Ion Regulatory Patterns of Mosquito Larvae Collected from Breeding Sites in the Amazon Rain Forest

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ABSTRACT

We examined the ion composition of mosquito breeding sites located in the Amazon rain forest and the ion regulatory patterns of larvae from these habitats. We found larvae of *Toxorhynchites haemorroidalis*, *Limatus durhamii*, *Culex* (*Carrollia*) *bonnei*, and *Culex* (*Culex*) sp. residing in fallen palm bracts, leaves, and tree holes that were filled with water. These breeding sites had micromolar levels of Na^+ (1.6–99 μmol L^{-1}), but K^+ and Cl^- concentrations were higher and varied over a large range (231–17,615 μmol L^{-1} K^+; 355–2,700 μmol L^{-1} Cl^-). Despite the variability in environmental ion levels and ratios, all four species maintain high hemolymph NaCl levels (80–120 mmol L^{-1} Na^+; 60–80 mmol L^{-1} Cl^-). However, the species differed in the means by which they maintain hemolymph ion balance, as indicated by the range of unidirectional Na^+ and Cl^- uptake rates. *Toxorhynchites haemorroidalis* had extremely low rates of Na^+ uptake and undetectable Cl^- uptake, whereas *L. durhamii* had high rates of uptake for both ions. This variability in rates of uptake may reflect species differences in rates of diffusive ion loss (i.e., permeability). We observed the same curious pattern of Na^+ inhibition and Cl^- stimulation by low-pH exposure in all four species of mosquitoes, as has been documented in other mosquitoes and aquatic insects. Kinetic analyses of Na^+ and Cl^- uptake in *C. bonnei* larvae revealed an unusual pattern of Na^+ uptake that increases linearly (nonsaturable) to extremely high rates, while Cl^- uptake is a low-affinity, low-capacity system. This pattern contrasts with *L. durhamii* and *Culex* (*Culex*) sp. larvae, which had large increases in both Na^+ and Cl^- uptake when external NaCl levels were increased. Our results suggest that although these rain forest mosquito larvae are residing in habitats with similar low Na^+, high Cl^- composition and maintain similar hemolymph NaCl levels, the underlying mechanisms of ion regulation differ among the species.

Introduction

Mosquito larvae are found in aquatic habitats that vary tremendously in ion composition, from essentially distilled water to hypersaline conditions (reviewed by Clements [1992]). In order to reside in these environments, larvae may possess either enormous plasticity in ion regulatory mechanisms to tolerate a wide range of conditions or specializations for particular water chemistry (e.g., low or high pH, ion poor). The majority of the research on ion regulation in mosquito larvae has focused on those few species that reside in habitats beyond the freshwater range (>5% seawater) and has resulted in well-defined models of the two specialized strategies involving powerful ion-transporting epithelia (osmoregulators; Bradley 1987) or the ability to accumulate organic osmolytes (osmoconformers; Patrick and Bradley 2000). These two strategies also appear to be distributed according to phylogeny in that a genus will have species employing only one of the two strategies (reviewed by Bradley [1994]).

Over 95% of all mosquito species are found in freshwater, and within these habitats, ion concentrations, ratios, and pH values vary enormously. It is not known whether all species possess plastic ion regulatory mechanisms that allow larvae to tolerate a large range of chemical environments or if, like the

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salt-tolerant larvae, certain species are specialized for specific freshwater habitats. Our previous study of a freshwater-restricted *Culex quinquefasciatus* and a euryhaline species, *Culex tarsalis*, found that the former species possesses a flexible Na^+ uptake mechanism that would better enable it to inhabit ion-poor environments (Patrick et al. 2001). In our companion article in this issue (Patrick et al. 2002), we found further support for plasticity in Na^+ uptake, suggesting that *C. quinquefasciatus* larvae modulate transport capacity (i.e., number of transporters) within 2 d and also express Na^+ transporters with enhanced Na^+ affinity when reared in a novel, low-Na^+ environment. In contrast, we found opposing characteristics in Cl^- uptake between Californian and Amazonian populations of *C. quinquefasciatus*, with the former displaying very low-capacity, nonsaturating, inflexible Cl^- uptake, whereas the latter exhibit very high-affinity, high-capacity uptake that was plastic. This contrast demonstrates population differences in mechanisms and strategies in maintaining Cl^- balance. Our work thus far has examined laboratory-reared mosquitoes that have been maintained in a constant environment for numerous generations. Despite this, we have found evidence of species- and population-based plasticity and specialization. We wanted to determine what role these two strategies play in wild mosquitoes that, from one generation to the next, face variable environmental conditions with unusual ionic concentrations and ratios.

The Amazon rain forest is a region with an impressive diversity of mosquitoes (400+ species) and also of breeding sites (Penny and Arias 1982). In general, the major bodies of water in this region have been typified as being ion poor (Furch 1984). However, mosquitoes also breed in sites within the rain forest (e.g., tree holes, bromeliads, fallen leaves), and the chemical composition of these sites has not been thoroughly examined. In the Northern Hemisphere, where mosquito breeding sites have been widely studied, an array of ionic concentrations and ratios have been documented (MacGregor 1921; Petersen and Chapman 1969, 1970; Vrtiska and Pappas 1984; Clements 1992). This study is the first to examine water chemistry of mosquito breeding sites and the ion regulatory patterns in mosquito larvae collected from various sites within the Amazon rain forest habitat. In addition to determining ion composition of rain forest breeding sites and the hemolymph from mosquito larvae residing in these habitats, we employed basic in vivo physiological techniques to quantify unidirectional Na^+ and Cl^- uptake rates. Our purpose was to determine whether different species of mosquito exhibit common in vivo ion regulatory characteristics or whether there are different physiological solutions to surviving in this habitat. To characterize patterns in unidirectional Na^+ and Cl^- uptake, we examined the larva's response to different environmental manipulations (low pH, high NaCl) that are known to affect ion balance in aquatic organisms.

