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Effects of rearing salinity on expression and function of ion motive ATPases and ion transport across the gastric caecum of *Aedes aegypti* larvae

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Abstract

Larvae of *Aedes aegypti*, the yellow fever vector, inhabit a variety of aquatic habitats ranging from fresh water to brackish water. This study focuses on the gastric caecum of the larvae, an organ that has not been widely studied. We provide the first measurements of H^+, K^+, and Na^+ fluxes at the distal and proximal gastric caecum, and have shown that they differ in the two regions, consistent with previously reported regionalization of ion transporters. Moreover we have shown that the regionalization of vacuolar H^+-ATPase and Na^+/K^+ -ATPase is altered when larvae are reared in brackish water (30% seawater) relative to fresh water. Measurements of luminal Na^+ and K^+ concentrations also show a 5-fold increase in Na^+/K^+ ratio in the caecal lumen in larvae reared in brackish water relative to fresh water, whereas transepithelial potential and luminal pH were unchanged. Calculated electrochemical potentials reveal changes in the active accumulation of Na^+ and K^+ in the lumen of the gastric caecum of fresh water versus brackish water larvae. Together with the results of previous studies of the larval midgut, our results show that the caecum is functionally distinct from the adjacent anterior midgut, and may play an important role in osmoregulation as well as uptake of nutrients.

Introduction

Aedes aegypti is responsible for the spread of dengue, Zika fever, chikungunya and yellow fever, all of which infect millions of humans. The *A. aegypti* larval stage inhabits a variety of aquatic habitats like marshes, drains, and rock pools. Although the preferred medium for *A. aegypti* larvae is fresh water, it has been reported that the adults also lay eggs in brackish water (5% to 30% seawater) and that the larvae survive to form adults (Jude et al., 2012; Ramasamy et al., 2011; Surendran et al., 2012). Current mosquito population control measures focus on larvae from fresh water habitats (Surendran et al., 2012), therefore understanding larval physiology and how they adapt to different rearing salinities can provide the foundation for development of novel larvicides.

Fresh water mosquito larvae face the challenge of dilution of body fluids through fresh water uptake during feeding or across the body surfaces, in addition to loss of ions from the body fluids into the surrounding water. Larvae of *A. aegypti* reared in fresh water maintain their haemolymph ion concentrations at levels above those in the external medium by hyper-regulating (Bradley, 1994; Wigglesworth, 1938). Hyper-regulation of the body fluids is achieved through reduction in drinking, production of dilute urine, and active ion absorption by the anal papillae, midgut, and rectum (Koch, 1938; Ramsay, 1950; Stobbart, 1974; Wigglesworth, 1933a; Wigglesworth, 1933b; Wigglesworth, 1938). By contrast, larvae reared in brackish water experience much smaller gradients for passive movements of ions and water. In the laboratory *A. aegypti* can tolerate a salinity of up to 30% seawater, which is roughly isosmotic to the larval haemolymph (Clark et al., 2004).

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Larvae possess eight blind sacs, called gastric caeca, which are situated immediately posterior to the cardia and open into the anterior region of the midgut (Volkmann and Peters, 1989a). Ingested fluid passes through the anterior midgut into the posterior midgut, and is then channeled into the ecto-peritrophic space and moves anteriorly towards the caeca via antidromic peristalsis (Ramsay, 1950; Volkmann and Peters, 1989b; Wigglesworth, 1933b). This countercurrent flow allows for better utilization of ingested nutrients (Volkmann and Peters, 1989b). Based on morphological characteristics, it was hypothesized that the gastric caeca in *A. aegypti* larvae are also important in digestion, resorption/storage of nutrients, fluid reabsorption and maintenance of ion balance (Jones and Zeve, 1968; Ramsay, 1950; Volkmann and Peters, 1989b; Wigglesworth, 1933b; Wigglesworth, 1942). The caecum carries out these functions using four types of cells: ion transporting cells, reabsorbing/secreting cells, imaginal cells, and cells which secrete the caecal membrane (Volkmann and Peters, 1989b).

Two ATPases, vacuolar-type H^+-ATPase (VA) and Na^+/K^+-ATPase (NKA) were hypothesized to be the main drivers of active transport and nutrient resorption in the gastric caeca of larval *A. aegypti*. An immunohistochemical study (Patrick et al., 2006) revealed striking regionalization of the two ATPases in the gastric caeca of larvae reared in fresh water. VA is expressed along the apical membrane throughout the length of the caeca, with expression on both the apical and basal membrane along the distal third. NKA is expressed only on the basal membrane of the proximal two-thirds of the caeca. Based on morphological characteristics, the most abundant type of cells in the gastric caeca are the resorbing/secreting cells located in the proximal two-thirds of the caeca

(Volkmann and Peters, 1989a). These cells are implicated in digestion, nutrient absorption and storage, and are hereafter referred to as digestive cells to avoid confusion with cells implicated in secretion and absorption of K^+, Na^+ and H^+ by the ion transporting cells located in the distal third of the caecum (Volkmann and Peters, 1989a). Cell size, the length and diameter of apical microvilli and the numbers of mitochondria in the ion transporting cells all decrease in response to increased water salinity (Volkmann and Peters, 1989b). These changes, in conjunction with the V-ATPase regionalization, implicate the ion transporting cells of the caecum in osmoregulation (Patrick et al., 2006; Volkmann and Peters, 1989b).

