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RESEARCH ARTICLE

Tissue-specific ionomotive enzyme activity and K^+ reabsorption reveal the rectum as an important ionoregulatory organ in larval *Chironomus riparius* exposed to varying salinity

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SUMMARY

A role for the rectum in the ionoregulatory homeostasis of larval *Chironomus riparius* was revealed by rearing animals in different saline environments and examining: (1) the spatial distribution and activity of keystone ionomotive enzymes Na^+-K^+-ATPase (NKA) and V-type H^+-ATPase (VA) in the alimentary canal, and (2) rectal K^+ transport with the scanning ion-selective electrode technique (SIET). NKA and VA activity were measured in four distinct regions of the alimentary canal as follows: the combined foregut and anterior midgut, the posterior midgut, the Malpighian tubules and the hindgut. Both enzymes exhibited 10–20 times greater activity in the hindgut relative to all other areas. When larvae were reared in either ion-poor water (IPW) or freshwater (FW), no significant difference in hindgut enzyme activity was observed. However, in larvae reared in brackish water (BW), NKA and VA activity in the hindgut significantly decreased. Immunolocalization of NKA and VA in the hindgut revealed that the bulk of protein was located in the rectum. Therefore, K^+ transport across the rectum was examined using SIET. Measurement of K^+ flux along the rectum revealed a net K^+ reabsorption that was reduced fourfold in BW-reared larvae *versus* larvae reared in FW or IPW. Inhibition of NKA with ouabain, VA with bafilomycin and K^+ channels with charybdotoxin diminished rectal K^+ reabsorption in FW- and IPW-reared larvae, but not BW-reared larvae. Data suggest that the rectum of *C. riparius* plays an important role in allowing these larvae to cope with dilute as well as salinated environmental conditions.

Key words: chironomid, transepithelial ion transport, Na^+-K^+-ATPase, V-type H^+-ATPase, salinity.

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INTRODUCTION

Homeostasis of hemolymph ionic and osmotic composition in aquatic insect larvae is achieved largely by regulation of material entry through the alimentary canal and elimination through the excretory system (Bradley, 1994; Dow, 1986; Phillips, 1981). The alimentary canal is structurally divided into the foregut, midgut, Malpighian tubules and hindgut. The foregut, encompassed by the esophagus, receives the food bolus and moves it to the midgut (Clements, 1992; Dow, 1986). The midgut, which is further divided into the gastric caeca, anterior midgut and posterior midgut, is important for maintaining ion, fluid and acid-base balance in aquatic insect larvae as it is the main uptake site of minerals, water and nutrients, which are ingested as or with food (Dow, 1986; Clark et al., 1999; Clark et al., 2005; Khodabandeh, 2006; Boudko, 2012; Okech et al., 2008a; Okech et al., 2008b; Linser et al., 2009; Jagadeshwaran et al., 2010). The hindgut, which includes an ileum and a rectum, and the Malpighian tubules together constitute the excretory system. The Malpighian tubules secrete a primary urine by actively transporting Na^+, K^+ and Cl^- from the hemolymph into the tubule lumen, which in turn generates a transepithelial osmotic gradient that facilitates fluid movement (Phillips, 1981; Clark and Bradley, 1997; Donini et al., 2006). The ionic composition of fluid secreted by the tubules is unlike that of the hemolymph and would upset hemolymph homeostasis were it not for a selective reabsorption of ions, water and nutrients in the rectum (Phillips et al., 1986; Bradley and Philips, 1977; Sutcliffe, 1961; Meredith and Phillips, 1973; Strange et al., 1984; Leader and Green, 1978; Bradley, 1994). In a freshwater (FW) environment, rectal reabsorption of ions (lumen to hemolymph) results in the production of a dilute urine, permitting larvae to conserve ions while eliminating excess water. Larvae of mosquitoes and chironomids also use externally protruding anal papillae as additional sites of ion uptake in habitats such as FW (Wigglesworth, 1933; Koch, 1983; Donini and O'Donnell, 2005; Nguyen and Donini, 2010).

Na^+-K^+-ATPase (NKA) and V-type H^+ ATPase (VA) are well-known membrane energizers implicated in driving a wide variety of epithelial transport processes in insects and other animals (Emery et al., 1998; Harvey et al., 1998). Depending on physiological requirements and/or cell type, the activity of NKA and/or VA leads to the secretion or absorption of fluid, mineral ions and amino acids (Emery et al., 1998; Harvey et al., 1998). VA functions as an electrogenic pump, transporting protons from the cytoplasm to extracellular or exterior fluid and generating cell-negative membrane voltages. The membrane voltage can then serve to drive ion transport through ion-specific channels, and the electrochemical proton potential can serve to drive secondary active transport processes such as cation/H^+ exchange or anion/H^+ cotransport (Harvey et al., 1998; Harvey, 2009). NKA is responsible for the maintenance of two electrochemical gradients across the plasma membrane by its electrogenic activity of exporting $3Na^+$ from the cell and importing $2K^+$ to the cell. This can power Na^+/H^+ exchange, Na^+ (or K^+):amino acid symport or give rise to K^+ and Na^+ diffusion

via channels (Emery et al., 1998; Boudko, 2012). The presence and localization of both ATPases has been established in the gut epithelia, Malpighian tubules and anal papillae of aquatic mosquito larvae, where they are proposed to play an integral role in the transepithelial movement of solutes (Patrick et al., 2006; Okech et al., 2008a; Smith et al., 2008; Xiang et al., 2012). However, to our knowledge, no studies have examined the tissue-specific activity of these keystone ionomotive enzymes in aquatic insect larvae in response to changes in environmental conditions. Nevertheless, it has been shown that there are comparatively higher levels of NKA activity in the hindgut *versus* foregut/midgut of damselfly and dragonfly larvae (Khodabandeh, 2006), which supports the idea that tissue-specific alterations in ionomotive enzyme activity may play an important role in how aquatic insect larvae respond to changes in environmental ion levels.

Larvae of the chironomid *Chironomus riparius* are ubiquitous benthic inhabitants of FW environments such as lakes, rivers and ponds (Pinder, 1986; Pinder, 1995). However, they are also known to thrive in bodies of water with increased salinity such as brackish water (BW) ditches, coastal rock pools and intertidal zones, as well as polluted FW habitats exposed to salinated industrial effluent (Driver, 1977; Colbo, 1996; Parma and Krebs, 1977; Bervoets et al., 1994; Bervoets et al., 1996). In these environments, parameters such as osmolarity and ionic milieu can vary greatly, and as a result, ion regulation is an important process for survival. Very little is known about the ionoregulatory responses of larval *C. riparius* to sustained changes in ambient salinity. Recently it has been shown that despite a substantial reduction in external ion levels, larvae of *C. riparius* reared in ion-poor water (IPW) maintain hemolymph NaCl and pH at the same levels as larvae reared in FW (Nguyen and Donini, 2010). This was partially attributed to the anal papillae, which are sites of net NaCl absorption and H^+ secretion under ion-poor conditions (Nguyen and Donini, 2010). In a more recent study that examined the effects of increased external salinity on ionoregulatory homeostasis in larval *C. riparius*, it was found that acute exposure to BW (20% seawater) increased hemolymph Na^+ and Cl^- and decreased hemolymph pH (Jonusaite et al., 2011). A decrease in whole-body NKA and VA activities in BW *versus* FW animals was also observed, and because the bulk of ionomotive enzyme activity was found to be in the alimentary canal of *C. riparius* (i.e. gut and Malpighian tubules), it was hypothesized that modulation of ionomotive enzyme activity in one or more regions of the alimentary canal may be important in order for larval *C. riparius* to acclimate to changes in environmental salinity (Jonusaite et al., 2011). With this background information in mind, the present study was aimed at investigating whether there was a role for specific segments of the gut as well as the Malpighian tubules in ion regulation of *C. riparius* larvae upon exposure to different ionic conditions. In order to do this, a novel approach was taken that focused on whether NKA and VA activity exhibited spatial variation along the alimentary canal of larval *C. riparius* and how enzyme activity might change when larvae were reared in environments that varied in ionic composition (i.e. IPW, FW and BW). To put biochemical observations into a functional context, the scanning ion-selective electrode technique (SIET), combined with the application of ion transport inhibitors, was used to characterize transepithelial ion flux.

