- (*B*) uptake rates of Amazonian *Culex quinquefasciatus* reared in 60 μM NaCl (*solid squares*), Californian *C. quinquefasciatus* reared in 60 μM NaCl (*solid circles*), 2 d in 250 μM NaCl (*gray circles*), and reared in 4,000 μM NaCl (*open circles*). Data for the latter two treatments are taken from Patrick et al. (2001). Values are means ± SEM. $N = 4–8$.

Na^+ uptake in *A. aegypti* (Fig. 2*B*). In contrast, the Na^+ uptake inhibition in *C. quinquefasciatus* in Rio Negro/pH 3.5 was not significantly different from control medium/pH 3.5 (Fig. 2*A*). Together, these results could indicate a species differences with regard to sensitivity of the osmoregulatory tissues to the complex mixture of organic compounds of Rio Negro water but only under acidic conditions. Recently, an interaction between low pH and Rio Negro water with ion uptake was reported in resident fish of the Rio Negro (Gonzalez et al. 2002). The impact on ion regulation noted in our study could be contributing to the paucity of mosquitoes in areas of the Rio Negro drainage where pH can be as low as 3 because of the release of organic acids from decaying forest litter (e.g., small tributaries, flooded forest; Val and Almeida-Val 1995). Perhaps a greater influence on mosquito populations is the aquatic bacterial community, a crucial food resource, which occurs at an extremely low density in the Rio Negro relative to other bodies of water in the Amazon (i.e., 1/500; Walker 1986).

The exposure to low pH resulted in an unusual response: concurrent inhibition of Na^+ uptake and stimulation of Cl^- uptake in *C. quinquefasciatus* (Fig. 2*A*). Only Na^+ uptake in the Amazonian *A. aegypti* larvae was affected by the Rio Negro/pH 3.5 treatment (Fig. 2*B*). Stobbart (1967), however, reported both Na^+ uptake inhibition and Cl^- uptake stimulation in *A. aegypti* larvae in pH <5. This contrast in populations of *A. aegypti* may indicate differences in pH sensitivities of ion transport mechanisms, with the Amazonian population being the least sensitive.

The inhibition of Na^+ uptake found in our study is typical of aquatic organisms exposed to acidic media, in both invertebrates (Lechleitner et al. 1985; Frisbie and Dunson 1988; Twitchen 1990) and vertebrates (McDonald 1983; Freda 1986) and is attributed to the inhibition of the exchange of Na^+ for H^+ through either an Na^+/H^+ antiporter or an Na^+ channel/H^+-ATPase moiety where the active extrusion of protons by a V-type H^+ ATPase results in an electrodiffusive gradient for Na^+ entry via the channel (Lin and Randall 1991; Potts 1994; Gonzalez and Wilson 2001). Our results therefore suggest that mosquito larvae possess a mechanism of Na^+ uptake similar to that of other aquatic animals. Conversely, the significant stimulation of Cl^- uptake during low-pH exposure found in *C. quinquefasciatus* larvae (Fig. 2*A*), *A. aegypti* larvae (Stobbart 1967), and also in water bugs (*Corixa dentipes, Corixa punctata*; Vangenechten et al. 1989) is unlike that of other freshwater animals (Wood 1989; McMahon and Stuart 1989), including the crustaceans (e.g., crayfish; Wood and Rogano 1986; Zanotto and Wheatley 1993). In these animals, there is evidence for a Cl^-/HCO_3^- exchanger, with the outward HCO_3^- concentration gradient driving Cl^- uptake (Kirschner 1997; O'Donnell 1997). The inhibition of Cl^- uptake by acidic environments is attributed to an increase in internal H^+ levels, which is buffered by internal HCO_3^- and consequently reduces its availability for the Cl^- uptake mechanism (Wood 1989). This mechanism does not explain the stimulation of Cl^- uptake in insects during low-pH exposure. Instead, our results suggest that Cl^- uptake by mosquitoes may be associated with inward H^+ movement (e.g., H^+-Cl^- cotransport), as described in locust hindgut (Phillips et al. 1996), or driven by an electrical gradient (e.g., Cl^- channel). Under acidic conditions, there would be a favorable gradient for H^+ influx that could increase the rate of H^+-Cl^- cotransport or, because of H^+ diffusion, the interior of the ion-transporting cells would become more positive, thereby enhancing inward Cl^- movement. Obviously, the increase in Cl^- uptake observed in *C. quinquefasciatus* did not avoid hemolymph Cl^- reduction (Fig. 1*A*), indicating that low-pH exposure must stimulate Cl^- efflux to an even greater extent. Indeed,

