Larvae of the midge *Chironomus riparius* possess two distinct mechanisms for ionoregulation in response to ion-poor conditions

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Nguyen H, Donini A. Larvae of the midge *Chironomus riparius* possess two distinct mechanisms for ionoregulation in response to ion-poor conditions. *Am J Physiol Regul Integr Comp Physiol* 299: R762–R773, 2010. First published July 14, 2010; doi:10.1152/ajpregu.00745.2009.—This study examined the role of the anal papillae of the freshwater (FW) chironomid larva *Chironomus riparius* in ionoregulation under ion-poor conditions. The scanning ion-selective electrode technique (SIET) was utilized to characterize the species, direction, and rates of inorganic ion transport by the anal papillae following acute and long-term exposure to ion-poor water (IPW). The major inorganic ions in the hemolymph of larvae treated as above were measured using standard ion-selective microelectrodes. The anal papillae of *C. riparius* are sites of net NaCl uptake and H+ secretion under FW and IPW conditions and are not likely to be a major contributor of K+ exchange. Acute and long-term exposure to IPW increased total net transport of Na+, Cl−, and H+ by the anal papillae, but the mechanisms underlying the increase under the two conditions were different. Acute IPW exposure increased the magnitude of net ion fluxes at sites along the anal papillae, while long-term IPW exposure resulted in increased size of the anal papillae with no change in the magnitude of net ion fluxes. The contribution of the anal papillae to observed alterations of hemolymph ion activities upon exposure to IPW is discussed. Inhibitors of the Na+/H+ exchangers (EIPA) and carbonic anhydrase (methazolamide) provide evidence for Na+/H+ and Cl−/HCO3− exchange mechanisms in the anal papillae. This study demonstrates that *C. riparius* larvae employ two different mechanisms to upregulate the total net transport of ions by the anal papillae, and these mechanisms are at least partially responsible for regulating hemolymph ion activity.

Chironomids; anal papilla; transepithelial ion transport; inorganic ions; homeostasis

**HOMEOSTATIC REGULATION OF the ionic and osmotic composition of the blood is critical for survival. Aquatic animals are common inhabitants of dilute freshwater (FW) environments, which are less concentrated than their blood, and thus, are faced with a number of ionic and osmotic challenges in the regulation of salt and water balance (25, 31, 35, 37, 41). The paucity of ions in the external medium establishes diffusional ion gradients that favor the osmotic influx of water and passive loss of ions. To counteract the water uptake and dilution of the blood, large amounts of dilute hypotonic urine are produced (6, 25, 31). To maintain ion balance, ions are actively absorbed from the external medium to replenish the ions lost through excretion and metabolic processes (25, 31, 44, 54, 66).

FW habitats are variable, and environmental salinity can fluctuate with changes in climate that can lead to dilution through rainfall or concentration through evaporation (43). One of the principal difficulties in assessing the effects of climate change on the environment is the lack of a complete understanding with regard to how aquatic organisms respond to these physiochemical changes. Current knowledge on hypoosmotic acclimation in FW fish and crustaceans suggests that the predominant strategies employed in ion-poor conditions are to 1) increase ion uptake from the external medium and 2) reduce passive ion loss (31, 47, 68). Increased ion uptake is facilitated by increased expression and activity of ion transporters in the osmoregulatory tissues (17, 18, 26, 32, 48, 59, 60, 71). In the euryhaline killifish *Fundulus heteroclitus*, increased Na+/K+-ATPase (NKA) activity and NKAmRNA expression are observed in the gill following FW transfer (48). Similar observations have also been made by McDonald and Rogano (32) who showed that a prolonged 1-wk exposure to ion-poor water (IPW) stimulates increased activity of Na+ and Cl− transporters in the trout *Salmo gairdneri*. In the striped bass (*Morone saxatilis*), there is increased mRNA expression of the V-type H+-ATPase in the gill after FW transfer (59). Like their fish counterparts, euryhaline and FW crabs also upregulate the activity of NKA and the V-type H+-ATPase in dilute environments, and these changes in transporter activity correspond to an increase in the rates of Na+ uptake at the gills (17, 26, 60, 71). Henry et al. (18) have shown that acclimation to a low salinity medium in the euryhaline crab *Carcinus maenas* involves increased activity and mRNA expression levels of NKA in the posterior gill. A reduction in passive ion loss is largely attained through a decrease in paracellular permeability via regulation of junctional proteins (7, 47). Studies on ion regulatory physiology of FW aquatic animals has been, for the most part, restricted to FW vertebrates and crustaceans leaving other larger aquatic invertebrate groups, such as insects, largely unexplored.

Chironomid larvae are ubiquitous benthic invertebrates and are often the most abundant group of insects in FW habitats (41, 42). Adult chironomids are known to breed in swarms near permanent and transient bodies of water, such as lakes, rivers, ponds, and saline rock pools (15, 42). Eggs are laid on the surface of the water or are attached to nearby substrate, and the aquatic larvae develop through several instar stages and a pupal stage before emerging as adults. In these environments, the water level and salinity are known to fluctuate (15). In the semipermanent and temporary ponds of Saskatchewan, larvae of the opportunistic midge *Chironomus riparius* thrive in conditions of varying salinity ranging from 0.062 parts per thousand (ppt) to 1.286 ppt (15). Within these environments, the water chemistry is also highly variable ([Na+], 0.043–9.613 mmol/l; [K+], 0.384–1.241 mmol/l; [Cl−], 0.056–1.241 mmol/l). Despite the dilute conditions, larval *C. riparius* maintain hemolymph ion levels well above that of the external environment ([Na+], 93–137 mmol/l; [K+], 5–9 mmol/l; [Cl−], 42–66 mmol/l) (3). The ion transport mechanisms...
The anal papillae are highly permeable to both water and ions (1, 22, 62, 66). Structurally, the anal papillae are composed of a thin external cuticle lined internally by a single cell layer syncytial epithelium surrounding an inner lumen, which is continuous with the internal environment of the animal (11). Functionally, they actively absorb ions from the external environment (22, 55, 62, 66, 70). Koch (22), Stobart (55), and Wright (70) have demonstrated that the anal papillae of mosquito and chironomid larvae are the predominant sites of NaCl uptake from the external environment; however, they could not conclusively eliminate the possibility of ion exchange through the general body surface or the gut. Morphological studies in chironomid and mosquito larvae have established that the size of the anal papillae correlate with the salinity of the external medium, becoming enlarged in dilute media and decreasing in size in relatively more concentrated conditions (29, 34, 58, 67). Sohal and Copeland (53) showed that rearing the larval mosquito Aedes aegypti in progressively more dilute conditions leads to changes in the ultrastructure of the anal papillae epithelium, in particular increased folding of the apical and basolateral membranes and number of mitochondria. Taken together, these profound morphological and ultrastructural changes have led to the hypothesis that the anal papillae increase ion transport in dilute external conditions; however, the growth of the anal papillae in dilute environments also represents an apparent paradox. Although the increased size of the anal papillae can facilitate increased ion transport by enhancing the surface area available for ion exchange, an enlarged surface area of the papillae may also be liable to increased water uptake and passive ion loss and thus raises questions regarding the modulation of ion transport by the anal papillae in dilute media.