MATERIALS AND METHODS

Experimental animals

Animals from a laboratory colony of *Chironomus riparius* (Meigen), maintained in the Department of Biology at York University, were used. Eggs were hatched in 6 liter aquaria containing a 2.54 cm deep mixture of fine and coarse grade industrial sand (K&E Industrial Sand, Wyoming, ON, Canada) and 3 liters of aerated dechlorinated municipal tap water (approximate composition of FW in $\mu mol\,l^{-1}$: $[Na^+]$ 590; $[Cl^-]$ 920; $[Ca^{2+}]$ 760; $[K^+]$ 43; pH 7.35). The aquaria were held at room temperature (RT, ~21°C), exposed to a 12 h:12 h light:dark regime and larvae were fed every second day with a dusting of ground TetraFin Goldfish Flake Food (Tetra Holding US, Blacksburg, VA, USA). The water in the aquaria was replaced weekly.

Rearing of experimental animals in IPW and BW

In aquaria identical to those outlined above, larvae were reared from first instar and allowed to develop to the fourth instar (~30 days) either in IPW (composition in $\mu mol\,l^{-1}$: $[Na^+]$ 20; $[Cl^-]$ 40; $[Ca^{2+}]$ 2; $[K^+]$ 0.4; pH 6.5) or BW ($7\,g\,l^{-1}$ Instant Ocean SeaSalt; United Pet Group, Blacksburg, VA, USA). FW control animals were reared in FW conditions (as outlined above) for the duration of the experimental period. Experiments were conducted on fourth instar larvae that had not been fed for 24 h before collection.

Measurement of Na^+ and K^+ in hemolymph and BW rearing medium

Larvae were placed on tissue paper, which absorbed moisture from the surface of the insect, and then transferred to a Petri dish filled with paraffin oil (Sigma-Aldrich, Oakville, Canada). Samples of hemolymph were collected by making a small tear in the cuticle with fine forceps, causing the hemolymph to pool into a droplet. Levels of K^+ and Na^+ in collected droplets as well as BW rearing medium samples were measured as ion activities using ion-selective microelectrodes (ISMEs). The K^+ and Na^+ ISMEs were constructed as previously described (Jonusaite et al., 2011). In brief, microelectrodes were backfilled with appropriate electrolyte solutions and front-loaded with the appropriate ionophore cocktail. The following ionophore cocktails (Fluka, Buchs, Switzerland) and back-fill solutions (in parentheses) were used: Na^+ Ionophore II Cocktail A ($100\,mmol\,l^{-1}$ NaCl) and K^+ Ionophore I Cocktail B ($100\,mmol\,l^{-1}$ KCl). The K^+ and Na^+ ISMEs were calibrated in 5 and $50\,mmol\,l^{-1}$ solutions of KCl and 30 and $300\,mmol\,l^{-1}$ solutions of NaCl, respectively. ISME slopes (mV) for a 10-fold change in ion concentration were (means ± s.e.m.): 54.8±1.56 (N=4) for K^+ and 55.9±0.53 (N=3) for Na^+. The circuit for voltage measurements was completed with a conventional reference electrode filled with $500\,mmol\,l^{-1}$ KCl. The electrodes were connected through an ML 165 pH Amp to a PowerLab 4/30 (ADInstruments, Colorado Springs, CO, USA) data acquisition system and the voltage recordings were analyzed using LabChart 6 Pro software (ADInstruments). Calculations of hemolymph and BW rearing medium ion levels were made using the following equation as described by Donini et al. (Donini et al., 2007):

$$a_h = a_c \times 10^{\Delta V/S}, \quad (1)$$

where a_h is the hemolymph or medium ion activity, a_c is the ion activity in one of the calibration solutions, ΔV is the difference in voltage between the hemolymph or medium and the calibration solution, and S is the slope of the electrode measured in response to a 10-fold change in ion activity.

Measurement of NKA and VA activity

The whole gut (complete with Malpighian tubules) or isolated regions of the gut were collected and quick-frozen in liquid nitrogen. Samples were stored at –80°C until further analysis. Four regions of the gut were isolated for examination as follows: (1) the combined foregut and anterior midgut, which included gastric cecae, (2) the posterior midgut, (3) the Malpighian tubules and (4) the hindgut.

NKA and VA activities were determined according to methods previously outlined for *C. riparius* tissues (Jonusaite et al., 2011).

Immunohistochemical localization of NKA and VA

Immunohistochemical localization of NKA was achieved using a mouse monoclonal antibody raised against the α-subunit of avian NKA (α5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA). This antibody has been used successfully to localize NKA in other dipteran species such as mosquitoes (Patrick et al., 2006; Okech et al., 2008a; Smith et al., 2008). To localize VA, a rabbit polyclonal serum antibody raised against the B subunit of the VA of *Culex quinquefasciatus* was employed (a kind gift from S. Gill, UC Riverside) (Filippova et al., 1998). The entire gut of fourth instar larva was isolated in ice-cold physiological saline (composition in mmol l^{-1}: 5 KCl, 74 NaCl, 1 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 8.6 HEPES, 20 glucose, 10 glutamine, pH 7.0) [saline adapted from Leonard et al. (Leonard et al., 2009)] and fixed in 2% paraformaldehyde for 2 h at RT. Fixed tissue was then washed three times (3×30 min) in phosphate-buffered saline (PBS; pH 7.4) at RT and blocked for 1 h at RT with 10% antibody dilution buffer (ADB; 10% goat serum, 3% BSA and 0.05% Triton X-100 in PBS). The tissue was then thoroughly rinsed in PBS and incubated for 48 h at 4°C with anti-NKA α-subunit antibody at a dilution of 1:10 with ADB and anti-VA B-subunit antibody at a dilution of 1:1000 with ADB. As negative controls, tissues were incubated for 48 h at 4°C with ADB alone. Following incubation, tissues were washed for 2 h at RT in PBS and probed for 18 h at 4°C with either fluorescein-isothiocyanate-labelled goat or Cy2-conjugated sheep anti-mouse secondary antibodies (1:500 in ADB; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). To remove unbound secondary antibody, tissues were washed twice in PBS (2×1 h) at RT and incubated with either tetramethylrhodamineisothiocyanate-labelled or Alexa Fluor 594-conjugated goat anti-rabbit secondary antibodies (1:500 in ADB; Jackson ImmunoResearch Laboratories) as described above. Tissues were rinsed again in PBS and mounted in ProLong Gold Antifade reagent (Invitrogen Canada, Burlington, ON, Canada). Images were captured using an Olympus IX71 inverted microscope (Olympus Canada, Richmond Hill, ON, Canada) equipped with an X-CITE 120XL fluorescent Illuminator (X-CITE, Mississauga, ON, Canada). Single confocal plane images were gathered using an Olympus BX-51 laser-scanning confocal microscope. All images were assembled using Adobe Photoshop CS2 software (Adobe Systems Canada, Toronto, ON, Canada).

SIET measurement of K^+ concentration gradient adjacent to rectum surface

The SIET methodology used in this study is described in detail elsewhere (Rheault and O'Donnell, 2001; Rheault and O'Donnell, 2004; Nguyen and Donini, 2010). In brief, a K^+ ISME was connected to the headstage with an Ag/AgCl wire electrode holder (World Precision Instruments) and the headstage was connected to an ion polarographic amplifier (IPA-2, Applicable Electronics, Forestdale, MA, USA). A reference electrode was a 3% agar in 3 mol l^{-1} KCl bridge connected to the headstage through an Ag/AgCl half-cell (WPI) and positioned in the bulk bathing medium to complete the circuit. The K^+ ISME was constructed as described above and calibrated in 1 and 10 mmol l^{-1} solutions of KCl. The ISME slope (mV) for a 10-fold change in ion concentration was 57.3±0.31 (mean ± s.e.m., N=34).