Material and Methods

Experimental Animals

Larvae of *Toxorhynchites haemorrhoidalis* (Fabricus), *Limatus durhamii* (Theobald), *Culex* (*Carrollia*) *bonnei* (Dyar), and an unidentified species of *Culex* (*Culex*) were collected from breeding sites in the rain forest near the shores of the Rio Negro in the Anavilhanas archipelago, approximately 100 km north of Manaus, Brazil. Collection took place in December 1999. Breeding sites were primarily water-filled palm bracts, but larvae were also located in fallen leaves or trees with water-filled depressions. Water samples (20 mL) were taken from each breeding site for pH and ion analysis. When possible, the entire content of the breeding site was poured into a plastic tray; otherwise, mosquito larvae were collected using a small mesh net (4 cm in diameter) and transferred to a container filled with water from the site. The larvae were transported back to the experimental site onboard the INPA (Instituto Nacional de Pesquisas da Amazonia) research vessel *Amanai II*. Taxonomic identification of the mosquitoes was performed based on larvae and also on adult forms that were allowed to eclose. The larvae were separated and placed into containers with water from their original breeding site. All experiments were conducted on fourth-instar larvae or large third-instar larvae.

Ion and pH Analyses of Larval Hemolymph and Breeding Site Water

Water Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cl^- concentrations were measured in 22 breeding sites where mosquito larvae were collected. Hemolymph Na^+ and Cl^- concentrations were measured in larvae from all four taxonomic groups. The larvae had been held in water from their original breeding site. The protocol for hemolymph collection followed that described in Patrick et al. (2002). Hemolymph and water Na^+ concentrations were determined using a flame photometer aboard the research vessel, or samples were stored and analyzed later using an atomic absorption spectrophotometer. Breeding site water K^+, Ca^{2+}, and Mg^{2+} concentrations were measured in diluted samples using an atomic absorption spectrophotometer. Hemolymph and water Cl^- concentrations were determined using a modified version of a colorimetric assay (Zall et al. 1956). The pH of breeding site water was analyzed using an Orion portable pH electrode and meter that is designed to measure the pH of low ionic strength solutions.

Na^+ and Cl^- Uptake Rates

Experimental Protocol. The general experimental protocol for measuring ion uptake follows that described in Patrick et al. (2002). In all experiments, Na^+ and Cl^- uptake rates were measured simultaneously in individual larva. Because of the larger size of the *T. haemorroidalis* larvae (about 15–40 mg

compared with 1–3 mg for other species), one larva was placed in an individual well in order to measure Na^+ and Cl^- uptake rates. All other larvae were placed in groups of six to seven per well.

Rates of 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:

$$J_{in} = cpm_{larva} \times \frac{1}{SA_{H_2O}} \times \frac{1}{mass_{larva}} \times \frac{1}{time},$$

where cpm_{larva} designated whole-body activity and SA_{H_2O} was the mean specific activity of the water (cpm $nmol^{-1}$). For Na^+ uptake calculations, cpm_{larva} and SA_{H_2O} were determined from the measurements of gamma radioactivity of ^{22}Na. For Cl^- uptake, cpm_{larva} and SA_{H_2O} refer to the ^{36}Cl beta radioactivity determined by dual label scintillation and gamma counting as described in Patrick et al. (2002).

Series 1. The first series of experiments examined patterns in Na^+ and Cl^- uptake in larvae of all four species in response to changes in environmental ion and pH levels. Ion uptake rates were determined in larvae following transfer to control (20 μmol L^{-1} Na^+, 25 μmol L^{-1} K^+, 25 μmol L^{-1} Ca^{2+}, 45 μmol L^{-1} Cl^-, pH 6.0–6.5), pH 3.5 (all other ions held constant), and 6 mmol L^{-1} NaCl (all other ions held constant) media. The pH value was adjusted using H_2SO_4 or KOH.

Series 2. The rates of Na^+ and Cl^- uptake in larvae of *C. bonnei* were measured in seven different NaCl concentrations ranging from 20 to 6,000 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 (20, 40, 80, 160, 1,000, 2,000, or 6,000, μmol L^{-1} NaCl; 25 μmol L^{-1} $Ca(NO_3)_2$, 25 μmol L^{-1} KCl, pH 6.5). As KCl salt was used in making the experimental media, the actual water Cl^- concentrations were 25 μmol L^{-1} higher than the reported Na^+ concentration.

Kinetic media were made up in batches with the appropriate pH and ion concentrations. Na^+ and Cl^- uptake rates were measured simultaneously. For the 20, 40, and 80 μmol L^{-1} NaCl treatment, 0.05 μCi of ^{22}Na and 0.1 μCi of ^{36}Cl were added to each well and for the 160, 1,000, 2,000, and 6,000 μmol L^{-1} NaCl treatments, 0.1 μCi of ^{22}Na and 0.2 μCi of ^{36}Cl were added to each well containing six to seven larvae.