The focus of the present study was to examine the role of the anal papillae of the FW chironomid larvae C. riparius in mediating the ionoregulatory response to ion-poor conditions. To this end, we used the scanning ion-selective electrode technique (SIET) to conclusively identify the major inorganic ions transported by the anal papillae and characterize the magnitude and direction of the net transepithelial ion fluxes at the anal papillae in FW and IPW. We hypothesized that (1) larvae acutely exposed to ion-poor conditions must increase the rate of net ion flux at the anal papillae; and (2) if morphological alterations in the anal papillae occurred in response to ion-poor conditions, they took place to facilitate ion uptake. We demonstrate here that acute and long-term IPW exposure stimulate the total net transport of Na⁺, Cl⁻, and H⁺ by the anal papillae of larval C. riparius and that these changes are at least, in part, responsible for regulating hemolymph ion levels. Our findings are the first to demonstrate that morphological changes of the papillae associated with long-term exposure to IPW are not associated with an increase in the magnitude of net ion fluxes at the papillae but that acute transfer to ion-poor conditions are.

**MATERIALS AND METHODS**

**Insects.** A laboratory colony of C. riparius M. was established at York University with egg ropes obtained from the Aquatic Laboratories Facility in the Department of Biology at McMaster University, Hamilton, ON, Canada. The eggs were hatched in FW (100 mmol/l NaCl); Cl⁻ 920; Ca²⁺ 760; K⁺ 43; pH 7.35 and then transferred into aerated 6-liter aquaria containing a 1-in. depth mixture of fine- and coarse-grade industrial sand (K&E Industrial Sand, Wyoming, ON, Canada) and 3-liter of either FW or IPW (100 mmol/l NaCl); Cl⁻ 20; Ca²⁺ 40; Cl⁻ 2; K⁺ 0.4; pH 6.5. The aquaria were held at room temperature (~21°C), exposed to a 12:12-h, light-dark regimen, and larvae were fed every second day with a dusting of ground TetraFin Goldfish Flake Food (Tetra Holding US, Blacksburg, VA). The water in the aquaria was renewed weekly with appropriate FW or IPW. Levels of Na⁺, K⁺, and Cl⁻ within the rearing media were monitored over a 30-day period to ensure consistent ionic rearing conditions. Experiments were conducted on third or fourth instar larvae that were unfed for 1 day prior to experiments and remained unfed during treatments.

**Acute and rearing exposures to IPW.** Acute exposure consisted of the following. Larvae that were reared from the first instar and allowed to develop for ~30 days in FW were transferred into and held in IPW (experimental) or FW (control) for a period of 48 h. Hemolymph ion activities and total body water content were measured at 5, 10, 24, 29, 34, and 48 h posttransfer. Ion fluxes at the anal papillae were recorded within two distinct time periods after transfer of larvae: 6–11 h posttransfer and 24–48 h posttransfer. The sizes of the anal papillae were measured at 48 h posttransfer to IPW. Rearing exposure consisted of the following: larvae were reared from first instar and allowed to develop for at least 30 days in IPW (experimental) or FW (control). Hemolymph ion activities, total body water content, sizes of anal papillae, and ion fluxes at the anal papillae were measured.

**Construction of ion-selective microelectrodes.** The protocol utilized for the construction of liquid ion-exchange membrane ion-selective microelectrodes (ISMEs) was adapted from Smith et al. (51). Borosilicate glass capillaries (model TW150-4; WPI, Sarasota, FL) were pulled on a P-97 Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA) to a tip diameter of ~5–8 μm. The micropipettes were exposed to vapors of NaNO₃, KNO₃, LiCl, and NaCl in tetrahydrofuran (Fluka, Buchs, Switzerland) at 300°C, allowed to cool, and backfilled with an appropriate electrolyte solution. The tips of the micropipettes were front-filled with the appropriate ionophore cocktail to a column length of ~150–300 μm. The following ionophore cocktails and backfill electrolyte solutions (in parentheses) were used (13): Na⁺ Ionophore II Cocktail A (100 mmol/l NaCl); K⁺ Ionophore I Cocktail B (100 mmol/l KCl); Cl⁻ Ionophore I Cocktail A (1 mol l⁻¹ NaCl); and H⁺ Ionophore I Cocktail B (100 mmol/l NaCl + 100 mmol/l sodium citrate, pH 6.0). To measure hemolymph activities of Na⁺, K⁺, and H⁺, the tips of the ISMEs were dipped in a solution of polynvinyl chloride (Fluka, Buchs, Switzerland) in tetrahydrofuran (Fluka) as described by Rheault and O’Donnell (46). Measurements of hemolymph Cl⁻ activity were carried out using a solid-state silver wire electrode as described by Donini et al. (12). Selectivity of the ionophores for their respective ions has been previously established (13, 51, 52).