An *in vitro* preparation of the rectum was constructed by first isolating the whole alimentary canal of the fourth instar larva in the physiological saline defined above (see Immunohistochemical localization of NKA and VA, above). The entire gut was then transferred to a 35 mm Petri dish containing 3 ml of fresh saline solution. All subsequent SIET measurements were performed on the posterior region of the hindgut that constitutes the rectal epithelium. For each single-point measurement of K^+ concentration gradient, the ISME was positioned 5–10 μm from the surface of the rectum and a voltage was recorded. The microelectrode was then moved a further 100 μm away, perpendicular to the tissue 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. For each site along the rectum the sampling protocol was repeated four times. The ISME was positioned and the recorded voltage gradient was calculated using Automated Scanning Electrode Technique (ASET) software (version 2.0, Science Wares, East Falmouth, MA, USA). Control measurements to account for the mechanical disturbances in the ion gradients that arise from the movement of the microelectrode were taken 3–4 mm away from the surface of the rectum. This sampling protocol was previously established and utilized for measuring ion gradients at the anal papillae of mosquitoes and midges (see Donini and O'Donnell, 2005; Del Duca et al., 2011; Nguyen and Donini, 2010). K^+ measurements were chosen in part because when altered by the presence of NKA and VA inhibitors, they can provide some insight into the movement of major ions (e.g. Na^+ and Cl^-) across the rectum. Direct Na^+ and Cl^- measurements with Na^+ and Cl^- ISMEs require substantial modification of the saline such that the background NaCl in the bath is reduced by approximately fivefold. These conditions would not allow the rectum to behave in a normal manner as they would be substantially different from hemolymph.

Calculation of K^+ flux

Voltage gradients recorded by the ASET software were converted into concentration gradients using the following equation as described by Donini and O'Donnell (Donini and O'Donnell, 2005):

$$\Delta C = C_B \times 10^{\Delta V/S} - C_B, \quad (2)$$

where ΔC is the concentration gradient between the two points measured in μmol l^{-1} cm^{-3}; C_B is the background ion concentration, calculated as the average of the concentration at each point measured in μmol l^{-1}; ΔV is the voltage gradient obtained from ASET in μV; 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:

$$J_I = D_I(\Delta C) / \Delta x, \quad (3)$$

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 (1.92×10^{-5} cm^2 s^{-1} for K^+); ΔC is the concentration gradient in pmol cm^{-3}; and Δx is the distance between the two points measured in cm.

Effect of ouabain, charybdotoxin or bafilomycin on SIET measurement of K^+ concentration gradient adjacent to rectum surface

The effects of 1 mmol l^{-1} ouabain (an NKA inhibitor; Sigma-Aldrich), 23 nmol l^{-1} charybdotoxin (ChTX; a K^+ channel blocker; Abcam, Cambridge, MA, USA) and 1 μmol l^{-1} bafilomycin (a VA inhibitor; LC Laboratories, Woburn, MA, USA) on K^+ flux at the rectum were assessed by recording K^+ concentration gradients adjacent the rectum with the SIET. The choice of ouabain and bafilomycin concentrations was based on our previous study on enzyme activity in *C. riparius* larva (see Jonusaite et al., 2011).

Table 1. K^+ and Na^+ levels (mmol l^{-1}) in hemolymph of *Chironomus riparius* larvae reared in ion-poor water (IPW), freshwater (FW) and brackish water (BW)

	IPW	FW	BW
K^+	7.9±0.6	8.7±1.2	8.5±0.4
Na^+	73.9±2.6	74.4±1.0	80.9±2.7

Hemolymph ion levels are expressed as means ± s.e.m. (*N*=11–18).

The dose of ChTX was selected such that it equalled the highest concentration used in other insect and mammalian tissue studies (Miller et al., 1985; MacKinnon et al., 1988; Grinstein and Smith, 1990; Bleich et al., 1996). Ouabain and bafilomycin were dissolved in DMSO (Sigma-Aldrich) and, prior to use, diluted to a desired concentration in saline (composition defined above in Immunohistochemical localization of NKA and VA). ChTX was dissolved and diluted in saline. Using the *in vitro* rectum preparation, initial measurements in a bath saline solution established the baseline K^+ gradient at the surface of the rectum. The ISME was removed from the saline bathing the preparation. Ouabain, ChTX or bafilomycin was added at the desired concentration and the preparation was incubated with the inhibitor for 5 min. The bathing solution with the inhibitor was replaced several times with fresh saline and the K^+ gradient at the same sites along the surface of the rectum was recorded. Controls were treated with the same protocol but received saline or DMSO (without the inhibitor). DMSO was added at a concentration of 0.1%, which equalled the DMSO concentration that resulted from the addition of the inhibitor.

Statistics

Data are expressed as means ± s.e.m. (*N*). Comparisons between treatment groups or tissues were assessed with a one-way ANOVA followed by a Tukey's or Dunn's comparison test. To examine the effects of inhibitors on the K^+ flux, data were subjected to Student's *t*-test. Statistical significance was allotted to differences with $P<0.05$. All statistical analyses were conducted using SigmaStat 3.5 software (Systat Software, San Jose, CA, USA).

RESULTS

Effect of rearing conditions on hemolymph K^+ and Na^+

Rearing the larvae in FW, IPW or BW had no effect on the hemolymph K^+ and Na^+ levels despite the changes in K^+ and Na^+ levels in the rearing media (Table 1). The levels of K^+ and Na^+ in the hemolymph of IPW-, FW- and BW-reared larvae ranged from 7.9 to 8.7 mmol l^{-1} and from 73.9 to 80.9 mmol l^{-1}, respectively (Table 1). BW rearing medium contained 7.3 mmol l^{-1} K^+ and 59.4 mmol l^{-1} Na^+.

NKA and VA activity profiles

In the alimentary canal of FW-reared *C. riparius* (Fig. 1A), NKA and VA activities of the combined foregut and anterior midgut (FAMG), the posterior midgut (PMG) and the Malpighian tubules (MT) were found to be quantitatively similar, ranging from 1.08 to 1.17 μmol ADP mg^{-1} protein h^{-1} and from 1.47 to 1.8 μmol ADP mg^{-1} protein h^{-1} for NKA and VA, respectively (Fig. 1B,C). In contrast, the activity of NKA and VA was ~10–20-fold higher in the hindgut at 22.76 μmol ADP mg^{-1} protein h^{-1} for NKA and 16 μmol ADP mg^{-1} protein h^{-1} for VA (Fig. 1B,C).

Effect of rearing conditions on NKA and VA activity in the alimentary canal

There was no difference in whole-gut NKA and VA activities between larvae reared in FW and IPW, but the activity of both enzymes was reduced in the whole gut of BW-reared larvae (Fig. 2A, Fig. 3A). At the level of the individual segments of the alimentary canal, the activities of both enzymes were found to be

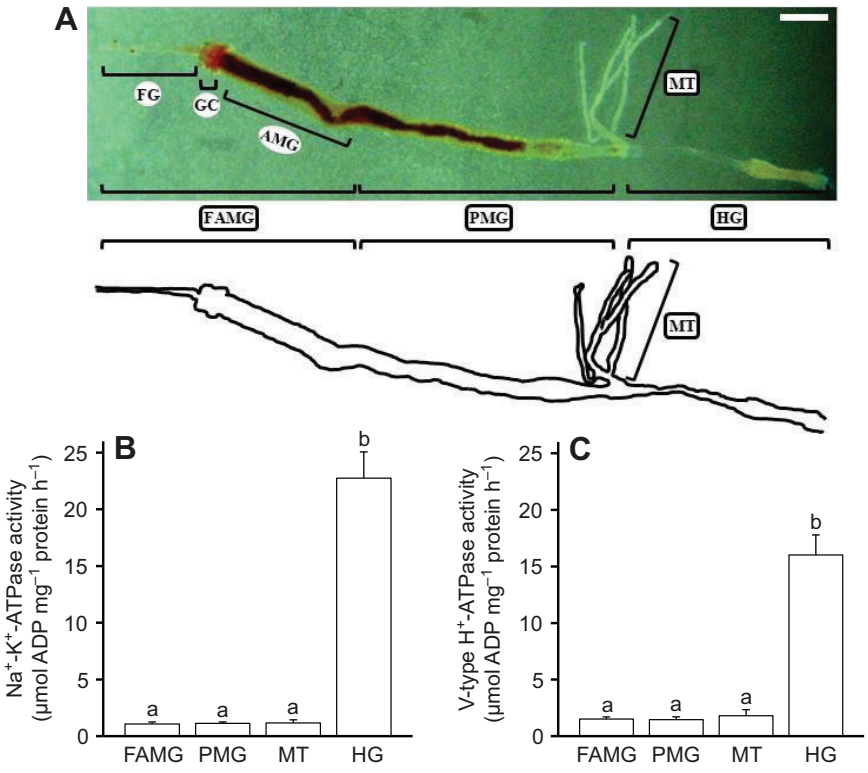

Fig. 1. The (A) alimentary canal and spatial distribution of (B) Na^+-K^+-ATPase (NKA) and (C) V-type H^+-ATPase (VA) in discrete alimentary canal regions of freshwater-reared *Chironomus riparius* larva. Brackets indicate regions of the gut used for NKA and VA activity assay: FAMG, foregut (FG), gastric caeca (GC) and anterior midgut (AMG); PMG, posterior midgut; MT, Malpighian tubules; HG, hindgut. All data are expressed as means ± s.e.m. (*N*=6). Letters denote statistically significant differences between the segments (one-way ANOVA, Tukey's multiple comparison, $P<0.05$). Scale bar, 1 mm.