In contrast to the extensive literature on ion transport and acid-base balance by the midgut (Clark et al., 2000; Corena et al., 2002; Izeirovski et al., 2009; Jagadeshwaran et al., 2010; Linser et al., 2009; Onken et al., 2008), Malpighian tubules (Clark and Bradley, 1997; Ramsay, 1950; Veenstra, 1988; Weng et al., 2003) and anal papillae (Donini et al., 2007; Donini and O'Donnell, 2005; Koch, 1938; Stobbart, 1971), very little is known about ion transport across the gastric caeca, with the exception of a single study reporting proton concentration gradients in the unstirred layer near the surface of the caecum (Boudko et al., 2001). Although (Boudko et al., 2001) measured H^+ gradients, they did not account for the effects of buffering on H^+ concentration gradients (Messerli et al., 2006).

In this study we have measured H^+ concentration gradients, corrected for buffering action, and used the corrected concentration gradients to calculate the fluxes of H^+ across the basal membranes of the caecum. This study is the first to measure H^+, Na^+ and K^+ transport rates along both the distal and proximal regions of

the gastric caeca of *A. aegypti*, and is also the first to examine the effects of rearing salinity on the rates of ion transport. We have also determined whether ions are passively or actively transported across the gastric caeca of fresh water and brackish water larvae by measuring the transepithelial potential difference (TEP), luminal pH, Na^+ concentration and K^+ concentration, and electrochemical potentials for H^+, Na^+ and K^+ across the caecal epithelium. Moreover, we have shown that rearing salinities alter VA and NKA regionalization and ATPase activity in the gastric caeca. This study thus provides functional correlates of the regionalization of ion transporters previously identified by immunohistochemical techniques (Filippov et al., 2003; Kang'ethe et al., 2007; Patrick et al., 2006; Pullikuth et al., 2006). Moreover, our results reveal the caecum to be an ion transporting region that is functionally distinct from the midgut.

Materials and methods

Rearing larvae

Aedes aegypti (Linnaeus) larvae were reared in fresh water (dechlorinated tap water) or brackish water (30% seawater; 10.5 g Instant Ocean® Sea Salt/L dechlorinated tap water). The eggs were hatched and held in rectangular tubs, and fed a 1:1 ground liver powder:dry yeast mixture *ad libitum*. Larvae were raised at room temperature (21°- 23° C) on a 12 h: 12 h light-dark cycle. Fourth instar larvae were used for all experiments.

Immunohistochemistry

VA and NKA were localized using two antibodies previously shown to cross-react with *Aedes aegypti* (Patrick et al., 2006). VA was localized using a polyclonal

serum antibody raised against the B subunit of *Culex quinquefasciatus* VA in rabbits (Filippov et al., 2003). NKA was localized using a monoclonal antibody, 'a5', raised against the α-subunit of avian P-type Na^+/K^+-ATPase in mice by Dr Douglas Fambrough, (Takeyasu et al., 1988). This antibody was obtained from the Developmental Studies Hybridoma Bank (DSHB); it was developed under the auspices of the National Institute of Child Health and Human Development and is maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. The secondary antibodies used were Alexa fluor 488 labelled goat anti-mouse and Cy3 labelled goat anti-rabbit (Jackson Immuno Research, CO, USA). These secondary antibodies were developed to have minimal cross reactivity with other species in the incubation medium.

Fourth instar larvae were rinsed and dissected in ice-cold phosphate buffered saline (PBS). The larvae were snipped on the posterior end to release the gut (caeca and midgut), and the gut contents and peritrophic membrane were removed. Whole mount immunostaining was carried out as described by (Patrick et al., 2006). The gut was fixed overnight in 4% paraformaldehyde (PFA)/PBS solution at 4°C. The tissues were rinsed in PBS followed by a methanol dehydration/rehydration series and rinsed in PBS. The gut tissues were then blocked with PBS/0.1% Triton X-100 (PBT) including 2% bovine serum albumin (BSA). Tissues were incubated overnight at 4°C in a 1:30 dilution of mAb □5 (NKA antibody) and a 1:1000 dilution of polyclonal serum antibody to VA made up in PBT/1%BSA. A 1:1000 dilution of rabbit pre-immune serum made up in PBT/1%BSA served as a control. Tissues were then rinsed in PBT/1%·BSA/2%·normal goat serum (NGS) to remove any unbound antibody, and incubated for 2 h at room temperature with secondary antibodies at a 1:2000 dilution in PBT/1%·BSA/2%·NGS, followed by rinses

in PBT/1%·BSA/2%·NGS. The tissues were mounted on slides using ProLong® Gold antifade reagent (Thermo Fisher Scientific, MA, USA), and stored at -20° C in the dark. Tissues were examined by laser scanning confocal microscopy using a Nikon A1R system located in the Shiley Center for Science & Technology Building, University of San Diego, CA, USA.

Basolateral surface areas (μm^2) for individual cells were estimated using the polygon selection tool in ImageJ software (https://imagej.nih.gov/ij/). For both rearing media, 3 VA-rich and 3 NKA-rich cells were measured for each of 5 tissue samples. Although the measurements were done on whole mounts, we assumed that the surfaces were planar. Since the caeca all have similar curvature, the degree of underestimation of surface area arising from this assumption would be similar in both groups. All measurements were done on cells that were clearly delineated, and which were away from the periphery of the caecum.