**Measurement of hemolymph ion activities.** Larvae were placed on a piece of tissue paper that absorbed any moisture from the exterior surface of the insect. The larvae were transferred into a dish filled with paraffin oil (Sigma, Oakville, ON, Canada). A small cut was made in the cuticle with microscissors releasing a droplet of hemolymph suspended in the paraffin oil. Na⁺, K⁺, Cl⁻, and H⁺ activities were measured from the hemolymph droplet using ISMEs calibrated in the following solutions: Na⁺ (30 mmol/l NaCl/270 mmol/l LiCl and 300 mmol/l NaCl); K⁺ (15 mmol/l KCl/285 mmol/l NaCl and 150 mmol/l KCl/150 mmol/l NaCl); Cl⁻ (30 mmol/l KCl/270 mmol/l KNO₃ and 300 mmol/l KCl); H⁺ (300 mmol/l NaCl containing 1 mmol/l HEPES...
at pH 7.5 and 8.5). ISME slopes (mV) for a 10-fold change in ion concentration were (means ± SE): 60.1 ± 0.16 for Na⁺, 58.3 ± 0.20 for K⁺, 59.4 ± 0.24 for Cl⁻, and 54.0 ± 0.34 for H⁺. The circuit for voltage measurements was completed with a conventional reference electrode constructed from borosilicate glass capillaries (model IB200F-4; WPI) filled with 500 mmol/l KCl. The electrodes were connected through an ML 165 pH Amp to a PowerLab 4/30 (AD Instruments, Colorado Springs, CO) data-acquisition system, and the voltage recordings were analyzed using LabChart 6 Pro software (AD Instruments). Calculations of hemolymph ion activities were made using the following equation as described by Donini et al. (12):

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

where \( a^h \) is the hemolymph ion concentration, \( a^c \) is the ion concentration in the calibration solution, \( \Delta V \) is the difference in voltage between the hemolymph and the calibration solution, and \( S \) is the slope of the electrode measured in response to a 10-fold change in ion activity.

**Total body water content of larvae.** Larvae were placed on a piece of tissue paper that absorbed any moisture from the exterior surface of the insect. Larvae were subsequently weighed to the nearest microgram on a microbalance (model UMXP; Mettler-Toledo International, Mississauga, ON, Canada), dehydrated in a conventional oven at 60°C for several days, and reweighed. The weight differential before and after dehydration served as an approximate measure of the total body water content. Values were expressed as a percentage of the total wet body weight.

**Measurement of ion gradients adjacent the surface of the anal papillae in vitro with the SIET.** The SIET system used in this study is described in detail by Rheault and O’Donnell (45, 46). In brief, ISMEs were connected to a headstage with an Ag/AgCl wire electrode holder (WPI), and the headstage was connected to an ion polarographic amplifier (IPA-2; Applicable Electronics, Forestdale, MA). A reference electrode was positioned in the bulk bathing medium to complete the circuit. Construction of the reference electrode involved heating a borosilicate microcapillary glass tube (model TW150-4; WPI) at one end until a slight 45° bend was created. The glass tubes were filled with 3 mol/l KCl solution containing 3–4% agar and were connected to the headstage through an Ag/AgCl half-cell (WPI). For simultaneous recording of two ion gradients at the same site, two headstages were mounted on an HD-2 dual headstage holder (Applicable Electronics) and both channels of the IPA-2 were employed.

The ISMEs were calibrated in 0.1, 1, and 10 mmol/l solutions of NaCl for Na⁺ and Cl⁻ selective electrodes and KCl for K⁺ electrodes. The H⁺ ISMEs were calibrated in solutions of 1 mmol/l NaCl containing 1 mmol/l HEPES adjusted to pH 7, 8, and 9 with NaOH or HCl. ISME slopes (mV) for a 10-fold change in ion concentration were (means ± SE): 58.5 ± 0.35 for Na⁺, 57.3 ± 0.52 for K⁺, 57.0 ± 0.27 for Cl⁻, and 57.7 ± 0.37 for H⁺. An in vitro preparation of the anal papillae was constructed by first pinching the larvae at the ninth abdominal segment (just anterior to the papillae) with a fine forcep, making a cut with microscissors and then sealing the open end with

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**Fig. 1.** Activities of major inorganic ions, Na⁺ (A), Cl⁻ (B), K⁺ (C), and the pH (D) of the hemolymph of larval Chironomus riparius at specified times after transfer from freshwater (FW) to ion-poor water (IPW). FW-to-FW transfers served as the control. Values are means ± SE of: \( n = 18–29 \) (Na⁺), \( n = 21–28 \) (Cl⁻), \( n = 22–29 \) (K⁺), and \( n = 21–29 \) (pH). *†Significant difference from the 0-h time point (pretransfer larvae) in the IPW and FW transfers, respectively (2-way ANOVA, Bonferroni posttest, \( P < 0.05 \)).
Chironomid larvae have four anal papillae: two dorsal and two ventral (the two dorsal are larger than the two ventral). Initial measurements with SIET showed no significant difference in ion fluxes between dorsal and ventral papillae of the same animal. All subsequent measurements were recorded at the dorsal anal papillae. A typical recording involved positioning the ISME 5–10 μm from the surface of the anal papillae and recording a voltage. The microelectrode was moved to a second position 100–140 μm from the surface where a second voltage was recorded. This sampling procedure employed a wait time of 4 s between movements of the ISME (where no recording took place) and a subsequent recording time of 1 s. The wait time of 4 s was determined by increasing the wait time in increments of 0.5 s until a plateau in the recorded signals was achieved. This guaranteed that the ISMEs recorded at 100% efficiency. For each site along the papilla, the sampling protocol was repeated four times. A voltage gradient was calculated by the Automated Scanning Electrode Technique software (Science Wares, East Falmouth, MA) by taking the difference in voltage between the two sites, and the reported voltage gradient is an average of the four recordings. After complete sampling of the surface of each papilla, control recordings were made to account for the mechanical disturbances in the ion gradients that arise from the movement of the microelectrodes. The ISME was moved 5–7 mm from the anal papilla preparation, and voltage gradients were measured using the same sampling protocols described above. The background control recordings were then subtracted from the signals at the papilla. This SIET sampling protocol was previously established for measuring ion gradients at the anal papillae of mosquitoes (13). The same protocol was used when recording with the simultaneous two headstage system.