The relationship between $[NaCl]_{ext}$ 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}) was calculated using the following equation:

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

Kinetic analysis of Na^+ uptake was not performed as it did not saturate within the range of media $[Na^+]$ tested.

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.

Results

Breeding Sites and Hemolymph Ion Concentrations

There was great variability in the ion concentrations measured in the 22 breeding sites in which mosquito larvae were collected (Fig. 1). Comparison of hemolymph Na^+ levels in three of the four species (no hemolymph Na^+ data were obtained for *Culex* (*Culex*) sp.) revealed a significant difference (one-way ANOVA, $P < 0.0001$; Fig. 2). *Culex bonnei* larvae Na^+ concentration was 35% ($P < 0.013$) to 55% ($P < 0.0001$) higher than that of *Limatus durhamii* and *Toxorhynchites haemorroidalis*, respectively. In contrast, hemolymph Cl^- levels were similar among all four species and averaged around 60 mmol L^{-1}.

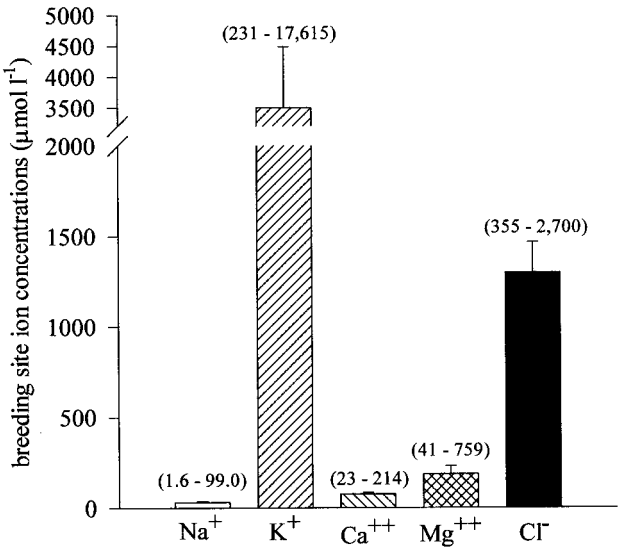

Figure 1. Water Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cl^- concentrations of breeding sites where mosquito larvae were collected in the Amazon rain forest. The numbers in parentheses are the minimum and maximum concentrations measured for each ion. Values are means ± SEM; $N = 22$.

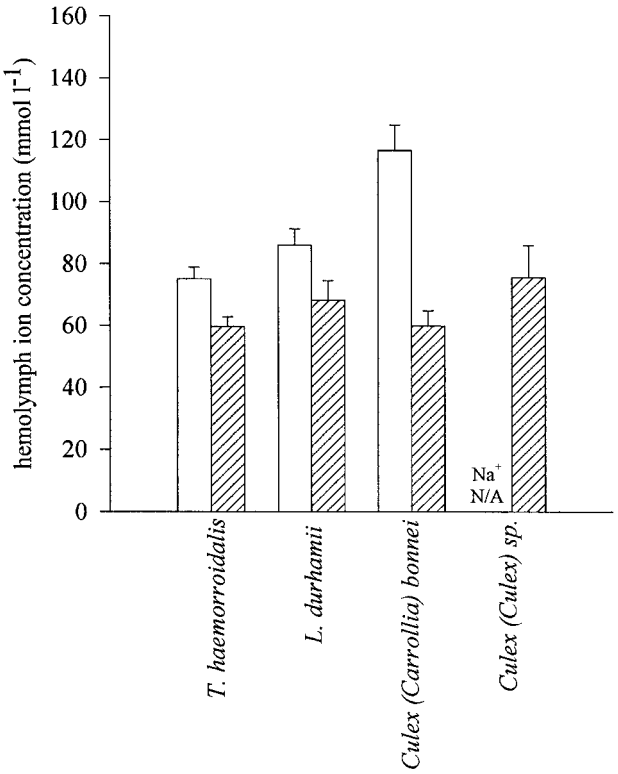

Figure 2. Hemolymph Na^+ (*open bars*) and Cl^- (*hatched bars*) concentrations of larval *Toxorhynchites haemorroidalis*, *Limatus durhamii*, *Culex* (*Carrollia*) *bonnei*, and *Culex* (*Culex*) sp. These larvae were collected from several breeding sites in the Amazon rain forest. Values are means ± SEM; $N = 5$–15. Hemolymph Na^+ concentration was not determined in *Culex* (*Culex*) sp.

Na^+ and Cl^- Uptake Rates

Series 1. The rates of Na^+ and Cl^- uptake in the four species varied tremendously when they were held in their original breeding site water and then tested in the control medium (one-way ANOVA, Na^+ $P < 0.0009$, Cl^- $P < 0.0003$; Fig. 3). *Toxorhynchites haemorroidalis* larvae had the lowest rate of Na^+ uptake of all four species, and Cl^- uptake was undetectable (Fig. 3*A*). Both *Culex* (*Culex*) sp. (Fig. 3*B*) and *C. bonnei* (Fig. 3*D*) had similar rates of Na^+ uptake, but Cl^- uptake was over threefold higher in the former species. *Limatus durhamii* had the highest rates of Na^+ and Cl^- uptake of the four species (Fig. 3*C*).