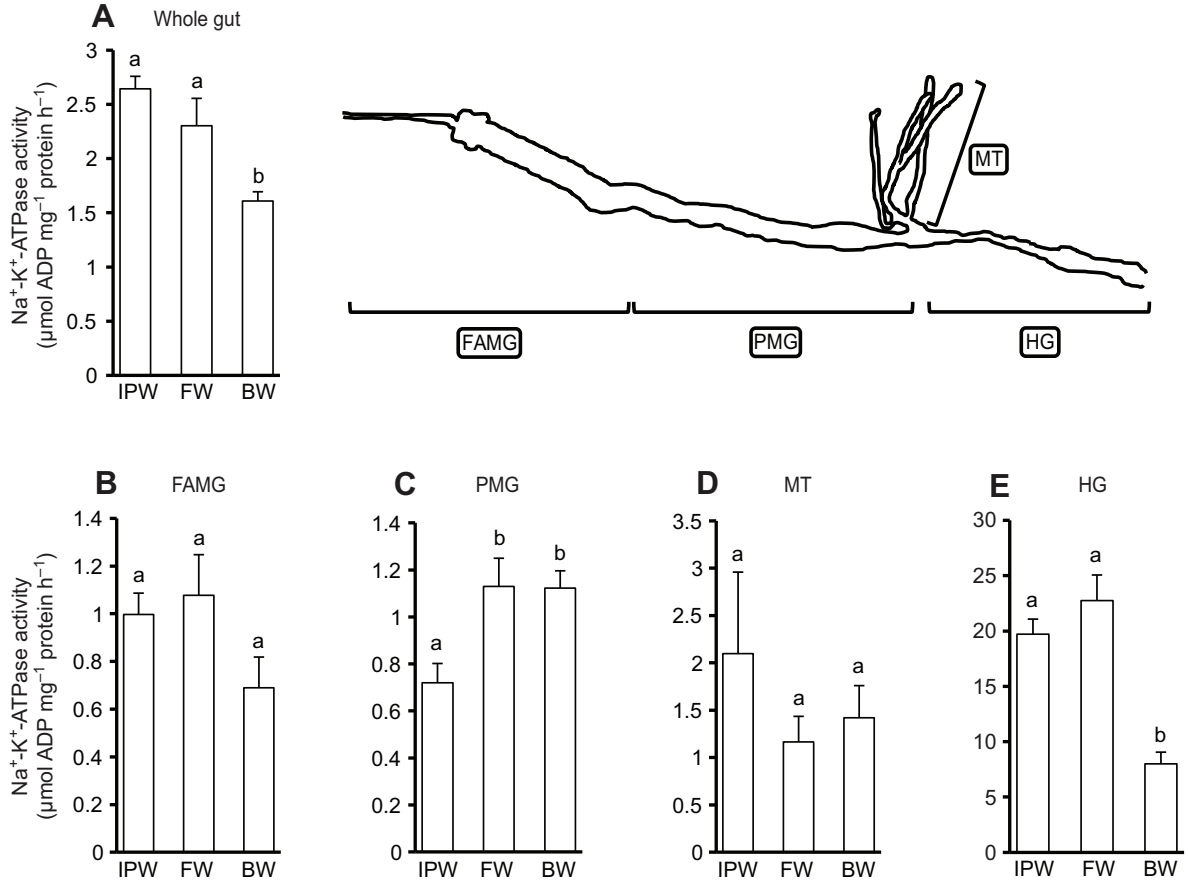

Fig. 2. The effect of varying the ionic strength of rearing conditions on Na^+-K^+-ATPase (NKA) activity in the (A) entire (intact) alimentary canal, (B) foregut and anterior midgut (with gastric caeca; FAMG), (C) posterior midgut (PMG), (D) Malpighian tubules (MT) and (E) hindgut (HG) of *Chironomus riparius* larvae. Larvae were reared in either ion-poor water (IPW), freshwater (FW) or brackish water (BW; 20% seawater). All data are expressed as means ± s.e.m. (N=6). Letters denote statistically significant differences between rearing groups (one-way ANOVA, Tukey's multiple comparison, P<0.05).

reduced by at least half in the hindgut of BW-reared larvae (Fig. 2E, Fig. 3E). The rearing treatments had no effect on the NKA and VA activities in the FAMG or the MT (Fig. 2B,D, Fig. 3B,D). In the PMG, the activities of both enzymes were lower in larvae reared in IPW when compared with their FW and BW counterparts (Fig. 2C, Fig. 3C).

Given that BW rearing reduced whole-gut and hindgut NKA and VA activity, and because a reduction in whole-gut enzyme activity most likely reflects a reduction in hindgut NKA and VA activity (which was found to be 10–20-fold higher than any other region of the alimentary canal), the hindgut became the focus of further experiments.

NKA and VA immunolocalization in the hindgut

Immunohistochemical localization of NKA and VA in the hindgut of *C. riparius* revealed the presence of both enzymes in the rectum (Fig. 4A,C). In contrast, both ATPases were absent in the ileum (Fig. 4A,C). In an optical section through the whole-mount rectum, rectal epithelium cells showed NKA localized to the basolateral regions of the plasma membrane (Fig. 4D). Immunostaining for VA was detected in subapical and cytoplasmic regions of the rectal epithelium (Fig. 4E). No co-localization of NKA and VA was found (Fig. 4F) and no signal was observed in control whole mounts that had been probed with secondary antibody only (Fig. 4G).

Effects of ouabain, ChTX and bafilomycin on K^+ efflux at the rectum

SIET measurements adjacent the hemolymph-side surface of the rectum detected K^+ efflux (from rectal lumen to bath). K^+ efflux did not vary to any great extent spatially along the length of the rectum (see Fig. 5A). Rearing the larvae in FW, IPW or BW had no effect on the direction of K^+ fluxes (efflux); however, BW rearing caused a substantial reduction (approximately fourfold) in the magnitude of K^+ efflux relative to FW and IPW rearing (Fig. 5B).

K^+ efflux across the rectum of *C. riparius* larvae reared in BW was unaltered following the addition of ouabain, ChTX or bafilomycin to solutions bathing the tissue (Fig. 6A–C). In contrast, all of the inhibitors reduced the K^+ efflux measured from the rectum of IPW- and FW-reared larvae (Fig. 6A–C). Ouabain application resulted in a ~3.6-fold decrease in K^+ efflux at the rectum of larvae reared in FW or IPW. ChTX and bafilomycin decreased K^+ efflux at the rectum of FW- and IPW-reared larvae ~2.7 and ~3.4 times, respectively (Fig. 6B,C). No change in K^+ efflux was found at the control tissues incubated with DMSO or saline only (Fig. 6D,E).