VA and NKA Activity Assay

The ATPase activity assay relies on the enzymatic coupling of ouabain- (SigmaAldrich Canada, Ltd.,) or bafilomycin- (LC Laboratories, Woburn, MA, USA) sensitive hydrolysis of adenosine triphosphate (ATP) to the oxidation of reduced nicotinamide adenine dinucleotide (NADH). Disappearance of NADH is directly measured in a microplate spectrophotometer. *Aedes aegypti* larval caeca were collected into 2 ml microcentrifuge tubes (20 sets of caeca per tube) containing an ice-cold solution of 150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA and 50 mmol l^{-1} imidazole (pH 7.3). VA and NKA ATPase activities were determined as previously described

(D'Silva et al., 2017), and enzyme activity was expressed as micromoles of ADP per milligram of protein per hour.

Dissections and Physiological Saline

The entire gut from each fourth instar larva was dissected out in *Aedes* larval saline (in mmol l^{-1}: 5 L-proline, 9.1 L-glutamine, 8.74 histidine, 14.4 leucine, 3.37 arginine-HCl, 10 glucose, 5 succinic acid, 5 malic acid, 10 citric acid (tri-sodium salt), 30 NaCl, 3 KCl, 5 $NaHCO_3$, 0.6 $MgSO_4$, 5 $CaCl_2$, 25 HEPES) titrated to pH 7 using NaOH. The dissected gut was then transferred from the dissection dish to a saline-filled 35 mm Petri dish which had been pre-coated with poly-L-lysine (0-150 kDa, Sigma-Aldrich Canada, Ltd.) to aid tissue adherence to the bottom of the dish, as described by (Naikkhwah and O'Donnell, 2011).

Ion-Selective Microelectrodes and the Scanning Ion-Selective Electrode Technique (SIET)

H^+, Na^+ and K^+ scans were carried out in *Aedes* larval saline. Since protons may diffuse freely or in association with buffers in the saline, the proton transport rates were corrected for buffering using equations described in Messerli et al. (2006). Methods for fabrication and calibration of K^+-selective, Na^+-selective and H^+-selective microelectrodes have been described previously (Pacey and O'Donnell, 2014). The gradients of ion concentrations formed in the unstirred layer by ion transport across the gastric caecum were measured using SIET, as described previously (Pacey and O'Donnell, 2014). Concentration gradients were then

converted to rates of ion secretion (from bath to lumen) or absorption (from lumen to bath) using Fick's equation. Measurements were made at three sites, 50 μm apart, along the distal end or proximal end of gastric caecum. All measurements were carried out within 3 minutes of dissection since rates of ion secretion and absorption decayed steadily after the first 3-6 minutes (D'Silva and O'Donnell, unpublished). Previous measurements have shown that 5-HT stimulates ion transport in the anterior and posterior midgut of *A. aegypti*, increasing TEP and preventing its decay (Clark et al., 1999).

Measurement of transepithelial potential (TEP) and luminal H^+, Na^+ and K^+ concentrations

Luminal H^+, Na^+ and K^+ concentrations and transepithelial potential were measured in the gastric caecum of *A. aegypti* larvae using ion-selective double-barrelled microelectrodes. Micropipettes were pulled from theta-glass borosilicate capillaries (TST150, WPI, New Haven, CT, USA). After pulling, one barrel was filled with a 2-3 cm column of distilled water and the open end of the capillary was inserted through a whole in the plastic lid of a 3 ml glass vial containing ~ 200 μl of dimethyldichlorosilane. After 15 – 20 seconds, the pipette was transferred onto the surface of a hot plate at 200°C for 10 - 15 seconds. A small volume (~ 100 nl) of the appropriate ion exchanger cocktail was introduced into the shank of the silanized barrel.

pH microelectrodes were first back-filled with hydrogen ionophore I, cocktail B (Fluka). The H^+-selective barrel was then backfilled with 100 mmol l^{-1} NaCl and 100 mmol l^{-1} sodium citrate at pH 6, and the reference barrel was filled with 1 mol l^{-1} KCl.

H^+-selective electrodes were calibrated in solutions of (in mmol l^{-1}) 130 NaCl, 5 KCl, 0.5 NaH_2PO_4, 0.1 Na_2HPO_4, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, adjusted to pH 6.5 and 7.5.

The tip of each Na^+-selective microelectrode was filled with the neutral carrier ETH227 (sodium ionophore I, cocktail A, Fluka). The Na^+-selective barrel was then backfilled with 150 mmol l^{-1} NaCl and the reference barrel was filled with 1 mol l^{-1} KCl. Na^+-selective electrodes were calibrated in solutions of (in mmol l^{-1}) 15 NaCl:135 KCl and 150 NaCl.

The tip of each K^+-selective microelectrode was filled with potassium ionophore I, cocktail B (Fluka). The K^+-selective barrel was then backfilled with 150 mmol l^{-1} KCl. The reference barrel was filled with 150 mmol l^{-1} sodium acetate near the tip and shank and 150 mmol l^{-1} KCl in the rest of the electrode. The K^+-selective electrode was calibrated in solutions of (in mmol l^{-1}) 150 KCl and 15 KCl:135 NaCl.