Calculation of ion fluxes. Voltage gradients recorded by the ASET software were converted into concentration gradients using the following equation as described by Donini and O’Donnell (13): 

\[ \Delta C = C_0 \times 10^{(\Delta V/D_i)} - C_0 \]

where \( \Delta C \) is the concentration gradient between the two points (measured in mmol·cm\(^{-2}\)·s\(^{-1}\)); \( C_0 \) is the background ion concentration, calculated as the average of the concentration at each point (measured in mmol/l); \( \Delta V \) is the voltage gradient obtained from ASET (in mV); and \( S \) is the slope of the electrode. Using the calculated concentration gradients, a corresponding flux value was then derived using Fick’s law of diffusion as follows: 

\[ J_i = D_i (\Delta C) / \Delta x \]

where \( J_i \) is the net flux of the ion (in pmol·cm\(^{-2}\)·s\(^{-1}\)); \( D_i \) is the diffusion coefficient of the ion (1.55 × 10\(^{-5}\) cm\(^2\)·s\(^{-1}\) for Na\(^+\) and Cl\(^-\); 1.92 × 10\(^{-5}\) cm\(^2\)·s\(^{-1}\) for K\(^+\); and 9.4 × 10\(^{-5}\) cm\(^2\)·s\(^{-1}\) for H\(^+\)); \( \Delta C \) is the concentration gradient (in mmol/cm\(^3\)); and \( \Delta x \) is the distance between the two points measured in centimeters. Proton gradients were measured in solutions containing HEPES buffer and were thus calculated by taking into account the buffering capacity of HEPES as previously described in detail (13, 23, 52).

Calculation of total Na\(^+\), Cl\(^-\), K\(^+\), and H\(^+\) transport by the anal papillae. The dorsal and ventral papillae were isolated from a number of larvae, and pictures were taken with an inverted microscope with camera attachment. Measurements of the length and width of each papilla were carried out using ImageJ Java-based image processing software (National Institutes of Health, Bethesda, MD). The surface area of the papillae was determined by assuming that their surface area can be represented by that of a cylinder with one open end and was calculated using the following equation: 

\[ SA = \pi r^2 + 2 \pi rh \]

where \( r \) is the width of the papilla and \( h \) is the length of the papilla, both measured in centimeters. Total ion transport by the anal papillae of a single larva was estimated by multiplying the average flux (pmol·cm\(^{-2}\)·s\(^{-1}\)) for a specific ion at any point along the surface of the papilla (hereafter referred to as a single-point flux) by the total surface area of the four anal papillae.

Effects of EIPA and methazolamide on ion gradients adjacent the surface of the anal papillae. The effects of EIPA [an Na\(^+\)/H\(^+\) exchanger (NHE) inhibitor; Sigma] on Na\(^+\) and H\(^+\) fluxes at the anal papillae were assessed by simultaneously recording Na\(^+\) and H\(^+\) gradients adjacent the papillae with the SIET. Similarly, the effects of methazolamide (a carbonic anhydrase inhibitor; Sigma) on Cl\(^-\) and H\(^+\) fluxes were assessed by simultaneously recording Cl\(^-\) and H\(^+\) fluxes. The inhibitors were dissolved in DMSO (Sigma) and diluted to the desired concentrations with a solution of 1 mmol/l NaCl + 1 mmol/l HEPES (pH 8), prior to use. Using the in vitro anal papilla preparation, initial measurements in a bath solution of 1 mmol/l NaCl + 1 mmol/l HEPES (pH 8), established the baseline ion gradients at the surface of the anal papilla. The ISMEs were removed from the solution bathing the preparation. Methazolamide or EIPA was added at the desired concentration, and the preparation was incubated with the inhibitor for 15 min. The bathing solution with the inhibitor was replaced several times with fresh bathing solution, and measurements at the same sites along the surface of the anal papillae were recorded. Gradients were converted to fluxes and the resulting fluxes after application of the inhibitor were expressed as a percentage of the baseline fluxes. Controls were treated with the same protocol but received DMSO (without the inhibitor) at a concentration, which equaled the highest DMSO concentration that resulted from the addition of the inhibitor.

Statistics. Data are expressed as means ± SE (n). Comparisons between two groups (e.g., FW-reared vs. IPW-reared) were evaluated with unpaired Student’s t-tests, whereas comparisons between two or more groups were assessed with a one-way ANOVA followed by a Tukey’s comparisons test or the nonparametric Mann-Whitney U-test with a Dunn’s comparisons test where appropriate (GraphPad Instat; GraphPad Software, San Diego, CA). Multiple group comparisons involving two independent factors (e.g., salinity and time) were conducted using a two-way ANOVA followed by a Bonferroni posttest (SPSS, SPSS, Chicago, IL). To assess the effects of the inhibitors on the ion fluxes, the normalized flux values (resultant flux values taken as a percentage of the initial baseline flux) were log transformed to give a normal distribution of the data and then subsequently compared using a one-way ANOVA. Differences with \( P < 0.05 \) were considered statistically significant.

![Fig. 2. Major inorganic ions in the hemolymph of larval C. riparius reared in FW and IPW. Values are means ± SE of: \( n = 38–44 \) (Na\(^+\)), \( n = 39–44 \) (Cl\(^-\)), \( n = 39–44 \) (K\(^+\)), and \( n = 36–41 \) (pH). *Significant difference from FW (Student’s t-test, \( P < 0.05 \)).](http://ajpregu.physiology.org/ by [http://ajpregu.org](http://ajpregu.org))
RESULTS

Hemolymph ion activities. Hemolymph Cl\(^{-}\) and K\(^{+}\) activities and pH ranged from \(~50\) to \(90\) mmol/l; \(~10\) to \(20\) mmol/l, and \(~7.7\) to \(8.0\), respectively. Transferring larvae from FW to IPW resulted in significant time-dependent changes in the activities of Cl\(^{-}\), K\(^{+}\), and H\(^{+}\) (pH) in the hemolymph, which differed from the control FW to FW-transferred larvae (Fig. 1). The Cl\(^{-}\) activity decreased \(~30\%\) between 5 and 10 h post-transfer and subsequently increased \(~50\%\) between 10 and 24 h posttransfer before returning to pretransfer levels. Cl\(^{-}\) activity of control larvae was unaltered. An increase in K\(^{+}\) hemolymph activity was significant at 5 h after transfer and was not apparent in the FW-to-IPW transferred larvae. There was no difference in K\(^{+}\) hemolymph activity between controls and experimental larvae at and after 10 h posttransfer. Hemolymph pH was significantly increased by 24 h posttransfer to IPW and remained elevated at 29 and 48 h posttransfer. Hemolymph pH of control larvae was unaltered. The Na\(^{+}\) activity of the hemolymph was unaltered in larvae transferred to IPW or FW. The K\(^{+}\) activity of larvae reared in IPW was elevated compared with larvae reared in FW (Fig. 2). There was no difference in the hemolymph Na\(^{+}\), Cl\(^{-}\), and H\(^{+}\) (pH) activities between larvae reared in FW or IPW.