DISCUSSION

Overview

This study demonstrates spatial variation in the activity of NKA and VA along the alimentary canal of aquatic *C. riparius* larvae and that: (1) observed differences in tissue-specific enzyme activity

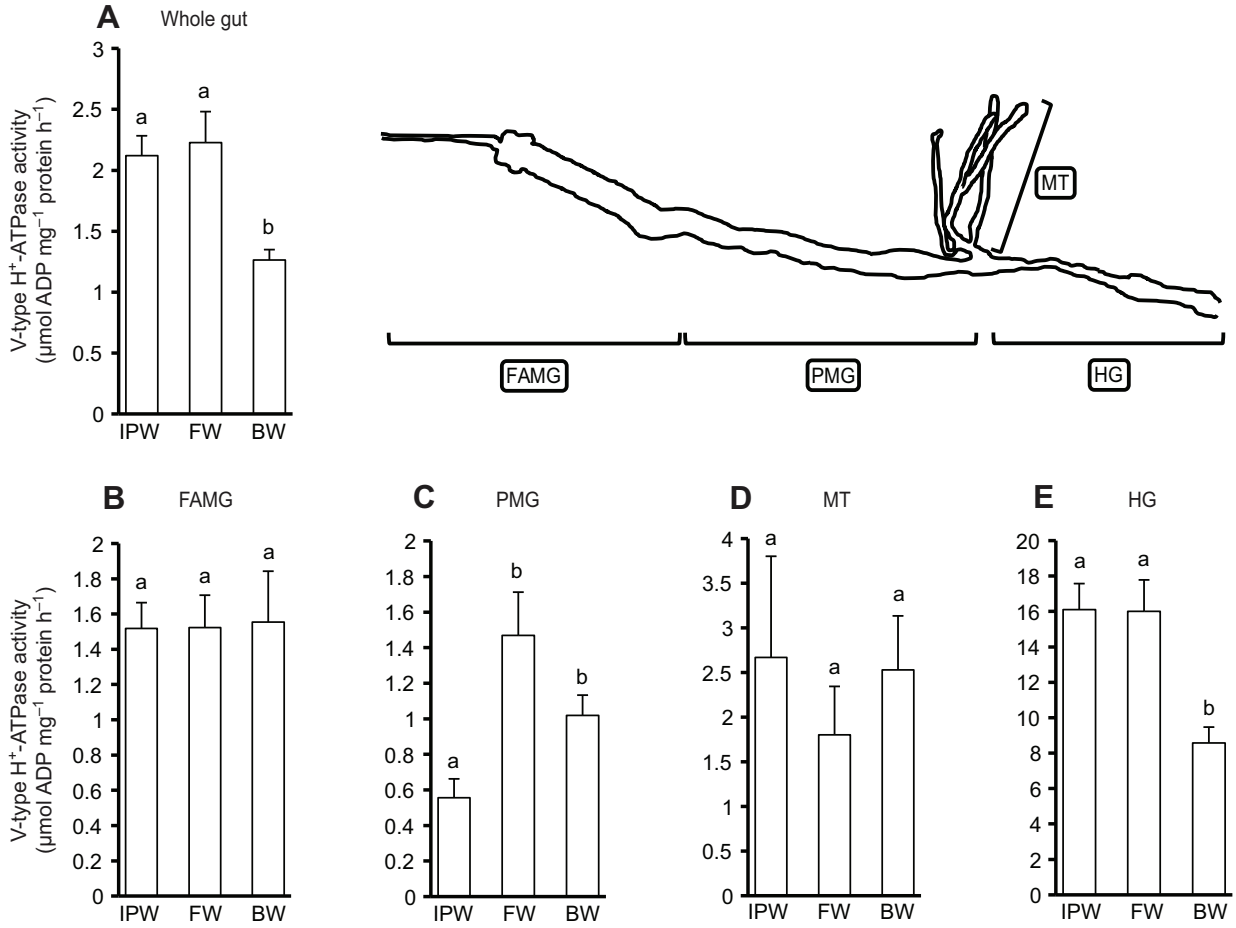

Fig. 3. The effect of varying the ionic strength of rearing conditions on V-type H^+-ATPase (VA) activity in the (A) entire (intact) alimentary canal, (B) foregut and anterior midgut (with gastric caeca; FAMG), (C) posterior midgut (PMG), (D) Malpighian tubules (MT) and (E) hindgut (HG) of *Chironomus riparius* larvae. Larvae were reared in either ion-poor water (IPW), freshwater (FW) or brackish water (BW; 20% seawater). All data are expressed as means ± s.e.m. (*N*=6). Letters denote statistically significant differences between rearing groups (one-way ANOVA, Tukey's multiple comparison, $P<0.05$).

and (2) environmentally induced changes in the NKA and VA activity of particular regions of the alimentary canal both point to the hindgut as an important site for iono/osmoregulation in *C. riparius* reared in water of differing ionic content. Immunolocalization of NKA and VA suggests that within the hindgut area, it is the rectum that appears to possess the bulk of ionomotive enzyme protein. Furthermore, *in situ* inhibition of NKA, VA and K^+ channels in the rectum reduces ion (K^+) reabsorption (efflux, rectal lumen to hemolymph). By providing direct insight into K^+ movement across the rectum of *C. riparius* and indirect insight into the movement of major ionic species such as Na^+ and Cl^-, these data collectively indicate overall ion reabsorption across the rectum of *C. riparius*. But inhibition of K^+ efflux can only be observed in tissues isolated from animals that are reared in hyposmotic surroundings, where ion retention is necessary in order to maintain ionoregulatory homeostasis. In contrast, animals reared in BW conditions exhibit the same pattern of spatial variation in NKA and VA activity as seen in FW- and IPW-reared *C. riparius*, but reduced NKA and VA activity in the hindgut compared with the aforementioned animals. The significance of this latter result is that these animals exhibit greatly reduced rectal K^+ efflux, which is suggestive of attenuated ion reabsorption. From a physiological perspective, this would be an appropriate strategy in saline conditions. In addition, the presence of NKA and VA inhibitors (ouabain and bafilomycin, respectively) did not further reduce K^+ efflux across the rectum of BW-reared *C. riparius*. This introduces the idea that, relative to other areas of the alimentary canal, elevated ionomotive enzyme activity in the rectum of BW-reared animals is no longer required for ion reabsorption/retention strategies such as those adopted by FW- or IPW-reared animals, but could nonetheless be involved in other important physiological processes (e.g. acid/base balance, ammonia secretion, etc.).

Spatial variation in NKA and VA activity along the alimentary canal

In the alimentary canal of FW-reared *C. riparius*, NKA as well as VA activity in the FAMG, PMG and MT were quite similar (Fig. 1B,C). However, the hindgut was found to possess ionomotive enzyme activity levels that were ~10–20 times higher than those found in other regions (Fig. 1B,C). To the best of our knowledge, no other study has reported on spatial differences in VA activity in the alimentary canal of insects. However, high levels of NKA activity in the hindgut of *C. riparius*, relative to the other gut regions, are consistent with previous studies on terrestrial insects (Peacock, 1976; Peacock, 1977; Peacock, 1981a; Peacock, 1981b; Tolman and Steele, 1976). Furthermore, one study has reported NKA activity in the gut of aquatic insect larvae (see Khodabandeh, 2006), and in this regard, a basic separation of the gut into the foregut/midgut and