Slopes of electrodes for a 10-fold change in ion concentration or 1 pH unit were, 57-60 mV, 52-54 mV and 58-61 mV for Na^+, K^+ and H^+, respectively. Microelectrode tip resistance and noise were reduced by submicron breakage of the tips that was accomplished by gently brushing the tip with tissue paper under saline (Ianowski and O'Donnell, 2006; Tripathi et al., 1985).

Voltages of the reference (V_{ref}) and ion-selective barrel (V_i) were measured by a high-input impedance differential electrometer (HiZ-223; Warner Instruments, CT, USA). V_i and V_{ref} were measured with respect to an Ag/AgCl electrode connected to the bath through a 0.5 mol l^{-1} KCl agar bridge. V_i was filtered through a low-pass RC filter at 2 Hz to minimize noise resulting from the high-input impedance (>10^9 Ω) of the ion-selective barrel. V_{ref} and the difference (V_i–V_{ref}) were recorded using an AD converter

and data acquisition system (PowerLab and LabChart software; ADInstruments Inc., CO, USA).

Calculation of electrochemical potentials

The electrochemical potential ($\Delta\mu/F$, in mV) of an ion is calculated using the equation:

$$\Delta\mu/F = 59\log([\text{ion}]_{\text{lumen}}/[\text{ion}]_{\text{bath}}) + z\text{TEP}$$

where $[\text{ion}]_{\text{lumen}}$ is the concentration of the ion in the lumen (mmol l^{-1}), $[\text{ion}]_{\text{bath}}$ is the concentration of the ion in the bath (mmol l^{-1}), z is the valency and TEP is the transepithelial potential difference (mV), caecal lumen relative to bath. A positive value indicates a luminal ion concentration in excess of equilibrium, *i.e.* passive movement from gut lumen to bath is favoured. A negative value indicates a luminal ion concentration below equilibrium, *i.e.* passive movement from bath to gut lumen is favoured.

Statistical analysis

Data were plotted using Graphpad InStat (Graphpad Software Inc., La Jolla, CA), and values are expressed as means ± sem. NKA and VA ATPase activity data within the caeca and across rearing salinity, were analyzed using a two-way ANOVA followed by Tukey's post-hoc test.

The rates of H^+, Na^+ and K^+ transport are expressed as mean ± sem for a number of caeca (N). Each mean value was based on three replicate scans at 3 sites

along the distal or proximal region of the caecum. H^+, Na^+ and K^+ transport rates were plotted against caecal region (proximal or distal) and rearing salinity and analyzed by two-way ANOVA followed by Tukey's post-hoc test. Differences were considered significant at $P<0.05$.

TEP, luminal H^+, Na^+ and K^+ concentrations and electrochemical potentials are expressed as mean ± sem (N). The parameters were plotted with respect to rearing salinity and analyzed by unpaired Student's *t*-test. Differences were considered significant at $P<0.05$.

Results

VA and NKA expression along the gastric caeca

Immunohistochemical localization revealed that both VA and NKA were expressed in the larval *A. aegypti* gastric caeca (Fig. 1). Controls in which the NKA and VA antisera were omitted from the immunostaining procedure showed no staining (not shown).

In larvae reared in fresh water, VA was expressed along the distal third of the caeca and NKA was expressed along the proximal two-thirds (Fig. 1A, 1B), as shown previously (Patrick et al., 2006). Clustering of VA-rich cells in the distal region of gastric caeca of larvae reared in brackish water was not as apparent as in fresh water (Fig. 1D), and VA-rich cells were interspersed between the NKA-rich cells in the proximal gastric caeca of larvae reared in brackish water (Fig. 1C, 1D). The basolateral surface area of the VA-rich cells was ~ 249 μm^2, approximately 9-fold lower than the corresponding surface

area of ~ 3102 μm^2 of the NKA-rich cells of larvae reared in brackish water (Figure 1C; Table 1).

NKA and VA activity profiles

ATPase activity (μmol ADP mg^{-1} protein h^{-1}) of NKA was similar to that of VA in the gastric caeca of larvae reared in fresh water (Fig. 2). ATPase activity of NKA was also similar to that of VA in the gastric caeca of larvae reared in brackish water (Fig. 2). However, both NKA and VA ATPase activities were significantly lower in gastric caeca of larvae reared in brackish water relative to the corresponding ATPase activities in larvae reared in fresh water (Fig. 2).

Fluxes of H^+, Na^+ and K^+ along the gastric caecum

Fluxes of H^+ (Fig. 3A, B), Na^+ (Fig. 3C) and K^+ (Fig. 3D) across the basolateral membrane were measured along the haemolymph-facing surface of the gastric caecum of larval *A. aegypti*. SIET measurements revealed that H^+ was absorbed (into the bath) at the distal gastric caecum and secreted (from bath to cell) at the proximal region (Fig. 3A, B). Na^+ and K^+ were secreted at both the distal and proximal regions of the gastric caecum (Fig. 3C, D).

At the distal gastric caecum, the rate of absorption of H^+ in larvae reared in fresh water was significantly higher than in larvae reared in brackish water (Fig. 3B). At the proximal gastric caecum, H^+ secretion rates were similar in larvae reared in fresh water compared to larvae reared in brackish water (Fig. 3B).