In vitro anal papillae ion fluxes. The SIET recordings demonstrated that the anal papillae of larval C. riparius are sites of inorganic ion exchange. Anal papillae from FW-reared larvae showed a net influx (pmol·cm\(^{-2}\)·s\(^{-1}\)) of Na\(^{+}\) (126.9 ± 26.2) and Cl\(^{-}\) (128.6 ± 10.1) from the external bath medium into the anal papillae lumen and an efflux of H\(^{+}\) (65.1 ± 18.7) and K\(^{+}\) (6.4 ± 12.1) from the papillae lumen into the external bath. The fluxes of these ions, measured as voltage gradients, were manifested in a widespread spatial distribution along the surface of the anal papillae with no apparent difference in magnitude (see Fig. 3, A and B; data not shown for Cl\(^{-}\) and

Fig. 3. Representative Scanning Ion-Selective Electrode Technique (SIET) sampling of the Na\(^{+}\) (A) and H\(^{+}\) (B) voltage gradients adjacent the surface of the anal papilla (AP) of a 3rd or 4th-instar C. riparius larva. Arrows represent the magnitude (arrow length) and direction (arrowhead) of Na\(^{+}\) or H\(^{+}\) flux recorded at the surface of the anal papilla. Horizontal scale bar = 90 \(\mu\)m, vertical scale bar = 5 mV. ISME, ion-selective microelectrode. C: average net fluxes of ions across the anal papillae of larval C. riparius reared in FW or IPW and transferred from FW to IPW for 6–11 h (IPW Acute, 6–11 h posttransfer) and 24–48 h (IPW Acute, 24–48 h posttransfer). Positive flux values signify the influx of ions from the external bath into the anal papilla lumen, while negative flux values indicate the efflux of ions from the papilla lumen into the external bath. Values are means ± SE. Na\(^{+}\) \((n = 9–13)\), Cl\(^{-}\) \((n = 8–9)\), K\(^{+}\) \((n = 7–10)\), and H\(^{+}\) \((n = 10–17)\). Letters indicate significant differences between the groups for each ion measured, a, similar to FW; b, similar to IPW 6–11 h; c, similar to IPW 24–48 h (1-way ANOVA, Tukey’s or Dunn’s comparison, \(P < 0.05\)).
K+). The direction of specific ion fluxes did not change when larvae were transferred to, or reared in, IPW. Acute exposure (FW-to-IPW transfer) of C. riparius larvae to IPW resulted in increased magnitude of the Na+ and Cl− influx at the anal papillae compared with FW-reared larvae (Fig. 3C). The Na+ and Cl− influx doubled by 6–11 and 24–48 h posttransfer into IPW, respectively. H+ efflux decreased to one-third by 6–11 h posttransplant and increased 2.6-fold compared with FW-reared larvae by 24–48 h posttransfer to IPW. The K+ fluxes were unaltered by acute exposure to IPW. The fluxes of Na+ and Cl− at the anal papillae of control larvae (FW-to-FW transfer) were similar when sampled prior to transfer, 6–11 h posttransplant, and 24–48 h posttransfer (data not shown). The Na+ and Cl− fluxes of control larvae ranged from ~100 to 140 pmol·cm−2·s−1 and ~120 to 170 pmol·cm−2·s−1, respectively. The magnitude of Na+, Cl−, and K+ fluxes from anal papillae of larvae reared in IPW were similar to the FW-reared larvae. In contrast, the H+ efflux doubled at the anal papillae of larvae reared in IPW.

**Total body water content.** The water content of C. riparius larvae ranged from ~82.5 to 84.5% of the wet body weight. The water content remained unaltered during acute exposure to IPW, and at 48 h posttransfer, this was different than control larvae, which had elevated water content (Fig. 4A). In contrast, larvae reared in IPW had a lower body water content than those reared in FW (Fig. 4B).

**Surface area of the anal papillae.** When larvae are reared in FW, the surface area of the dorsal and ventral papillae are 0.11 mm² and 0.09 mm², respectively. Rearing larvae in IPW results in a ~1.8-fold increase in the surface area of the anal papillae with respect to FW-reared larvae (Fig. 5). Both the dorsal and ventral papillae surface area increases by ~0.09 mm². The surface area of the anal papillae of larvae acutely exposed to IPW (48-h exposure) is similar to that of FW-reared larvae.

**Total ion transport by the anal papillae.** Total ion transport attributed solely to the combined action of the four anal papillae of larval C. riparius was calculated by multiplying the average single-point ion fluxes for a specific ion by the total surface area of the two ventral and two dorsal papillae. For a single larva reared in FW, the anal papillae are accountable for the total transport (uptake) of Na+ and Cl− at rates (pmol/s cm²) of 0.51 ± 0.11 and 0.52 ± 0.04, respectively, and the total transport (secretion) of K+ and H+ at rates of 0.03 ± 0.19 and 0.26 ± 0.08, respectively (Fig. 6). When larval C. riparius were acutely transferred from FW to IPW, the total transport rates of Na+ and Cl− by the anal papillae increased ~2.4-fold but, at different times after transfer to IPW (Fig. 6). Na+ uptake increased by 6–11 h posttransfer, whereas Cl− uptake increased after 24–48 h posttransfer. There was an initial decrease in H+ secretion at the anal papillae to 2/5 of initial rates by 6–11 h posttransfer followed by an increase of ~2.6-fold over initial rates by 24–48 h posttransfer. K+ secretion was unaltered by transfer into IPW. Rearing larvae in IPW resulted in increased uptake of Na+ (~2-fold) and Cl− (~2.5-fold) with increased secretion of H+ (~3.7-fold) compared with FW-reared larvae. K+ secretion by anal papillae of IPW-reared larvae was similar to that of FW-reared larvae.