Exposure to pH 3.5 media resulted in reductions in Na^+ uptake rates of all four species (*T. haemorroidalis*: 42%, $P < 0.0224$; *Culex* (*Culex*), sp. 82.7%, $P < 0.001$; *L. durhamii*, 86.3%, $P < 0.0117$; *C. bonnei*, 80.6%, $P < 0.0001$). In contrast, the low-pH exposure caused stimulation of Cl^- uptake. For larvae of *T. haemorroidalis*, the increase was from undetectable levels to 0.011 nmol mg^{-1} h^{-1} ($P < 0.0007$; Fig. 3*A*), and *Culex* (*Culex*) sp. experienced a 74.6% increase ($P < 0.0048$; Fig. 3*B*). *Limatus durhamii* (Fig. 3*C*) and *C. bonnei* (Fig. 3*D*) had increases of 51.3% and 68.9%, respectively, in Cl^- uptake, but these increases were not statistically significant.

Rates of Na^+ and Cl^- uptake were both stimulated dramatically when three of the four species were exposed to the 6 mmol L^{-1} NaCl medium (*T. haemorroidalis* not tested). *Culex* (*Culex*) sp. larvae showed 11.4-fold ($P < 0.0001$) and 5.4-fold ($P < 0.0001$) stimulation of Na^+ and Cl^- uptake, respectively (Fig. 3*B*). *Limatus durhamii* experienced sixfold ($P < 0.0001$) and fourfold ($P < 0.0001$) increases in Na^+ and Cl^- uptake (Fig. 3*C*). *Culex bonnei* larvae exhibited the greatest response, with a 28-fold ($P < 0.0001$) elevation in Na^+ uptake, but Cl^- uptake increased by only 7.4-fold ($P < 0.0001$; Fig. 3*D*).

Series 2. From figure 4, it is apparent that Na^+ uptake in *C. bonnei* larvae did not exhibit saturation within the range of water Na^+ concentration tested. Na^+ uptake increased in a linear fashion to very high rates. In contrast, Cl^- uptake did saturate, but at a much lower rate—approximately one-sixth of the corresponding Na^+ uptake at 5,000 μmol L^{-1}. Nonlinear regression analysis was performed for Cl^- uptake, yielding K_m and J_{max} values of 134.0 ± 68.0 μmol L^{-1} Cl^- and 1.39 ± 0.17 nmol mg^{-1} h^{-1}.

Discussion

Our examination of ion regulation of mosquito larvae collected from breeding sites in the Amazon rain forest has revealed several intriguing features. First, the responses of the four wild-caught species to environmental manipulations share qualities with those of laboratory-reared species from the same region (Patrick et al. 2002) and populations from other parts of the world (Stobbart 1967; Patrick et al. 2001). This pattern suggests that there are common attributes in mechanisms of ion uptake. At the same time, however, there were distinctions among the species in how hemolymph ions levels are regulated, suggesting that there are species-specific solutions to the same environmental challenges. Our present findings, together with our previous work, suggest that there is not a clear relationship between environmental NaCl levels and kinetic properties of ion uptake. Possible reasons for this are explored below.

Our analysis of 22 mosquito breeding sites indicate that while Na^+ levels are extremely low, varying between only 1.6 and 99 μmol L^{-1}, K and Cl^- concentrations can be extremely high, averaging 3,500 and 1,300 μmol L^{-1}, respectively (Fig. 1). This water chemistry, particularly the disparity in water Na^+ and Cl^- levels found in our study (Fig. 1), is not unlike that previously reported for some other temperate mosquito breeding sites (Paradise and Dunson 1997). In fact, there have been numerous studies of ion composition of mosquito breeding sites, primarily in the Northern Hemisphere, and all have indicated that ion levels and ratios can vary tremendously