Na^+ secretion rates at the distal gastric caecum were higher in larvae reared in fresh water in comparison to larvae reared in brackish water (Fig. 3C). At the proximal gastric caecum, the rate of secretion of Na^+ of larvae reared in fresh water was similar to larvae reared in brackish water (Fig. 3C). In larvae reared in fresh water, the rate of secretion of Na^+ at the distal end was significantly higher than the rate of secretion along the proximal region (Fig. 3C), whereas in larvae reared in brackish water, Na^+ secretion rates were similar at both the distal and proximal regions (Fig. 3C).

The K^+ secretion rate at both the distal and proximal gastric caecum was significantly higher in larvae reared in fresh water than the corresponding rates for larvae reared in brackish water (Fig. 3D). In larvae reared in fresh water, the rate of K^+ secretion at the distal gastric caecum was significantly higher than the K^+ secretion rate at the proximal gastric caecum (Fig. 3D). In larvae reared in brackish water, the K^+ secretion rate at the distal gastric caecum was similar to K^+ secretion at the proximal gastric caecum (Fig. 3D).

Transepithelial potential and transepithelial electrochemical potentials for H^+, Na^+ and K^+

The lumen of the gastric caecum was at a positive potential with respect to the bathing saline (Fig. 4A). The TEP of the gastric caecum of fresh water-reared larvae was not significantly different than that of larvae reared in brackish water. The TEP of the caecum was +29 mV, in contrast to the lumen negative TEP (-11.6 ± 5.3 mV, N=10) of the adjacent anterior midgut measured within 10 minutes of impalement. Previous studies in which the anterior midgut was perfused with

symmetrical saline also showed a lumen negative TEP of -66 mV which decayed to -10 mV within 10 – 15 minutes (Clark et al., 1999).

Luminal pH of the gastric caecum of fresh water-reared larvae was not significantly different from that of larvae reared in brackish water (Fig. 4B). Luminal pH was 7.6 ± 0.09, in agreement with previously reported luminal pH values (7.5 – 8) obtained by semi-quantitative colorimetric methods (Dadd, 1975; Zhuang et al., 1999). The luminal concentration of Na^+ of the gastric caecum of larvae reared in fresh water (Fig. 4D) was lower than the concentration in the bathing saline (65 mmol l^{-1}), and significantly lower than the luminal Na^+ concentration of larvae reared in brackish water (Fig. 4D). Luminal K^+ concentration for both treatments (Fig. 4F) was higher than that of the bathing saline (3 mmol l^{-1}). The luminal K^+ of the gastric caecum of larvae reared in fresh water (Fig. 4F) was significantly higher than larvae reared in brackish water (Fig. 4). The ratio of luminal Na^+:K^+ concentrations was reversed for the caecum of larvae reared in brackish water when compared to fresh water ratios (Fig. 4D, F), however, the sum of luminal Na^+ and K^+ was consistent for both rearing mediums.

The electrochemical potentials ($\Delta\mu/F$) for H^+, Na^+ and K^+ were calculated using the luminal ion concentrations of H^+, Na^+ and K^+, and the TEP. A positive value of the electrochemical potential for the ion indicates active transport from the bathing saline into the lumen. H^+ and K^+ were actively transported into the lumen in both rearing conditions (Fig. 4C, G). Na^+ was actively transported into the gastric caecal lumen of larvae reared in brackish water (Fig. 4E), and was near electrochemical equilibrium in the gastric caecum of larvae reared in fresh water (Fig. 4E).

Discussion

This study of the larval gastric caecum reports the first SIET measurements of H^+, Na^+, and K^+ fluxes across the basolateral membrane of the gastric caecum, as well as corresponding transepithelial electrochemical potentials and ion motive ATPase activities. It is important to note that we have assumed that flux across the basolateral membrane measured by SIET is equal to transepithelial flux across the caecum in the steady state. Basolateral fluxes into, or out of, the cells of the caecum might be altered after transfer into saline, particularly since the TEP is declining. A parallel study (D'Silva and O'Donnell, unpublished) demonstrates that the ion transport rates and TEP at the gastric caecum are restored and maintained for > 20 min by stimulation with the biogenic amine 5-hydroxytryptamine (5-HT, serotonin; 1 μmol l^{-1}). The directions of transport across the caeca are the same in the presence or absence of 5-HT (D'Silva and O'Donnell, unpublished), supporting our assumption that the fluxes we have measured are indicative of transepithelial ion transport. We have shown that the measured fluxes of H^+, K^+, and Na^+ differ in the proximal and distal regions of the caecum, consistent with the pronounced regionalization of ion transporters revealed in previous immunohistochemical studies (Patrick et al., 2006; Kang'ethe et al., 2007; Pullikuth et al., 2006; Filippova et al., 1998). Moreover we have shown that the regionalization of VA and NKA is dependent upon rearing salinity. Our measurements of the electrochemical gradients and transepithelial potentials for H^+, Na^+ and K^+ in larvae reared in fresh water and brackish water also implicate the caecum in ionoregulation.