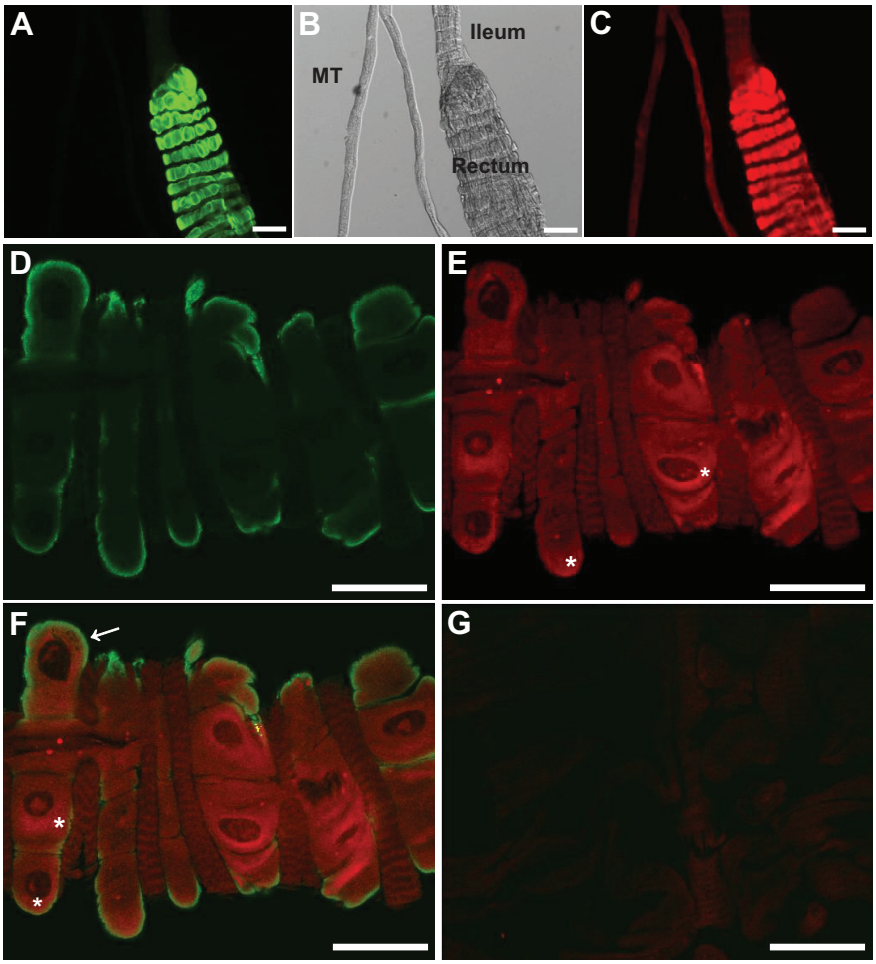

Fig. 4. Immunolocalization of Na^+-K^+-ATPase (NKA, green) and V-type H^+-ATPase (VA, red) in the hindgut (HG) of fourth instar *Chironomus riparius* larva reared in freshwater (FW). The HG expressed high levels of NKA (A) and VA (C) in the rectum and showed little to no expression of either ATPase in the ileum (A,C). (B) Brightfield image of A and C. NKA was localized to the basolateral membrane of rectal epithelium (D,F; white arrows) whereas VA exhibited subapical and cytoplasmic staining (E,F; asterisks). A merged image of NKA and VA immunoreactivity can be seen in F. Control rectal tissue processed identically to experimental tissues but probed with secondary antibody only is shown in G. Scale bars, (A–C) 100 µm, (D–G) 50 µm. MT, Malpighian tubules.

the hindgut also revealed higher levels of NKA activity in the hindgut of FW damselfly and dragonfly larvae (Khodabandeh, 2006). Therefore, the results of the present study are also consistent with these observations.

In the hindgut of *C. riparius*, NKA and VA immunoreactivity (staining) were found to be restricted to the rectal segment (Fig. 4A,C), allowing us to conclude that NKA and VA activity in the hindgut of *C. riparius* reflects ionomotive enzyme activity in the rectum. Our immunohistochemical observation of NKA expression on the basolateral membrane of the rectum of *C. riparius* (Fig. 4D) is similar to observations made in mosquito and other FW insect larvae, which also exhibit basolateral NKA in the hindgut (Patrick et al., 2006; Smith et al., 2008; Khodabandeh, 2006). However, subapical and cytoplasmic immunostaining of VA in the rectal epithelial cells (Fig. 4E) may be indicative of V_1 subunits (for which the antibody was used) that have dissociated from their membrane-bound V_0 anchors (Sumner et al., 1995).

Differences in NKA and VA activity in different rearing environments

Epithelia of the gut may contribute to the regulation of hemolymph ionic composition either through modulated absorption of ions from water ingested along with food, or through secretion of ions from the hemolymph into the gut lumen for subsequent elimination. For example, ion transport mechanisms of the gut epithelia of larval *Drosophila* are reconfigured during dietary salt stress so that there are reductions in K^+ and Na^+ absorption and increased K^+ and Na^+ secretion (Naikkhwah and O'Donnell, 2012). In the present study, we showed that NKA and VA activity in the FAMG of *C. riparius* larvae acclimated to varying salinity remain largely unaltered (Fig. 2B, Fig. 3B). These findings seem to suggest that alteration in external salt content did not trigger changes in the active transport machinery along the midgut epithelia of *C. riparius*, although this does not preclude changes in secondary ion transport processes or ultrastructural alterations of the epithelium, which may lead to alterations in ion transport function. For example, varying salinity may alter passive ion movement across the midgut because of the modulation of paracellular permeability, which is controlled by septate junctions (see Lane and Skaer, 1980). In this regard, the effects of environmental salinity on the permeability of intestinal epithelia in aquatic vertebrates such as fishes are well documented (see Marshall and Grosell, 2005). In addition, increased permeability of the anterior intestinal epithelium following FW to BW acclimation (without alteration in active transcellular transport processes) has also been suggested to occur in amphibians (Chasiotis and Kelly, 2009).

The physiological consequences of decreased NKA and VA activity in the PMG of larval *C. riparius* in response to IPW rearing are unclear (see Fig. 2C, Fig. 3C). In mosquitoes, the PMG is implicated in Na^+ absorption through VA-driven cation/amino acid symport across the apical membrane and subsequent NKA-driven Na^+ transport across the basal membrane into the hemolymph (Patrick et al., 2006; Okech et al., 2008b; Boudko et al., 2005; Rheault et al., 2007). If similar transport processes were present in the PMG of *C. riparius*, then the observed enzymatic activity decrease in IPW rearing conditions would not be consistent with conserving ions or nutrient uptake in dilute conditions.

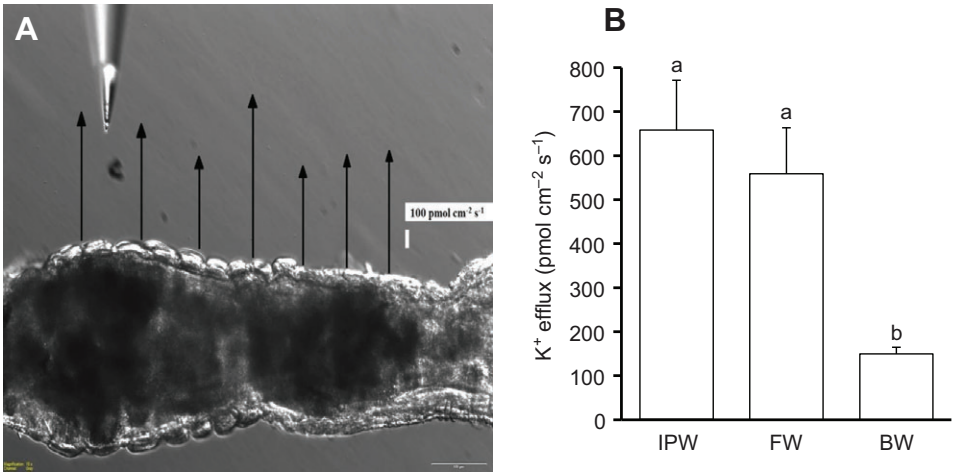

Fig. 5. (A) Representative scanning ion-selective electrode technique (SIET) measurements of the K^+ voltage gradients along the surface of the rectum. K^+ voltage gradients were measured using fourth instar *Chironomus riparius* larva reared in freshwater (FW). Arrows and arrow length represent the direction and magnitude of recorded K^+ fluxes, respectively, that were sampled at the base of the arrow. The scale for the magnitude of K^+ flux is denoted by the length of the thick bar below the 100 pm cm^{-2} s^{-1} label. Horizontal scale bar, 100 µm. (B) The effect of varying the ionic strength of rearing conditions on the average of single-point K^+ fluxes across the rectum of larval *C. riparius* reared in ion-poor water (IPW), FW or brackish water (BW; 20% seawater). Values signify the efflux of K^+ from the rectum lumen into the external bath saline. All data are expressed as means ± s.e.m. (*N*=27–29). Letters denote statistically significant differences between K^+ flux in different rearing conditions (one-way ANOVA, Dunn's multiple comparison, $P<0.05$).

NKA and VA activities in the MT of *C. riparius* larvae were consistent across the three salinity rearing conditions tested (Fig. 2D, Fig. 3D). Because the activity of these pumps is thought to regulate the rate of MT secretion, the results suggest that rates of secretion are unaltered by the rearing conditions. Indeed, this is the case in the mosquito *Aedes aegypti*, where fluid secretion rates are similar between FW- and BW-reared larvae (Donini et al., 2006). This does not exclude the MT as important ionoregulatory organs involved in acclimation to different salinities because the tubules of *A. aegypti* larvae reared in BW secrete more Na^+ at the expense of K^+ to help counteract the elevated Na^+ levels in the hemolymph relative to their FW-reared counterparts (Donini et al., 2006). In contrast to the observations in *C. riparius* and *A. aegypti*, a salt-stress-induced alteration in VA activity has been shown in larval *Drosophila* (Naikkhwah and O'Donnell, 2011). Specifically, rearing *Drosophila* on a KCl-rich diet results in increased VA activity in the MT, which increases the capacity of tubules to eliminate K^+ (Naikkhwah and O'Donnell, 2011).