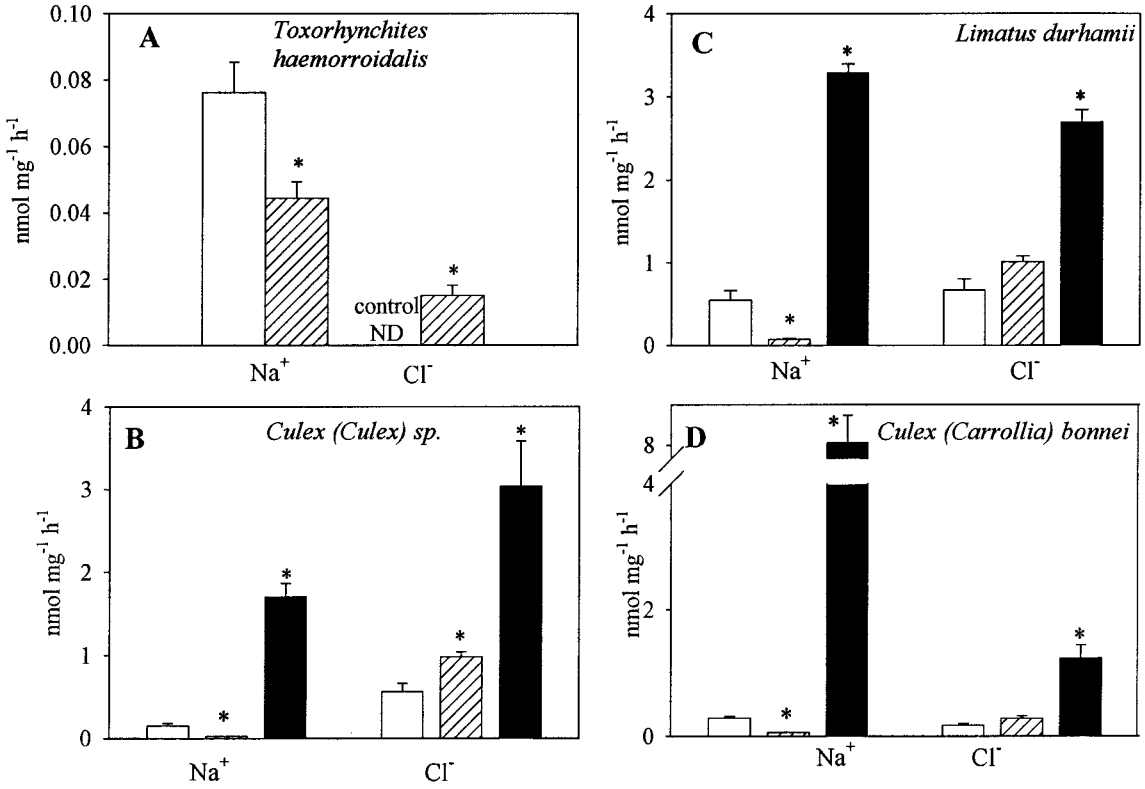

Figure 3. Unidirectional, whole-body Na^+ and Cl^- uptake rates of (*A*) *Toxorhynchites haemorroidalis*, (*B*) *Culex* (*Culex*) sp., (*C*) *Limatus durhamii*, and (*D*) *Culex* (*Carrollia*) *bonnei* measured in control medium (*open bars*), pH 3.5 medium (*hatched bars*), and 6 mmol L^{-1} NaCl medium (*solid bars*). Values are means ± SEM; $N = 3$–6. *ND* indicates that Cl^- uptake was not detectable in *T. haemorroidalis* tested in control water. *Toxorhynchites haemorroidalis* were not tested in 6 mmol L^{-1} NaCl medium. An asterisk denotes a significant difference from control value ($P = 0.05$).

(MacGregor 1921; Petersen and Chapman 1969, 1970; Vrtiska and Pappas 1984). For example, in a survey of tree hole habitats, Na^+ and Cl^- concentrations varied between 0.003 and 6 mmol L^{-1}, Ca^{2+} concentrations between 0.004 and 2.4 mmol L^{-1}, and K^+ concentrations between 0.001 and 0.333 mmol L^{-1} (Petersen and Chapman 1970). Williams and Linam (1967) reported tree hole breeding sites with Na^+ levels ranging between 21.5 and 68 mmol L^{-1}, while Cl^- was present in trace amounts, a pattern opposite that found for rain forest breeding sites (Fig. 1). The variation in breeding site water chemistry is attributed to the leaching of ions from the plant holding the rainwater (e.g., palm bract) and/or plant material that has fallen into the water.

Despite the enormous disparity in environmental Na^+ and Cl^- levels, all four species had hemolymph Na^+ and Cl^- concentrations (Fig. 2) that were not unlike previously published values for a variety of species with means near 100 and 50 mmol L^{-1}, respectively (Clements 1992). *Toxorhynchites haemorroidalis* and *Limatus durhamii* larvae had Na^+ concentrations that were slightly lower, whereas *Culex bonnei* hemolymph Na^+ was higher but similar to values we measured in Amazonian *Culex quinquefasciatus* and *Aedes aegypti* (Patrick et al. 2002). Species residing in breeding sites with micromolar amounts of Na^+, and laboratory-reared populations maintained in low-Na^+ tap water (20 μmol L^{-1}; Patrick et al. 2002), can maintain hemolymph Na^+ levels comparable to those of larvae maintained in higher Na^+ media (Stobbart 1959; Patrick et al. 2001). These observations confirm that mosquito larvae do tightly regulate hemolymph ion status in the face of extremely dilute concentrations. Our results contrast with a report by Stobbart (1960) in which *A. aegypti* larvae reared in 2 μmol L^{-1} Na^+ had reduced hemolymph Na^+ concentrations of 30 mmol L^{-1}. Other studies also reported substantial drops in hemolymph ion levels when fourth-instar larvae were transferred into distilled water for several days (Wigglesworth 1938; Ramsay 1953). These findings of compromised ion balance in larval *A. aegypti* suggest that this particular species may lack plasticity in its ion regulatory abilities. We found evidence of this when Amazonian *A. aegypti* were transferred from the rearing medium of 20 μmol L^{-1} Na^+ to 4 mmol L^{-1} NaCl