**Effects of EIPA and methazolamide on ion fluxes at the anal papillae.** Incubation of anal papillae in methazolamide resulted in dose-dependent decreases in Cl− influx and H+ efflux, which became statistically significant at a dose of 100 µmol/1−1 (Fig. 7). The mean Cl− influx became an efflux when anal papillae were incubated in the higher doses of methazolamide. Incubation of anal papillae in EIPA (1 and 10 µmol/l) resulted in a dose-dependent decrease of Na+ influx (Fig. 8). This was accompanied by a decrease in H+ efflux when anal papillae were incubated in 10 µmol/l EIPA.

**DISCUSSION**

The anal papillae of C. riparius are sites of net NaCl absorption and H+ secretion. Exposure to IPW increases the total net NaCl absorption and H+ secretion by the anal papillae (see Fig. 6), but there are two different mechanisms with which this occurs, dependent on whether the exposure is acute (e.g., 48 h) or long-term (e.g., 30 days). Larvae reared in FW or IPW (long-term exposure) have similar magnitudes of ion fluxes at sites along the surface of the anal papillae (see Fig. 3C FW- vs.
IPW-reared larvae; and 2) the SIET measures net flux, a product of both influx and efflux, regardless of passive or active transport processes. In the absence of a paracellular pathway to permit passive ion loss, similar observed magnitudes of net fluxes at sites along the anal papillae between IPW- and FW-reared larvae (Fig. 3C) would suggest that the efflux and influx components remain unaltered between the two conditions.

The anal papillae of both mosquitoes and midges are permeable to water (1, 22, 66), and an increase in papillae surface area is likely to result in an increase of water uptake at the papillae. This is counterproductive to the increase in ion uptake and could lead to dilution of the hemolymph. Results indicate that this is not the case since total body water content significantly decreased, albeit by only 1% (Fig. 4B) and hemolymph ion activities were unaltered, with the exception of an increase in K⁺ levels, which cannot be attributed to the anal papillae (Figs. 2, 3, and 6) when larvae were reared in IPW. At least two explanations for these results are possible. The first is that permeability of the anal papillae to water may be reduced when larvae are reared in IPW. The alternative is that other osmoregulatory tissues remove the excess water load. The syncytial epithelium of the anal papillae suggests that water uptake occurs through a transcellular route (11). Water uptake at the anal papillae may be mediated by putative aquaporin-like water channels. Aquaporin-like proteins have been characterized in the midge, Polypedilum vanderplanki (20); however, these water channels serve in the process of anhydrobiosis. Regulation of aquaporin-like channels in the Malpighian tubules of Rhodnius prolixus has been demonstrated (30), and aquaporin-like proteins are involved in osmoregulation in an aphid (49). The combined action of the Malpighian tubules and rectum of FW mosquito larvae produce a dilute urine, which serves to eliminate excess water, while conserving ions (6). In the FW mosquito A. aegypti the underlying transport mechanisms of the Malpighian tubules are altered with changes in the ionic composition of the rearing medium (14), and an increase in external salinity leads to elevated hemolymph serotonin titres,

![Graph](image-url)
Fig. 7. A: simultaneous probing of H\(^+\) and Cl\(^-\) voltage gradients using a double ISME system in conjunction with the SIET. Solid and dotted arrows represent the voltage gradients of H\(^+\) and Cl\(^-\) at the same sites adjacent to the surface of the anal papilla, respectively. Arrowheads signify the direction of ion movement, while the length of the arrow denotes the magnitude of the ion flux recorded at the surface of the anal papilla. SME, selective microelectrode. Horizontal scale bar = 9 mV, vertical scale bar = 100 \(\mu\)m. Shown are the effects of 10, 100, 1,000 \(\mu\)M methazolamide on the fluxes of Cl\(^-\) (B) and H\(^+\)/Cl\(^-/HCO_3^-\) (C) at the anal papillae of larval C. riparius reared in FW. Positive flux values indicate the influx of ions from the external bath medium into the papilla lumen, while negative flux values denote the efflux of ions from the lumen into the external bath. Flux values are expressed as %baseline fluxes. Values are means ± SE of \(n = 8–14\). *Significant difference from the control (DMSO) (1-way ANOVA, Dunn’s comparison, \(P < 0.05\)).

which stimulates fluid secretion by the Malpighian tubules (8). With the exception of a study on the transport and sequestering of cadmium, nothing is known about Malpighian tubule physiology in C. riparius (24). Future studies on the permeability of anal papillae to water and Malpighian tubule function in chironomid larvae reared in FW and IPW are warranted.