Fresh water

Our study confirms the regionalization of VA and NKA along the basal membrane of the gastric caeca of larvae reared in fresh water (Fig. 1A, B), consistent with the findings of Patrick et al. (2006). In addition, our measurements of ATPase activity shows that VA and NKA are functional ATPases in the caeca, and that ATPase activity is higher in the caeca of larvae reared in fresh water relative to those reared in brackish water (Fig. 2). Figure 5 summarizes the immunohistochemical evidence for ion transporters on the apical and basal membranes of the distal and proximal gastric caecum (Filippov et al., 2003; Kang'ethe et al., 2007; Patrick et al., 2006; Pullikuth et al., 2006). This schematic provides a framework for our discussion of H^+, Na^+ and K^+ transport rates across the distal and proximal regions of the caecum, in the sections below.

H^+ transport rates measured by SIET were consistent with the expected directions of H^+ transport based on previous VA expression patterns along the gastric caeca in larvae reared in fresh water by Patrick et al. (2006). Although VA was expressed on both apical and basal membranes of the distal gastric caecum (Patrick et al., 2006), we measured H^+ absorption (from lumen to bath) in this region (Fig. 3B). We hypothesize that the Na^+/H^+ exchangers, AeNHE3 and AeNHE8, expressed in the distal gastric caeca, contribute to this H^+ absorption (Fig. 5) (Kang'ethe et al., 2007; Pullikuth et al., 2006). AeNHE3 is present on the basal membrane and aids in cellular alkalinization, thus transporting H^+ from the cytoplasm to the bathing saline (Pullikuth et al., 2006). AeNHE3 and basal VA (Patrick et al., 2006; Pullikuth et al., 2006) thus function together in H^+ absorption across the basal surface of the distal gastric caeca (Fig. 5). Across the

apical membrane, AeNHE8 transports H^+ from the lumen into the cytoplasm, in exchange for Na^+ or K^+ (Fig. 5) (Kang'ethe et al., 2007). The effects of apical VA on transepithelial H^+ transport may therefore be minimal due to the cycling of H^+ across the apical membrane, into the lumen by VA and into the cell by AeNHE8 (Fig. 5) (Kang'ethe et al., 2007; Patrick et al., 2006).

In contrast to absorption of H^+ at the distal gastric caecum, we measured transepithelial H^+ secretion in the proximal region (Fig. 3B), consistent with the expression of VA only on the apical membrane (Fig. 5) (Patrick et al., 2006). Although some cycling of H^+ would occur at the apical membrane due to an apically expressed AeNHE8 (Kang'ethe et al., 2007) and VA (Patrick et al., 2006), two factors may contribute to transepithelial secretion of H^+ into the lumen in the proximal gastric caecum (Fig. 5). First, there is no evidence for a basal VA or NHE3 in this region that would drive H^+ from cell to bath. Second, overall secretion of H^+ at the proximal gastric caecum may be enhanced through production of high levels of cellular H^+ by the actions of cytoplasmic carbonic anhydrase in this region (Seron et al., 2004).

Although H^+ is absorbed across the distal caecum and secreted into the lumen in the proximal region, the positive electrochemical potential for H^+ between the lumen of the caecum and the bath (Fig. 4C) indicates that, for the caecum as a whole, secretion of H^+ dominates. The electrochemical potential ($\Delta\mu/F$) of +15 mV indicates that H^+ secretion is an active process, and that H^+ moves down its concentration gradient from the bathing saline (pH 7.0) into the lumen (pH 7.6 ± 0.09), but against an opposing lumen-positive TEP of +29 mV (Fig. 4).

Our measurements show that the pH of the lumen of the gastric caecum is dramatically lower (pH 7.6) than that of the adjacent highly alkaline (pH 10.5 - 11) anterior midgut (Dadd, 1975; Zhuang et al., 1999). The latter pH values were based on semi-quantitative colorimetric tests, which are complicated by the natural yellow to brown colouration of caecal contents. We therefore used double-barreled ion-selective electrodes to obtain quantitative measurements of luminal pH, as well as Na^+ and K^+ concentrations.

We propose that three transporters, AeNHE3, AeNHE8 and cation chloride cotransporter (CCC), account for transepithelial secretion of Na^+ and K^+ at the distal gastric caecum (Fig. 3C, D, 5). At the distal caecum, basally expressed AeNHE3 and CCC transport Na^+ and/or K^+ from the haemolymph-side into the cytoplasm (Fig. 5) (Filippov et al., 2003; Pullikuth et al., 2006), and apically expressed AeNHE8 transports Na^+ or K^+ from the cytoplasm into the lumen (Fig. 5) (Kang'ethe et al., 2007).

At the proximal gastric caecum, Na^+ and K^+ secretion (Fig. 3C, D) is consistent with the actions of apically expressed AeNHE8 (Kang'ethe et al., 2007), and basally expressed CCC (Filippov et al., 2003) and NKA (Patrick et al., 2006). We propose that NKA activity establishes a sodium gradient across the basal membrane and that this gradient energizes secondary active transport of Na^+, K^+ and Cl^- into the cell through CCC. The electrochemical potential for Na^+ (Fig. 3E) indicates that it is near equilibrium across the caecal epithelium in fresh water larvae. K^+ is actively secreted into the lumen, as indicated by a lumen positive TEP and positive electrochemical potential for K^+ (Fig. 3A, G).