NKA and VA activity were greatly reduced in the hindgut of BW-reared larvae relative to corresponding activity in FW- and IPW-reared animals (Fig. 2E, Fig. 3E). As discussed above, the hindgut region has previously been shown to possess high NKA activity in both aquatic insect larvae and terrestrial insects (Khodabandeh, 2006; Peacock, 1976; Peacock, 1977; Peacock, 1981a; Peacock, 1981b; Tolman, 1976). However, we are unaware of any report on changes in hindgut ionomotive enzyme activity either in response to environmental change or alterations in systemic salt and water balance. Considering the high NKA and VA activity in the hindgut and that enzyme activity was reduced by ~50% when larvae were reared in BW, it is likely that the observed decrease in NKA and VA activities found in whole guts of BW-reared larvae represent changes occurring in the hindgut. When taken together with immunohistochemical observations of the hindgut, where the rectum appears to be the principal site of enzyme immunoreactivity, these data suggest that the rectum plays an important role in the ability of larval *C. riparius* to cope with alterations in environmental salinity. Therefore, to address the physiological role of the rectum of larval *C. riparius* with respect to salt and water balance, *C. riparius* larvae were reared in IPW, FW or BW and K^+ fluxes were measured with SIET. In addition to providing information on transepithelial K^+ movement, alterations in K^+ flux rates in the presence of NKA and VA inhibitors can also serve as a proxy for major ion movement (for details, see Materials and methods, SIET measurement of K^+ concentration gradient adjacent to rectum surface) across the rectal epithelium.

Rearing of *C. riparius* larvae in IPW, FW or BW and SIET measurement of K^+ concentration gradient adjacent to the rectum surface

Consistent with the notion that the rectum of FW insects selectively reabsorbs ions and metabolites to produce a dilute urine, we measured K^+ efflux (reabsorption) along the entire length of the rectum (Fig. 5A). Although we did not directly measure Na^+ or Cl^- fluxes, the measurements of K^+ flux could also be indicative of the general movement of NaCl across the rectum. The BW condition imposes a significant challenge to both K^+ and Na^+ regulation in the larvae. The level of K^+ in BW is ~167 times greater than its levels in FW whereas Na^+ levels increase ~100 times in BW compared with FW. Therefore, there is a greater change in external K^+ levels from FW to BW conditions relative to the change in the levels of Na^+ and as such, a greater insult to the hemolymph K^+ levels. Our observation of a fourfold reduction in K^+ efflux at the rectum of BW-reared larvae with respect to larvae reared in FW or IPW (Fig. 5B) suggests an important role for this tissue in the regulation of K^+ homeostasis in *C. riparius*. Hemolymph K^+ levels in FW-reared larval *C. riparius* are ~8.7 mmol l^{-1} (Table 1), and based on the assumption that the MT lose at least some K^+ during the production of primary urine, under IPW and FW conditions, the larvae would require a mechanism to reabsorb K^+. Our data suggests that the rectum at least partially fulfills this role. Interestingly, the anal papillae of *C. riparius*, which is an important site of salt uptake in FW and IPW, does not take up K^+ (Nguyen and Donini, 2010) and therefore reabsorption of K^+ at the rectum would be of particular importance. Under conditions of BW

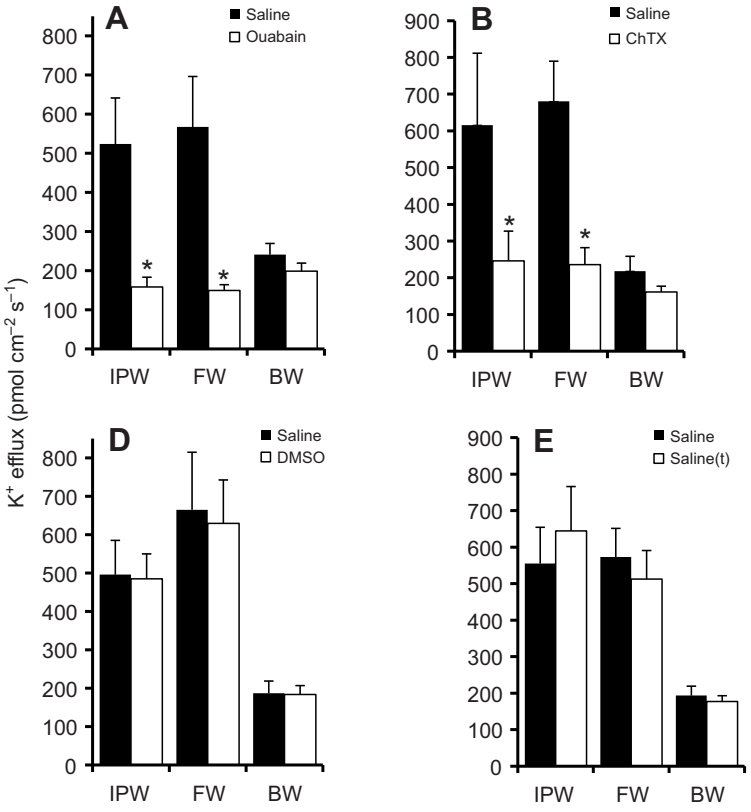

Fig. 6. Effects of (A) 1 mmol l^{-1} ouabain, (B) 23 nmol l^{-1} charybdotoxin (ChTX) or (C) 1 µmol l^{-1} bafilomycin on K^+ efflux at the rectum of larval *Chironomus riparius* reared in ion-poor water (IPW), freshwater (FW) or brackish water (BW; 20% seawater). Values indicate K^+ efflux from the rectum lumen into the external saline bath measured immediately after the rectum was prepared and mounted on the SIET apparatus (denoted as saline) and 5 min after incubation with an inhibitor. Control measurements at the rectum incubated with DMSO and saline [saline(t)] instead of inhibitors are shown in D and E, respectively. All data are expressed as means ± s.e.m. (*N*=9–19), except for BW animals in C, where data are means ± s.e.m. (*N*=2). Asterisks denote a statistically significant difference from the initial measurements (paired Student's *t*-test, $P<0.05$).

rearing, the amount of K^+ from imbibed medium would tend to increase K^+ hemolymph levels; however, there was no change in hemolymph K^+ levels in BW-reared larvae compared with FW- or IPW-reared animals (Table 1). We propose that a decrease in K^+ absorption by the rectum, as seen in BW-reared larvae, plays an important role in maintaining appropriate K^+ hemolymph levels. Furthermore, the NKA and VA activities in the rectum of IPW- and FW-reared larvae coupled with the observed decrease in enzyme activities in the rectum of BW-reared larvae suggests that NKA and VA drive K^+ reabsorption in the rectal epithelium of *C. riparius* larvae. To assess the role of NKA and VA in K^+ reabsorption by the rectum we applied pharmacological transport inhibitors in conjunction with the SIET.

The presence of ouabain in tissue bathing solutions reduced K^+ reabsorption (i.e. lumen to hemolymph K^+ movement) in the rectum of both FW- and IPW-reared *C. riparius* larvae (Fig. 6A). Because basolateral NKA transports K^+ from the hemolymph into the cell in exchange for Na^+, and SIET detected net K^+ movement from cell to hemolymph, the latter observation suggests the presence of K^+ transport mechanisms coupled to the membrane-energizing properties of NKA. In turn, this coupling would support the lumen-to-hemolymph movement of K^+ across the epithelium. A functional link between the activities of basolateral NKA and K^+ channels in resorptive epithelia is well documented (Ehrenfeld and Klein, 1997; Hurst et al., 1991; Matsumura et al., 1984; Messner et al., 1985; Kawahara et al., 1987; Sackin and Palmer, 1987; Hebert et al., 2005; Warth and Bleich, 2000; Hanrahan et al., 1986). In addition, ouabain inhibition of NKA has been shown to inhibit K^+ movement through basolateral K^+ channels in the amphibian proximal tubules (Matsumura et al., 1984; Messner et al., 1985).