Transferring larvae from FW to IPW (acute exposure) results in increased total net NaCl uptake without alterations in the size of the anal papillae (see Figs. 5 and 6). The increased NaCl uptake is caused by measureable, significant increases in net Na\(^+\) and Cl\(^-\) fluxes at sites along the surface of the anal papillae, despite the use of a common bath solution with equal NaCl concentration (Fig. 3). These results suggest that acute exposure to IPW leads to changes in the underlying ion transport mechanisms in the anal papillae epithelium. Whether these changes are a result of the recruitment of more transporters or in a change in the identity of the transporters remains to be studied. In the mosquito A. aegypti, transferring larvae from FW to 30% sea water and vice versa causes significant changes in the transport kinetics of both Na\(^+\) and Cl\(^-\) influx at the anal papillae, which favor increased NaCl uptake in dilute conditions (12). Similarly, in another FW mosquito, Culex quinquefasciatus, an increase in the maximal rate of total body Na\(^+\) uptake occurs in dilute conditions (39). Assuming that the anal papillae are the major sites of Na\(^+\) uptake, then the changes observed in C. quinquefasciatus may be representative of the recruitment of more transporters in the epithelium of the anal papillae. To date, only a few studies have examined the identity of underlying ion transport mechanisms at the anal papillae, and all of these have been conducted on mosquito larvae. The V-type H\(^+\)-ATPase and P-type Na\(^+\)/K\(^+\)-ATPase have been localized to the apical and basal membranes of the anal papillae epithelium in A. aegypti (38). Functional studies on whole larvae of A. aegypti suggest a role for the V-type H\(^+\)-ATPase in mediating Na\(^+\) influx, and the authors have proposed an apical V-type H\(^+\)-ATPase/Na\(^+\) channel moiety (38, 40). In addition, a novel mechanism for Cl\(^-\) uptake has been implicated, whereby Cl\(^-\) uptake is dependent on an electrodiffusive gradient, which is at least in part established by the outward pumping of H\(^+\) by the apical V-type H\(^+\)-ATPase (40). The P-type Na\(^+\)/K\(^+\)-ATPase is thought to mediate Na\(^+\) secretion across the basal membrane in the FW fish gill and anal papillae of A. aegypti (19, 38). Our results with C. riparius contrast with the model proposed for A. aegypti. Preincubation of the in vitro anal papillae preparation with the NHE inhibitor EIPA resulted in a decrease in net Na\(^+\) influx and net H\(^+\) efflux, when measured simultaneously, at sites along the anal papillae (Fig. 8). In addition, preincubation with the carbonic anhydrase inhibitor methazolamide decreased the net Cl\(^-\) influx and H\(^+\) efflux, when measured simultaneously, at sites along the anal papillae (Fig. 7). These results directly implicate NHE and indirectly implicate Cl\(^-\)/HCO\(_3^-\) exchange as routes for NaCl uptake at the anal papillae of C. riparius. In this case, we propose that carbonic anhydrase would supply H\(^+\) and HCO\(_3^-\)
to the NHE and Cl-/HCO₃⁻ exchange through the hydration of CO₂. Inhibiting carboxic anhydrase would reduce the availability of both H⁺ and HCO₃⁻, which are available for exchange with Na⁺ at the NHE, and Cl⁻ at a putative Cl⁻/HCO₃⁻ exchanger, which would explain the resulting inhibition of net Cl⁻ influx and H⁺ efflux. Mechanisms of NaCl uptake that are coupled to H⁺ and HCO₃⁻ have been implicated in FW fish, crustaceans, and in frog skin (see Refs. 5, 19, 21, and 71). In these instances Na⁺ uptake is thought to be indirectly coupled to H⁺ extrusion via V-type H⁺-ATPase, as suggested for the anal papillae of A. aegypti, since the low Na⁺ levels in a FW environment are not likely to provide a sufficient gradient for Na⁺ uptake through direct Na⁺/H⁺ exchange via NHEs. In fish, the involvement of the V-type H⁺-ATPase in both Na⁺ and Cl⁻ uptake is well established; however, it is debatable whether this aids Cl⁻ uptake across the apical or basal membranes (28, 63). In the gills of crustaceans an apically located V-type H⁺-ATPase can drive the uptake of Na⁺ through Na⁺-selective channels; however, Na⁺ entry can also be facilitated by electroneutral Na⁺/H⁺ exchange through NHE in marine and brackish water environments (61, 71). The larvae of C. riparius are predominantly FW inhabitants; however, they have been found inhabiting brackish water (4, 10). In these environments, it would be beneficial for Na⁺ uptake to occur via an electroneutral NHE. Our studies with EIPA do not preclude the presence of additional Na⁺ uptake mechanisms in the anal papillae of C. riparius. Indeed, the presence of an additional mechanism for Na⁺ uptake, which is not directly dependent on H⁺ exchange is supported by the negative correlation between changes in net Na⁺ influx and net H⁺ efflux at the anal papillae taken from larvae transferred from FW to IPW (see Fig. 3, IPW Acute). The pharmacological studies that employed EIPA were performed on the anal papillae of larvae reared in FW. It is possible that acute transfer into IPW preferentially activates a different transport mechanism for Na⁺ uptake than the Na⁺/H⁺ exchange mechanism. Evidence for plasticity in the Na⁺ transport mechanisms exists for the anal papillae of A. aegypti, where there is a change in transporter affinity when larvae are acutely transferred between FW and 30% sea water (12). Moreover, an increased transport affinity and maximal transport capacity are common features observed in many FW fish and crustaceans (studied to date) that have been acclimated to IPW (31, 32, 50). Our future studies will further elucidate the transport mechanisms of the anal papillae of C. riparius in both FW and IPW exposure.

Examination of hemolymph ion activities with the net ion fluxes and net ion transport at the anal papillae suggests that the anal papillae are partially responsible for maintaining hemolymph ion levels in C. riparius. The larvae reared in IPW maintain hemolymph NaCl and pH at the same levels as larvae reared in FW despite a substantial reduction in external ion levels (Fig. 2). The increased net NaCl uptake at the anal papillae observed in IPW-reared larvae would also serve to maintain hemolymph ion levels in the face of passive ion loss at other sites. In addition, the observed ~1% reduction in total body water content of IPW-reared larvae would also serve to maintain hemolymph ion levels in the face of passive ion loss (Fig. 4B). As explained previously, changes in body water content are not likely to be associated with processes at the anal papillae and are more likely to reflect on the activity of other osmoregulatory organs, such as the Malpighian tubules and rectum. The increase in net H⁺ secretion at the anal papillae of IPW-reared larvae is not associated with a corresponding increase in net H⁺ efflux at sites along the papillae (see Figs. 3 and 6); thus we propose that the Na⁺/H⁺ exchange mechanism for Na⁺ uptake is utilized under long-term exposure to IPW, consistent with the notion that increased surface area of the papillae is the underlying mechanism employed under long-term exposure to IPW. Hemolymph pH is not compromised, because the increased net H⁺ secretion is at least partially balanced by an increase in HCO₃⁻ secretion as a result of increased Cl⁻ uptake. In addition, other osmoregulatory organs, the Malpighian tubules, and rectum may be major sites of acid/base homeostasis as has been shown in mosquito larvae (9, 56, 57).