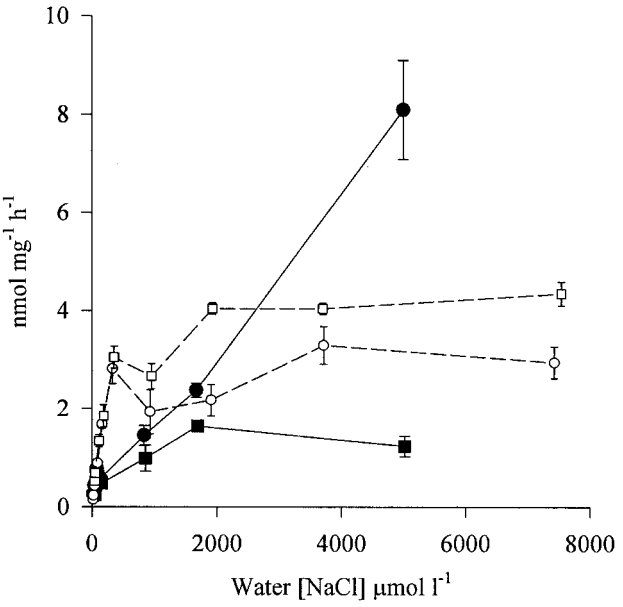

Figure 4. Effect of water NaCl concentration on whole-body Na^+ (*solid circles*) and Cl^- (*solid squares*) uptake rates of *Culex bonnei*. These values are compared to Na^+ (*open circles*) and Cl^- (*solid circles*) uptake rates of Amazonian *Culex quinquefasciatus* (data from Patrick et al. 2002). Values are means ± SEM; $N = 6–7$ for *C. bonnei*, and $N = 5–7$ for *C. quinquefasciatus*.

media for 3 d without any compensatory change in Na^+ and Cl^- uptake rates (Patrick et al. 2002).

Although the hemolymph Na^+ and Cl^- levels are similar in these four species of wild mosquitoes (Fig. 2), the means by which they regulate hemolymph ions are diverse, as indicated by the range of Na^+ and Cl^- uptake rates (Fig. 3). *Toxorhynchites haemorroidalis* larvae had a very low rate of Na^+ uptake, and Cl^- uptake was not detectable (Fig. 3*A*). In contrast, *L. durhamii* larvae had the highest rates of Na^+ and Cl^- uptake (Fig. 3*C*). The two *Culex* species were intermediate (Fig. 3*B*, 3*D*). Assuming that larvae are maintaining ion balance, these observed patterns in ion uptake suggest that there are species differences in the rate of ion loss. Ion efflux could occur through diffusive pathways (i.e., paracellular) or exchange diffusion mechanisms (Na^+/Na^+ Cl^-/Cl^- exchange). Although this aspect of ion regulation has not been addressed in mosquito larvae, it is possible that a significant proportion of unidirectional efflux may be linked on a 1 : 1 basis with unidirectional influx (Stobbart 1959; Fletcher 1980). Wigglesworth (1933*a*, 1933*b*) described the general body surface of mosquito larvae as being highly impermeable to salts and water, whereas the anal papillae, being more permeable, are the site of water and salt absorption (Wigglesworth 1938; Ramsay 1953) and the majority of the diffusive ion loss (Stobbart 1965). Despite being over 20-fold larger than the other three species, *T. haemorroidalis* larvae had anal papillae that were so small that they were difficult to discern under the microscope. Based on this qualitative observation, by having extremely reduced anal papillae, *T. haemorroidalis* would have lower overall surface area for ion loss and hence decrease the necessity for active Na^+ and Cl^- uptake. Indeed, this is the argument put forth for mosquito larvae held in high-salinity media in order to avoid water loss and salt gain (Wigglesworth 1938; Meredith and Phillips 1973), but it has not been addressed as a strategy to avoid ion loss in dilute freshwater. A quantitative analysis of the relationship between the morphology of the anal papillae and rates of ion flux in several freshwater species would allow us to better understand the connection between ion regulatory patterns and anal papillae structure.

The response of Na^+ and Cl^- uptake to low-pH exposure by all four species (Fig. 3) followed the same pattern as previously reported for laboratory-bred larvae (Stobbart 1967; Patrick et al. 2002) and other insects (Vangenechten et al. 1989). All species showed significant inhibition of Na^+ uptake, but *T. haemorroidalis* larvae did not have as great a reduction in rates (42%) as did *Culex* (*Culex*) sp. (83%), *L. durhamii* (86%), and *C. bonnei* (81%). This lower sensitivity in *T. haemorroidalis* could be a consequence of its low cuticular permeability that would in turn reduce access by external protons to Na^+ transport sites. Such detrimental effects of H^+ on Na^+ transport could include competitive inhibition or diminution of the electrochemical gradient for Na^+ entry (Potts 1994; Gonzalez and Wilson 2001). Alternatively, a reduced H^+ sensitivity could reflect a high-affinity Na^+ uptake mechanism such as those described in acid-tolerant stone fly nymphs (*Amphinemura sulcicollus*; Twitchen 1990) and an acid-insensitive fish, the neon tetra (*Paracheirodon innesi*; Gonzalez and Preest 1999).