In sum, H^+ and K^+ are actively secreted into the lumen of the gastric caecum lumen, with spatial differences in ion transport rates across the distal and proximal regions. Together, these observations provide functional correlates of the regionalization of ion transporters previously identified by immunohistochemical techniques in larvae reared in fresh water (Filippov et al., 2003; Kang'ethe et al., 2007; Patrick et al., 2006; Pullikuth et al., 2006). Our results clearly show that the caecum is functionally distinct from the adjacent highly alkaline (pH 10.5 – 11) (Dadd, 1975; Onken and Moffett, 2009; Onken et al., 2008; Zhuang et al., 1999) and lumen-negative (Clark et al., 1999) anterior midgut.

Brackish water

Comparisons of larvae reared in brackish versus fresh water show that changes in rearing salinity have multiple effects on the structure and function of the gastric caecum. Regionalization of VA and NKA in the caecum and rates of ion transport across the distal caecum are clearly different in larvae from brackish versus fresh water. In addition, NKA and VA activity, Na^+ and K^+ concentrations in the lumen of the caecum, and the electrochemical potential for Na^+ between lumen and bath vary with rearing salinity. Previous studies have shown that caecal cell morphology also changes when larvae are reared in different salinities (Volkmann and Peters, 1989b).

Decreased ATPase activities in brackish water larvae (Fig. 2) are correlated with a decreased mitochondrial density in higher rearing salinities (Volkmann and Peters, 1989b). Decreased ATPase activities may also account for decreased rates of H^+, Na^+ and K^+ transport at the distal gastric caecum (Fig. 3B, C, D). We hypothesize that in brackish water the distal gastric caecum may require lower

ATPase activity and consequently fewer mitochondria (Volkmann and Peters, 1989b), as the larvae do not face an osmotic challenge (Clark et al., 2004). On the other hand, in larvae reared in fresh water, a higher mitochondrial density and higher ATPase activity may be necessary for the larvae to hyper-regulate (Bradley, 1994; Wigglesworth, 1938). When fresh water larvae ingest large amounts of dilute media, the osmotic gradient will favour movement of water from the midgut into the haemolymph, thus reducing the volume of luminal fluid. Higher secretion of Na^+ and K^+ into the lumen (Fig. 3C, D) may be correlated with fluid secretion from haemolymph to lumen. Maintaining adequate fluid secretion into the gut lumen is important to support digestion and luminal fluid circulation. An increased number of mitochondria in the caecum of larvae reared in fresh water would help fuel the ion motive ATPases, which would in turn maintain fluid secretion into the caecal lumen despite intake of dilute medium.

We propose that the ion transporting cells and digestive cells observed in previous studies (Volkmann and Peters, 1989a; Volkmann and Peters, 1989b) correspond to VA-rich and NKA-rich cells, respectively (Fig. 1 C, D). The area occupied by VA-rich cells in the distal caecum is reduced in larvae reared in brackish water, and small VA-rich cells were dispersed throughout the proximal gastric caecum of larvae reared in brackish but not fresh water (Fig. 1C; Table 1). An apparent decrease in the size of the cap of VA-rich cells in the distal caecum of brackish water larvae (Fig. 1) is consistent with a previous ultrastructural study that showed that there was a decrease in size of ion transporting cells in the distal caecum of mosquito larvae reared in higher salinities (Volkmann and Peters, 1989b). The VA-rich cells of the distal gastric caecum (Fig. 1) may therefore be

involved in hyper-regulation in larvae reared in fresh water, and hence reduced in size in brackish water.

At the proximal caecum of *A. aegypti* larvae, the distribution of cell types is morphologically similar to the caecum of *Anopheles stephensi*, where the digestive cells are interspersed with ion transporting cells (Volkmann and Peters, 1989a). *Anopheles stephensi* breeds predominantly in fresh water, but it has also been reported to readily breed in water of high salinity (100% seawater; (Manouchehri et al., 1976). At the proximal gastric caecum, the size and location of the NKA-rich cells (Fig. 1C, D; Table 1) suggests that they correspond to the digestive cells described by (Volkmann and Peters, 1989a; Volkmann and Peters, 1989b). The smaller ion transporting cells (VA-rich cells) may play a role in driving ion-dependent nutrient transport by the adjacent digestive cells (NKA-rich cells). Similar rates of H^+ and Na^+ secretion across the proximal caecum of larvae reared in both fresh and brackish water (Fig. 3B, C) suggest that ion transport in this region may be essential for nutrient transport rather than ionoregulation.

In brackish water, the larvae ingest NaCl along with the food, and there is an increased Na^+ concentration in the lumen of the caecum (Fig. 4D) in comparison to larvae reared in fresh water. On the other hand, luminal K^+ concentration is significantly lower in the caecum of larvae reared in brackish water compared to fresh water (Fig. 4F). The decrease in luminal K^+ concentration is consistent with decreased secretion of K^+ along both the distal and proximal gastric caecum of larvae reared in brackish water. It is worth noting that the sum of Na^+ and K^+ concentrations is similar (~ 70 mmol l^{-1}) in the lumen of larvae reared in both fresh and brackish water and this value is also similar to

the sum of the concentrations of these ions in the bathing saline. The shift to higher levels of Na^+ in the lumen of the gastric caecum may be energetically favourable given the ready availability of Na^+ in the food and ingested water for brackish water larvae.