If the anal papillae are ion regulatory organs then one might expect changes in ion fluxes at the papillae to occur in response to changes in hemolymph ion levels. Transfer of larvae from FW to IPW resulted in a transient decrease in Cl⁻ hemolymph activity at 10 h posttransfer, followed by a transient increase at 24 h posttransfer (Fig. 1B). These changes, as well as those observed for K⁺ and pH, were not a result of dilution of the hemolymph from increased osmotic water uptake in the larvae, since there were no significant changes in total body water content (Fig. 4A). We did not measure increases in net Cl⁻.
influx at sites along the papillae until between 24–48 h posttransfer, which, as a result, lead to increased Cl\(^{-}\) uptake by the papillae (Figs. 3 and 6). This may suggest that the failure to upregulate Cl\(^{-}\) influx at the anal papillae over the initial ~11 h posttransfer into IPW contributed to the observed decrease in hemolymph Cl\(^{-}\) levels. Furthermore, the upregulation of Cl\(^{-}\) influx at the papillae seen 24–48 h posttransfer may have contributed to the observed increase in hemolymph Cl\(^{-}\) levels at this time. The data also indicate that upregulation of Cl\(^{-}\) influx occurred sometime between 11 and 24 h post-transfer and is a relatively slow process that requires a significant drop in Cl\(^{-}\) hemolymph levels. Under the same conditions, hemolymph Na\(^{+}\) levels were unaltered throughout the 48 h exposure to IPW (Fig. 1A). We recorded increased net Na\(^{+}\) influx at sites along the papillae that lead to increased Na\(^{+}\) uptake at the papillae 6–11 h posttransfer to IPW (Figs. 3 and 6). Thus, upregulation of Na\(^{+}\) influx occurred prior to 6 h posttransfer and appears to be a rapid process, which may not require significant decreases in hemolymph Na\(^{+}\) levels. This upregulated Na\(^{+}\) influx involves an aforementioned H\(^{+}\)-independent mechanism. Furthermore, it is only transient since the increased net Na\(^{+}\) influx, and, consequently, uptake at the anal papillae, returns to FW values at 24–48 h posttransfer. This suggests that upon abrupt transfer to IPW, increased Na\(^{+}\) uptake by the anal papillae can contribute to stabilizing Na\(^{+}\) hemolymph levels but, sometime between 11 and 24 h post-transfer is no longer necessary. Although we have no data for C. riparius, this may signify a tightening of other ion-exchange epithelia in the larvae, reducing passive Na\(^{+}\) losses. Reduced permeability in animals exposed to dilute conditions is well documented. Goldfish exposed to IPW undergo changes in tight junction composition, which is thought to reduce solute movement through the paracellular pathway in the gill (7). Larvae of C. quiniquefasciatus transferred from tap water to a low NaCl medium showed an apparent decrease in unidirectional Na\(^{+}\) efflux rates without alterations in Cl\(^{-}\) efflux rates (39). Stingrays inhabiting IPW also exhibit reduced NaCl efflux rates in these conditions compared with when they are exposed to ion-rich hard water (69). The rapid upregulation of Na\(^{+}\) uptake has been observed in the crayfish Astacus pallipes and the mosquito A. aegypti (50, 55). Interestingly, slower upregulation of Na\(^{+}\) uptake has been reported for other chironomids, C. tentans and C. dorsalis, where a drop in hemolymph Na\(^{+}\) concentration of ~36–40% is required. (70). Our results suggest that in this respect, C. riparius larvae are similar to mosquito larvae. Although some of our data suggest that the anal papillae are important in regulating hemolymph NaCl levels, this does not preclude the actions of other ion regulatory organs in this process.

The present study suggests that regulation of hemolymph K\(^{+}\) levels does not involve the anal papillae for two reasons: 1) negligible efflux of K\(^{+}\) was recorded at sites along the anal papillae, and 2) these fluxes of K\(^{+}\) remained unaltered during acute and long-term exposure to IPW. In Rhodnius prolixus, hemolymph K\(^{+}\) is regulated by the Malphigian tubules that secrete less K\(^{+}\) in response to a drop in K\(^{+}\) concentration within the hemolymph (27). The Malphigian tubules of larval mosquitoes alter the ratio of Na\(^{+}/K^{+}\) transported in response to external salinity (14). In addition, regulation of hemolymph K\(^{+}\) levels may involve exchange of K\(^{+}\) between the hemolymph and the K\(^{+}\)-rich intracellular compartment (44).

Transfer of larvae to IPW eventually leads to an increase in hemolymph pH by 24 h posttransfer. Changes in blood/hemolymph pH are common in aquatic animals, including mosquito larvae, subjected to changes in external ion levels (12, 36, 64). The documented inverse relationship between pH and external ion concentration is thought to result from the regulation of ion levels since ion transport is often associated with H\(^{+}\) and HCO\(_3\)\(^{-}\) exchange (65). The net H\(^{+}\) fluxes recorded at sites along the papillae and subsequent net H\(^{+}\) secretion at the papillae can only partially explain the recorded hemolymph pH levels. The increased H\(^{+}\) secretion at 24–48 h posttransfer may contribute to the elevated hemolymph pH at the same time; however, an earlier reduction in H\(^{+}\) secretion at the papillae did not correspond to changes in hemolymph pH. In this respect, our data support the notion that anal papillae are not actively involved in acid/base regulation (9).

**Perspectives and Significance**

Plasticity in ion transport mechanisms of the anal papillae are a feature of the larval chironomid’s (C. riparius) response to ion-poor conditions. Two distinct mechanisms are employed to increase net NaCl uptake, and under FW conditions, NaCl uptake is associated with H\(^{+}\) and HCO\(_3\)\(^{-}\) exchange. The function of the anal papillae, nature of the ion transport mechanisms, and plasticity that they exhibit under varying external ion levels is reminiscent of the FW fish and crustacean gills. This study provides the basis for future research that will characterize ion transport mechanisms in FW and IPW and explore the presence, localization, and role of carbonic anhydrase in the anal papillae.

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**DISCLOSURES**

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