The curious stimulatory effect of low pH on Cl^- uptake has now been repeated in several species of mosquitoes (*A. aegypti*, *C. quinquefasciatus*, *T. haemorroidalis*, *Culex* (*Culex*) sp., *L. durhamii*, *C. bonnei*; Stobbart 1967; Patrick et al. 2002) and water bugs (*Corixa dentipes*, *Corixa punctata*; Vangenechten et al. 1989). This pattern departs from aquatic vertebrates (Wood 1989) and any other aquatic invertebrates (McMahon and Stuart 1989), signifying a very different Cl^- absorption mechanism. In our companion article (Patrick et al. 2002), we propose that aquatic insects have a Cl^- transport system that is either driven electrically or associated with inward H^+ movement (i.e., H^+-Cl^- cotransporter). At pH 3.5, there is a large gradient for inward H^+ movement. This could be coupled to Cl^- transport as in the latter model or could result in a more positive charge across the apical membrane (inside positive) of the transporting epithelium (i.e., anal papillae), thereby enhancing an inward electrodiffusive gradient for Cl^-. Until transporters are identified in the anal papillae of mosquito larvae, we can only speculate on the underlying mechanisms responsible for the patterns we have observed in mosquito larvae. The increase in Cl^- absorption could be a means to counteract a larger Cl^-

efflux, as reported in water bugs at low pH (Vangenechten et al. 1989), or an attempt to maintain electroneutrality (i.e., match H^+ influx). The magnitude of change in Cl^- uptake during low-pH exposure in this study does not follow the same trend observed in Na^+ uptake inhibition by low pH (Fig. 4). These differences in pH sensitivity in Na^+ and Cl^- uptake may indicate differences in the affinities of these transporters for the respective ions.

The kinetic analysis of Na^+ and Cl^- uptake in *C. bonnei* revealed very disparate patterns in Na^+ and Cl^- uptake as water [NaCl] increased (Fig. 4). Na^+ uptake rose dramatically to very high rates, the highest that we have measured in mosquito larvae. In addition, Na^+ uptake showed no evidence of saturation, whereas Cl^- uptake did plateau at a very low rate. These findings indicate that there are fundamental differences in how Na^+ and Cl^- are transported in *C. bonnei.* Na^+ uptake appears to have an extremely high capacity and very low affinity, which is opposite to the low K_m and J_{max} values estimated for Cl^- uptake (see "Results").

Initially, we believed that the divergent patterns of Na^+ and Cl^- uptake in *C. bonnei* reflected the low Na^+ and high Cl^- levels of their breeding sites (Fig. 1). For example, *C. bonnei* larvae living in low-Na^+ water would increase transport capacity and/or affinity to ensure adequate Na^+ uptake rate and at the same time would not require a high transport capacity or affinity for Cl^- when external Cl^- ions are abundant. However, our examination of the other species held in various NaCl concentrations are not supportive of this hypothesis for mosquito larvae. Although we could not perform a complete kinetic analysis of uptake because of the limited number of available larvae, our estimates of the capacities for Na^+ and Cl^- uptake in *Culex* (*Culex*) sp. and *L. durhamii* did not follow the same pattern as *C. bonnei. Limatus durhamii* had similar rates of Na^+ and Cl^- uptake in the 6 mmol L^{-1} NaCl medium (Fig. 3*C*), whereas *Culex* (*Culex*) sp. transported Cl^- at a rate higher than Na^+ (Fig. 3*B*). In addition, California populations of *C. quinquefasciatus* and *C. tarsalis* in media with similar high Na^+ and Cl^- concentrations (4 mmol L^{-1}) had saturable, high-capacity Na^+ uptake and low, nonsaturating Cl^- uptake (Patrick et al. 2001). Finally, in our companion study of Amazonian *C. quinquefasciatus* and *A. aegypti* larvae that were reared in media with similar low Na^+ and Cl^- concentrations (20 μmol L^{-1}), we reported that both Na^+ and Cl^- uptake were saturable and exhibited high transport capacities and affinities (Patrick et al. 2002). The kinetic properties of ion uptake do not vary with rearing media NaCl levels. Taken together, these observations indicate dissimilarities in ion regulatory mechanisms, not only between genera but also between species of the same genus (*Culex quinquefasciatus*, *C. bonnei*, *Culex* (*Culex*) sp.) and even populations within a species (*C. quinquefasciatus*; Patrick et al. 2002). It implies that factors other than environmental ion concentrations are involved. These many patterns of ion uptake may reflect differences in underlying transport mechanisms (e.g., channels, exchangers, cotransporters), relative cuticular permeability to Na^+ and Cl^- loss (e.g., cuticular thickness, anal papillae size), or the extent to which Na^+ and Cl^- uptake components play a role in acid-base regulation (Na^+/H, Cl^-/HCO_3^- exchange) or nitrogenous waste excretion (Na^+/NH_4^+ association), all of which may involve a phylogenetic component and/or be influenced by selective forces unknown to us at this time. More in-depth examination of the species and populations described in this study and our companion article will help to explain the nature of these disparate ion regulatory patterns.

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Literature Cited

Bradley T.J. 1987. Physiology of osmoregulation in mosquitoes. Annu Rev Entomol 32:439–62.

———. 1994. The role of physiological capacity, morphology, and phylogeny in determining habitat use in mosquitoes. Pp. 303–318 in P.C. Wainwright and S.M. Reilly, eds. Ecological Morphology: Integrative Organismal Biology. University of Chicago Press, Chicago.

Clements A.N. 1992. The Biology of Mosquitoes. Vol. 1. Development, Nutrition and Reproduction. Chapman & Hall, London.

Fletcher C.R. 1980. The relationship between active transport and the exchange diffusion effect. J Theor Biol 82:643–661.

Furch K. 1984. Water chemistry of the Amazon basin: the distribution of chemical elements among freshwaters. Pp. 167–199 in H. Sioli, ed. The Amazon: Limnology and Landscape Ecology of a Mighty Tropical River and Its Basin. Junk, Dordrecht.