both Cl^- loss and uptake are stimulated in water bugs in pH 3.3 water; however, these insects are able to maintain Cl^- balance (Vangenechten et al. 1989).

The same pattern of inhibition of Na^+ uptake and stimulation of Cl^- uptake was observed in *A. aegypti* larvae exposed to 2 μmol L^{-1} bafilomycin A_1, the V-type H^+-ATPase inhibitor (Fig. 4*B*). The inhibition of Na^+ uptake in *A. aegypti* larvae further suggests the presence of an Na^+ transport mechanism that is associated with an apical H^+ ATPase, a configuration similar to that described above. Bafilomycin A_1 has been used to demonstrate indirectly that proton secretion drives Na^+ absorption in freshwater fish (Burg and Wood 1999; Fenwick et al. 1999) and amphibians (Ehrenfeld and Klein 1997). In these studies, bafilomycin A_1 inhibits a large fraction of Na^+ uptake by blocking proton pumping by the H^+ V-ATPase, thereby reducing the inward electrical gradient for Na^+ entry. Only in the in vitro studies of amphibian skin has both the concurrent blockage of H^+ secretion and Na^+ influx by bafilomycin A_1 been measured (Ehrenfeld and Klein 1997).

Unlike the inhibition of Na^+ uptake, the increased Cl^- uptake in *A. aegypti* larvae by bafilomycin A_1 contrasts with all other animals, including insects. We suggest that stimulatory effect could be attributed to reduced outward transport of internal H^+, thereby enhancing the electrodiffusive gradient for Cl^- entry. In the locust hindgut, the coupling of Cl^- absorption to H^+ secretion is through a proton recycling mechanism by which the extruded protons (via an apical V-ATPase) reenter the epithelium via H^+-Cl^- cotransport. Upon exposure to bafilomycin A_1, the lack of luminal protons to drive the symporter results in an even greater reduction in Cl^- absorption rate relative to the DMSO control (Phillips et al. 1996). This pattern is similar to the inhibitory effects of bafilomycin A_1 on Cl^- uptake in freshwater fish (Fenwick et al. 1999) and crustaceans (Onken and Putzenlechner 1995). Larsen et al. (1992) using oligomycin, a structural analogue of bafilomycin (Zeiske 1992), also reported a reduction in active inward Cl^- movement in toad skin preparations. In these systems, the authors argue that the blocking of apical H^+ secretion reduces the availability of HCO_3^- for exchange much as low pH does. Together, the low-pH and bafilomycin A_1 results support the Cl^-/HCO_3^- association in fish, amphibians, and crustaceans. Neither the locust hindgut proton-recycling mechanism nor the Cl^-/HCO_3^- model can explain the stimulated Cl^- uptake in *A. aegypti* larvae by bafilomycin A_1 (Fig. 4*B*). Further evidence against the presence of a Cl^-/HCO_3^- association in *A. aegypti* larvae was provided by the lack of effect on Cl^- uptake by ACZ, which blocks HCO_3^- production from CO_2 hydration reaction (Fig. 4*B*).