The addition of the K^+ channel blocker ChTX to tissue bathing solutions also inhibited K^+ absorption at the rectum of FW- and IPW-reared *C. riparius* (Fig. 6B). These results provide evidence for the presence of basolateral K^+ channels and suggest that basolateral NKA establishes an electrochemical gradient that supports outward movement of K^+ through these channels. ChTX is a small basic protein purified from the venom of the scorpion *Leiurus quinquestriatus* (Smith et al., 1986). It has been shown to block both large- and small-conductance Ca^{2+}-activated K^+ channels as well as Ca^{2+}-insensitive, voltage-dependent K^+ channels (Miller et al., 1985; Hermann and Erxleben, 1987; MacKinnon et al., 1988; Grinstein and Smith, 1990; Bleich et al., 1996). Interestingly, expression of a gene encoding the Ca^+-activated K^+ channel has been localized in the ion-transporting midgut epithelial cells of *Drosophila* (Brenner and Atkinson, 1997). Further characterization of the putative K^+ channels in the basolateral membrane of rectal epithelial cells of *C. riparius* will require further studies that focus on voltage-dependent and Ca^{2+}-activated K^+ channels.

Application of the VA inhibitor bafilomycin also reduced K^+ absorption by the rectum of IPW- and FW-reared larvae (Fig. 6C). Although we could not conclusively demonstrate apical membrane localization of VA in the rectal epithelial cells, its conspicuous absence on the basolateral membrane suggests that the pump may reside apically. If so, the observed reduction in K^+ efflux with VA inhibition suggests that K^+ transport across the apical membrane is at least in part dependent on the activity of apical VA. We propose that hyperpolarization of the apical membrane by VA drives K^+ uptake across this membrane *via* apical K^+ channels. Absorption of K^+ through channels with different properties at the apical and basolateral membranes is well documented in the desert locust (*Schistocerca gregaria*), whose rectal epithelium has both apical VA and basal NKA (Hanrahan and Phillips, 1983; Hanrahan et al., 1986; Peacock, 1977; Phillips et al., 1996). Furthermore, in the hindgut of both larval and adult *Drosophila*, transcripts encoding for inwardly rectifying K^+ channels (K_{ir}) are found in abundance (Luan and Li, 2012). K_{ir} channels are a special subset of K^+ channels

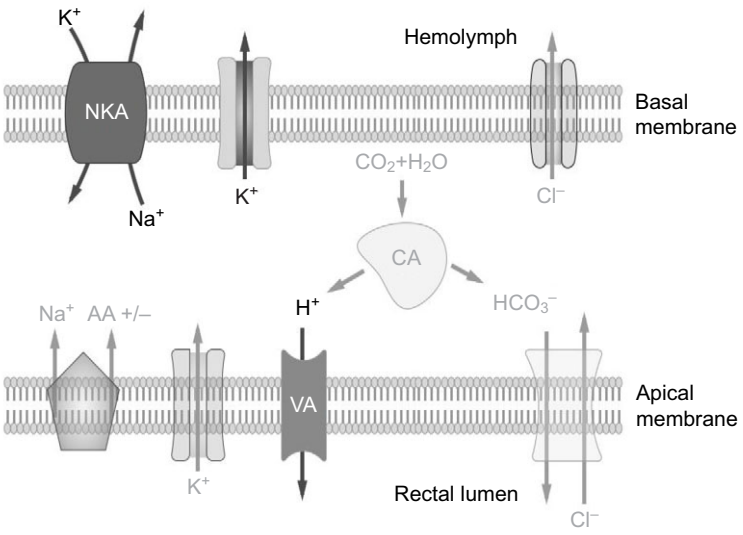

Fig. 7. Proposed model for ion transport mechanisms across the rectum of *Chironomus riparius*. Darker shaded transporters indicate proteins localized in this study; lighter shaded transporters indicate postulated proteins. The V-type H^+-ATPase (VA) hyperpolarizes the apical membrane and the voltage is used to drive K^+ diffusion into the cells *via* the putative apical K^+ channels. The voltage may also be used by the postulated Na^+:amino acid transporter (NAT) to drive Na^+ and amino acids into the cell. At the basolateral membrane, Na^+-K^+-ATPase (NKA) mediates Na^+ transport from the cell into the hemolymph and establishes an electrochemical gradient for K^+ diffusion to the hemolymph *via* basolateral K^+ channels. It is also suggested that Cl^- uptake may occur at the apical membrane in exchange for HCO_3^- and at the basolateral membrane *via* Cl^- channels driven by the cytosol negative potential established by basal NKA. Cytosolic carbonic anhydrase (CA) would supply H^+ to the VA and HCO_3^- to HCO_3^-/Cl^- exchangers in the apical membrane.

that pass K^+ more easily into, rather than out of, the cell and are highly expressed in renal epithelial cells for ion transport (Hibino et al., 2010). The presence of such K^+ channels in the apical membrane of epithelium involved in K^+ absorption, such as the rectum of *C. riparius*, seems reasonable but does not preclude the presence of other K^+-transporting mechanisms.

The results also demonstrate that ouabain, bafilomycin and ChTX have no effect on the low K^+ absorption by the rectum of BW-reared larvae (Fig. 6A–C), which suggests that under saline conditions: (1) rectal K^+ absorption no longer requires basal membrane energization by NKA; (2) rectal K^+ absorption no longer requires apical membrane energization by VA; and (3) K^+ channels that are insensitive to ChTX are also present, the dose of ChTX used was insufficient to block all K^+ channels, or K^+ flux across K^+ channels is no longer occurring in the rectum of BW-reared larvae. With regard to this final point, K^+ flux may occur through the paracellular route.

Based on the results of this study, a model for transcellular ion absorption across the rectum of *C. riparius* can be proposed as follows (see Fig. 7). Hyperpolarization of the apical membrane by VA drives the passive absorption of K^+ through putative K^+ channels at the apical membrane. VA-generated voltage may also be used to drive Na^+ and amino acids into the cells through a Na^+:amino acid transporter (NAT). Although we have no direct data to support the presence of NATs in the rectum of *C. riparius*, an observation that offers indirect support is that such proteins have been localized to the apical membrane of the FW mosquito rectum (Okech et al., 2008a). Basolateral NKA transports Na^+ from the cell into the hemolymph and generates an electrochemical gradient for the passive outward diffusion of K^+ *via* basolateral K^+ channels. Although not directly measured for the reasons stated above (see Materials and methods, SIET measurement of K^+ concentration gradient adjacent to rectum surface), Cl^- is also likely to be absorbed at the rectum of *C. riparius* and, based on studies with mosquito larvae, Cl^- most likely enters the cells at the apical membrane through apical Cl^-/HCO_3^- exchangers (Strange and Phillips, 1984; Strange et al., 1984) with hydration of CO_2 by cytosolic carbonic anhydrase providing HCO_3^- for the Cl^-/HCO_3^- exchanger and H^+ for VA (Smith et al., 2008). Cl^- may leave the cell at the basal membrane through Cl^- channels driven by the cytosol negative potential established by the activity of the basolateral NKA.

Perspectives and significance

The larvae of *C. riparius* are ubiquitous FW benthic inhabitants that play an important role in aquatic ecosystems by feeding on detritus and thereby recycling nutrients and acting as a food source for other animals. Interestingly, studies have found larval *C. riparius* thriving in salinated bodies of water such as coastal rock pools and FW bodies that have been salinated by industrial effluent. Climate change and anthropogenic factors such as road salting are predicted to continue damaging FW ecosystems and as a result, understanding the physiological mechanisms that permit larval *C. riparius* to thrive in different environmental conditions is important. In this study we demonstrated that the rectum is of particular importance in regulating ion homeostasis by absorbing relatively high amounts of K^+ into the hemolymph under IPW and FW conditions and that the rectum responds to larval BW exposure by significantly decreasing K^+ absorption. We further demonstrated that K^+ absorption by the rectum is dependent on the activities of both NKA and VA and is at least partially mediated by K^+ channels. These findings provide a strong impetus for further identification and characterization of the major transport mechanisms in the apical and basolateral membranes of the rectal epithelium of aquatic insects. We anticipate that the epithelial model initiated in this study will serve as a prototype for other K^+-absorptive epithelia of aquatic insects, as the frog skin has done for absorptive epithelia of vertebrates.

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AUTHOR CONTRIBUTIONS

S.J., S.P.K. and A.D. designed the study. S.J. executed all of the experiments. S.J., S.P.K. and A.D. interpreted the results and S.J. wrote the manuscript with editorial support from S.P.K. and A.D.

COMPETING INTERESTS

No competing interests declared.

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