Gonzalez R.J. and M.R. Preest. 1999. Ionoregulatory speciali-

zations for exceptional tolerance of ion-poor, acidic waters in the neon tetra (*Paracheirodon innesi*). Physiol Biochem Zool 72:156–163.

Gonzalez R.J. and R.W. Wilson. 2001. Patterns of ion regulation in acidophilic fish native to the ion-poor, acidic Rio Negro. J Fish Biol 58:1680–1690.

MacGregor M.E. 1921. The influence of the hydrogen-ion concentration in the development of mosquito larvae. Parasitology 13:348–351.

McMahon B. and S. Stuart. 1989. The physiological problems of crayfish in acid waters. Pp. 171–200 in R. Morris, E.W. Taylor, D.J.A. Brown, and J.A. Brown, eds. Acid Toxicity and Aquatic Animals. Cambridge University Press, Cambridge.

Meredith J. and J.E. Phillips. 1973. Ultrastructure of the anal papillae of a salt-water mosquito larva, *Aedes campestris*. J Insect Physiol 19:1157–1172.

Paradise C.J. and W.A. Dunson. 1997. Effects of pH and sulfate on insects and protozoans inhabiting treeholes. Arch Environ Contam Toxicol 33:182–187.

Patrick M.L. and T.J. Bradley. 2000. The physiology for salinity tolerance in larvae of two species of *Culex* mosquitoes: the role of compatible solutes. J Exp Biol 203:821–830.

Patrick M.L, R.J. Gonzalez, and T.J. Bradley. 2001. Sodium and chloride regulation in freshwater and osmoconforming larvae of *Culex* mosquitoes. J Exp Biol 204:3345–3354.

Patrick M.L, R.J. Gonzalez, C.M. Wood, R.W. Wilson, T.J. Bradley, and A.L. Val. 2002. The characterization of ion regulation in Amazonian mosquito larvae: evidence of phenotypic plasticity, population-based disparity, and novel mechanisms of ion uptake. Physiol Biochem Zool 75:000–000.

Penny N.D. and J.R. Arias. 1982. Insects of the Amazon Forest. Columbia University Press, New York

Petersen J.J. and H.C. Chapman. 1969. Chemical factors of water in tree holes and related breeding of mosquitoes. Mosq News 29:29–37.

———. 1970. Chemical characteristics of habitats producing larvae of *Aedes sollicitans*, *Aedes taeniorhynchus*, and *Psorophora confinnis* in Louisiana. Mosq News 30:156–161.

Potts W.T.W. 1994. Kinetics of sodium uptake in freshwater animals: a comparison of ion-exchange and proton pump hypotheses. Am J Physiol 266:R315–R320.

Ramsay J.A. 1953. Exchanges of sodium and potassium in mosquito larvae. J Exp Biol 30:79–89.

Stobbart R.H. 1959. Studies on the exchange and regulation of sodium in the larva of *Aedes aegypti* (L). I. The steady state exchange. J Exp Biol 36:641–653.

———. 1960. Studies on the exchange and regulation of sodium in the larva of *Aedes aegypti* (L). II. The net transport and the fluxes associated with it. J Exp Biol 37:594–608.

———. 1965. The effect of some anions and cations upon the fluxes and net uptake of sodium in the larva of *Aedes aegypti* (L). J Exp Biol 42:29–43.

———. 1967. The effect of some anions and cations upon the fluxes and net uptake of chloride in the larva of *Aedes aegypti* (L), and the nature of the uptake mechanisms for sodium and chloride. J Exp Biol 47:35–57.

Twitchen I.D. 1990. The physiological bases of resistance to low pH among aquatic insect larvae. Pp. 413–419 in B.J. Mason, ed. The Surface Waters Acidification Programme. Cambridge University Press, Cambridge.

Vangenechten J.H.D., H. Witters, and O.L.J. Vanderborght. 1989. Laboratory studies on invertebrate survival and physiology in acid waters. Pp. 153–169 in R. Morris, E.W. Taylor, D.J.A. Brown, and J.A. Brown, eds. Acid Toxicity and Aquatic Animals. Cambridge University Press, Cambridge.

Vrtiska L.A. and L.G. Pappas. 1984. Chemical analysis of mosquito larval habitats in southeastern Nebraska. Mosq News 44:506–509.

Wigglesworth V.B. 1933*a*. The effect of salts on the anal gills of the mosquito larva. J Exp Biol 10:1–14.

———. 1933*b*. The function of the anal gills of the mosquito larva. J Exp Biol 10:16–26.

———. 1938. The regulation of osmotic pressure and chloride concentration in the hemolymph of mosquito larvae. J Exp Biol 15:235–247.

Williams F.M. and J.H. Linam. 1967. Some physical and chemical factors of tree rot holes. Proceedings from the Utah Abatement Association.

Wood C.M. 1989. The physiological problems of fish in acid waters. Pp. 125–152 in R. Morris, E.W. Taylor, D.J.A. Brown, and J.A. Brown, eds. Acid Toxicity and Aquatic Animals. Cambridge University Press, Cambridge.

Zall D.M., M.D. Fisher, and Q.M. Garner. 1956. Photometric determination of chloride in water. Anal Chem 28: 1665–1678.