Unlike *A. aegypti*, bafilomycin A_1 had no effect on Na^+ and Cl^- uptake in *C. quinquefasciatus* relative to DMSO controls (Fig. 4*A*), suggesting that proton pumping is not associated with ion uptake. Interestingly, the DMSO control performed on the in vitro preparation of the locust hindgut resulted in a reduction in Cl^- absorption rate (Phillips et al. 1996), similar to what we observed in *C. quinquefasciatus* (Fig. 4*A*). In vitro electrophysiological studies of neuroblastomas/glioma hybrid cells found that DMSO (0.5%–1.0%) reversibly blocks Na^+, K^+, and Ca^{2+} currents but that there is variability in how other cell types respond to the same or higher-percentage solution (Jourdon et al. 1986). The authors state that these differences may reflect variation in membrane properties of different cell types and also that the DMSO effects on membranes themselves is unknown. At this point, we cannot judge exactly why the DMSO control in our study and Phillips et al. (1996) caused an inhibitory effect on Cl^- transport, but these effects do illustrate the necessity of performing a DMSO control. Nevertheless, Phillips et al. (1996) did see an additional significant inhibitory effect of bafilomycin on Cl^- transport relative to their DMSO control whereas there was no effect noted in *C. quinquefasciatus* but both an inhibitory (Na^+) and stimulatory (Cl^-) effect in *A. aegypti* (Fig. 4).

Species differences with respect to the role of V-ATPases in ion transport of crabs were recently reported. Weihrauch et al. (2001) documented the presence of the V_1 catalytic subunit of the H^+ V-ATPase in the gills of *Carcinus maenas*. Application of bafilomycin A_1 did not alter the potential difference across this osmoregulating tissue, indicating that proton pumping is not involved in transbranchial NaCl transport but may serve to regulate intracellular pH. In contrast, Onken and Putzenlechner (1995) reported that bafilomycin A_1 decreased short-circuit current (i.e., Cl^- influx) in isolated gills of the freshwater *Eriocheir sinensis*. V-type H^+ ATPases have been located in several ion-transporting tissues of larval *C. quinquefasciatus* (e.g., gastric cecae, midgut, hindgut, Malpighian tubules; Filippova et al. 1998). The anal papillae, which have been determined to be the site of active Na^+ and Cl^- absorption from the environment (Koch 1938; Wigglesworth 1938; Ramsay 1953), were not examined in the study by Filippova et al. (1998). Ultrastructural studies have indicated the presence of portasomes on the anal papillae of mosquito larvae (Garrett and Bradley 1984), which are believed to be the V_1 part of the H^+ V-ATPase (Zhuang et al. 1999). Future studies are planned to investigate the presence and location of V-ATPases in various species of mosquitoes in an attempt to determine the role of the proton pump in ion regulation.

The lack of an effect by bafilomycin A_1, together with the inhibitory effect of low pH on Na^+ uptake in *C. quinquefasciatus* (Fig. 2*A*), indicates that Na^+ uptake could be via a Na^+/H^+ exchanger rather than an Na^+channel/H^+-ATPase configuration. An Na^+/H^+ exchanger has been identified in fish (Claiborne et al. 2001) and a $2H^+/1Na^+$ exchanger has been characterized in freshwater invertebrates (Shetlar and Towle 1989). Unlike V-ATPase, there has been no examination of Na^+/H^+ exchangers in ion-transporting tissue in mosquito larvae.

Na^+ and Cl^- uptake in both *C. quinquefasciatus* and *A. aegypti* did not respond to external application of amiloride, bumetanide, and furosemide (Fig. 3). Previously, we have found

Na^+ uptake to be completely refractory to a similar 100 μmol L^{-1} dose of amiloride in Californian populations of *C. quinquefasciatus* and *C. tarsalis* (M. L. Patrick and R. J. Gonzalez, unpublished results). In other in vivo studies, freshwater vertebrates and invertebrates have exhibited amiloride-sensitive Na^+ uptake mechanisms, with the latter group showing a reduced sensitivity to the drug (O'Donnell 1997). This may indicate phylogenetic differences with respect to binding affinity of the Na^+ transporter for amiloride or could be attributed to the presence of cuticle on the exterior of the ion-transporting tissues in crustaceans (gills) and mosquito larvae (anal papillae). The lack of effect by bumetanide and furosemide could indicate insensitivity like amiloride, or that the $Na^+/K^+/2Cl^-$ cotransporters that these drugs inhibit are either basolaterally located or completely absent. This cotransporter plays a role at the basolateral membrane in ion uptake in fiddler crabs (*Uca tangeri*), as demonstrated by the serosal, not apical, effect of furosemide (Drews and Graszynski 1987). The $Na^+/K^+/2Cl^-$ cotransporter has not been implicated in salt absorption in freshwater fish but is involved in NaCl excretion, again at the basolateral membrane, in seawater teleosts (Wood and Marshall 1994).

It is interesting to note that the anal papillae of these Amazonian mosquitoes differed in size, with *A. aegypti* possessing much longer and wider anal papillae than *C. quinquefasciatus* (M. L. Patrick, personal observation). Although qualitative, this is intriguing because the two species have similar rates of ion uptake and body mass (average mass for each species ~1.7 mg), and anal papillae have been determined to be the major site of ion uptake in these freshwater species (Wigglesworth 1938; Treherne 1954; Stobbart 1965). In addition, previous studies indicated that the anal papillae length, maximum width, and the ultrastructural characteristics were the same in *A. aegypti* and *C. quinquefasciatus* (Wigglesworth 1933*a*, 1933*b*, 1933*c*; Copeland 1964; Sohal and Copeland 1966). However, Stobbart (1960) reported greatly hypertrophied anal papillae in larval *A. aegypti* reared in 2 μmol L^{-1} Na^+ relative to the original stock raised in 2,000 μmol L^{-1} NaCl. Based on these findings and our observation, we propose that the differences noted in the Amazonian populations may reflect plasticity in *A. aegypti* larvae to modulate the size of the anal papillae whereas *C. quinquefasciatus* does not exhibit such dramatic morphological change. It would be of interest to quantify the gross morphology and examine the ultrastructural properties of the anal papillae in these Amazonian mosquitoes in order to determine the contrasts from the published values and how this relates to the present findings of divergent mechanisms of Na^+ and Cl^- uptake.

The kinetic analysis of Na^+ uptake in *C. quinquefasciatus*, *A. aegypti*, and *Anopheles nuneztovari* (Fig. 5) and Cl^- uptake of the former two species (Fig. 6*A*, 6*B*) indicate that mosquito larvae inhabiting the Amazon region possess high-affinity transporters and high transport capacities (Table 1), but differences exist. Although *C. quinquefasciatus* and *A. aegypti* had similar J_{max} values for Na^+ uptake, the former species had a 30% greater Cl^- transport capacity, which could be realized through a greater number of transport sites, thereby compensating for the lower affinity of the system. In contrast, *A. aegypti* larvae had almost identical K_m and J_{max} values for Na^+ and Cl^- uptake (Table 1), a trend that differs from the results of Stobbart (1965, 1967), who reported differences in the K_m and J_{max} values for Na^+ and Cl^- uptake. In addition, values for both kinetic parameters estimated by Stobbart (1965, 1967) were much higher (K_m = 200–600 μmol L^{-1} NaCl, J_{max} = 4–12 nmol mg^{-1} h^{-1}) than reported in our study (Table 1). Stobbart (1965, 1967) also found that despite using different stocks of *A. aegypti* and various manipulations of the larvae and their environment, the K_m value remained unaltered and high relative to other freshwater invertebrates ($\lesssim$200 μmol L^{-1}; Shaw 1961; Wright 1975; Taylor and Harris 1986). On the basis of his results, Stobbart (1967) proposed that mosquito larvae could not increase the affinity of Na^+ and Cl^- uptake mechanisms and that an increase in J_{max} was the only avenue available to enhance ion uptake.

The finding of both phenotypic plasticity and population-based disparities in Na^+ and Cl^- uptake mechanism is the first, to our knowledge, to be documented in a single species. Both populations of *C. quinquefasciatus* exhibited plasticity in Na^+ uptake (Figs. 2*A*, 5*A*). The Amazonian *C. quinquefasciatus*, when held in 4 mmol L^{-1} NaCl media for 3 d, significantly reduced Na^+ uptake (Fig. 2*A*) to a rate similar to the California group reared in the same media (Patrick et al. 2001). The most parsimonious means to accomplish this would be to decrease uptake capacity (i.e., J_{max}) by reducing the number of Na^+ transport sites. In the reverse experiment, the Californian population substantially increased Na^+ J_{max} after only 2 d in 250 μmol L^{-1} NaCl and was able to further increase capacity and, more dramatically, enhance the affinity for Na^+ uptake when hatched and reared in the 60 μmol L^{-1} NaCl medium (Fig. 5*A*; Table 1). Although the Na^+ uptake capacity of the Californian group was only 60% of the J_{max} of Amazonian larvae, K_m values were comparable (Table 1). This indicates that larval development in dilute media not only involves increased capacity but the expression of high-affinity Na^+ transporters. Our results contrast with the conclusion of Stobbart (1967) who stated that the only way mosquito larvae could enhance Na^+ uptake was through changing capacity (i.e., J_{max}). *Culex quinquefasciatus* larvae were able to modulate both K_m and J_{max} of Na^+ uptake within a generation. For *A. aegypti*, however, it may require several generations in dilute media to observe changes in transport affinity.

Amazonian larvae demonstrated plasticity, whereas the Californian *C. quinquefasciatus* possesses an inflexible Cl^- uptake mechanism (Fig. 5*B*). The Amazonian population has the ability to downregulate Cl^- uptake when placed in a high-NaCl medium, a pattern similar to Na^+ uptake (Fig. 2*A*). In contrast, when the Californian population was placed in more dilute

media, there was no modulation of Cl^- uptake, not even when reared in 60 μmol L^{-1} Cl^- (Fig. 5*B*). Rates of Cl^- uptake in the Californian group were very low with extremely low affinity and capacity (estimated from 60 μmol L^{-1} reared larvae; no saturation in other treatments; Table 1). These results demonstrate not only population differences with respect to Cl^- transport in *C. quinquefasciatus* but also a disparity in how Na^+ and Cl^- uptake are regulated by the varying environmental conditions. The Californian *C. quinquefasciatus* did not suffer a drop in hemolymph Cl^- levels when reared in the 60 μmol L^{-1} NaCl (data not shown). Maintenance of constant hemolymph Cl^- levels while residing in an ion-poor environment without the ability to upregulate Cl^- absorption suggests that this population must have a fairly low rate of Cl^- loss. Our previous measurements of Cl^- efflux in this group support this idea (Patrick et al. 2001). Other aquatic animals have also demonstrated low ionic permeabilities as a strategy to tolerate challenges to ion balance (Gonzalez and Dunson 1987; Patrick et al. 1997), including certain species of mosquito larvae that live in ion-poor habitats found in the Amazon rain forest (Patrick et al. 2002, in this issue).

We can only speculate as to whether the inflexible Cl^- uptake mechanism of the Californian *C. quinquefasciatus* indicates a genetic distinction from the Amazon population. Perhaps to observe changes in Cl^- regulation (i.e., upregulation of uptake) would require more than one generation in dilute media or other environmental conditions that challenge ionic homeostasis (e.g., low pH). It is possible that the flexible Cl^- uptake mechanism in Amazonian populations could be a requirement for or a consequence of other environmental challenges (e.g., low/high pH) or metabolic demands (e.g., acid-base regulation). Additional studies of these groups or other distinct populations of *C. quinquefasciatus* could lead to an explanation of the disparities described for Cl^- uptake.

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