Conclusion

Our results reveal that the caecum is functionally distinct from the adjacent anterior midgut. In addition, the VA-rich and NKA-rich cells along the gastric caecum may play an important role in osmoregulation and uptake of nutrients in the two rearing conditions, as is indicated by the change in regionalization of the two types of cells in fresh water and brackish water.

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References

Boudko, D. Y., Moroz, L. L., Linser, P. J., Trimarchi, J. R., Smith, P. J. and Harvey, W. R. (2001). In situ analysis of pH gradients in mosquito larvae using non-invasive, self-referencing, pH-sensitive microelectrodes. *J Exp Biol* **204**, 691-9.

Bradley, T. J. (1994). The role of physiological capacity, morphology, and phylogeny in determining habitat use in mosquitoes. In Ecological Morphology: Integrative Organismal Biology. Chicago: University of Chicago Press.

Clark, T. M. and Bradley, T. J. (1997). Malpighian tubules of larval *Aedes aegypti* are hormonally stimulated by 5-hydroxytryptamine in response to increased salinity. *Archives of Insect Biochemistry and Physiology* **34**, 123-141.

Clark, T. M., Flis, B. J. and Remold, S. K. (2004). Differences in the effects of salinity on larval growth and developmental programs of a freshwater and a euryhaline mosquito species (Insecta: Diptera, Culicidae). *J Exp Biol* **207**, 2289-95.

Clark, T. M., Koch, A. and Moffett, D. F. (1999). The anterior and posterior 'stomach' regions of larval *Aedes aegypti* midgut: regional specialization of ion transport and stimulation by 5-hydroxytryptamine. *J Exp Biol* **202**, 247-52.

Clark, T. M., Koch, A. and Moffett, D. F. (2000). The electrical properties of the anterior stomach of the larval mosquito (*Aedes aegypti*). *J Exp Biol* **203**, 1093-101.

Corena, M., Seron, T. J., Lehman, H. K., Ochrietor, J. D., Kohn, A., Tu, C. and Linser, P. J. (2002). Carbonic anhydrase in the midgut of larval *Aedes aegypti*: cloning, localization and inhibition. *Journal of Experimental Biology* **205**, 591-602.

D'Silva, N. M., Donini, A. and O'Donnell, M. J. (2017). The roles of V-type H^+-ATPase and Na^+/K^+-ATPase in energizing K^+ and H^+ transport in larval *Drosophila* gut epithelia. *J Insect Physiol* **98**, 284-290.

Dadd, R. H. (1975). Alkalinity within the midgut of mosquito larvae with alkaline-active digestive enzymes. *J Insect Physiol* **21**, 1847-53.

Donini, A., Gaidhu, M. P., Strasberg, D. R. and O'Donnell M, J. (2007). Changing salinity induces alterations in hemolymph ion concentrations and Na^+ and Cl^- transport kinetics of the anal papillae in the larval mosquito, *Aedes aegypti*. *J Exp Biol* **210**, 983-92.

Donini, A. and O'Donnell, M. J. (2005). Analysis of Na^+, Cl^-, K^+, H^+ and NH_4^+ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes. *J Exp Biol* **208**, 603-10.

Filippov, V., Aimanova, K. and Gill, S. S. (2003). Expression of an *Aedes aegypti* cation-chloride cotransporter and its *Drosophila* homologues. *Insect Mol Biol* **12**, 319-31.

Ianowski, J. P. and O'Donnell, M. J. (2006). Electrochemical gradients for Na^+, K^+, Cl^- and H^+ across the apical membrane in Malpighian (renal) tubule cells of *Rhodnius prolixus*. *J Exp Biol* **209**, 1964-75.

Izeirovski, S., Moffett, S. B., Moffett, D. F. and Onken, H. (2009). The anterior midgut of larval yellow fever mosquitoes (*Aedes aegypti*): effects of amino acids, dicarboxylic acids, and glucose on the transepithelial voltage and strong lumenal alkalinization. *J Exp Zool A Ecol Genet Physiol* **311**, 719-26.

Jagadeshwaran, U., Onken, H., Hardy, M., Moffett, S. B. and Moffett, D. F. (2010). Cellular mechanisms of acid secretion in the posterior midgut of the larval mosquito (*Aedes aegypti*). *J Exp Biol* **213**, 295-300.

Jones, J. C. and Zeve, V. H. (1968). The fine structure of the gastric caeca of *Aedes aegypti* larvae. *J Insect Physiol* **14**, 1567–1575.

Jude, P. J., Tharmasegaram, T., Sivasubramaniyam, G., Senthilnanthanan, M., Kannathasan, S., Raveendran, S., Ramasamy, R. and Surendran, S. N. (2012). Salinity-tolerant larvae of mosquito vectors in the tropical coast of Jaffna, Sri Lanka and the effect of salinity on the toxicity of *Bacillus thuringiensis* to *Aedes aegypti* larvae. *Parasit Vectors* **5**, 269.

Kang'ethe, W., Aimanova, K. G., Pullikuth, A. K. and Gill, S. S. (2007). NHE8 mediates amiloride-sensitive Na^+/H^+ exchange across mosquito Malpighian tubules and catalyzes Na^+ and K^+ transport in reconstituted proteoliposomes. *Am J Physiol Renal Physiol* **292**, F1501-12.

Koch, H. J. (1938). The absorption of chloride ions by the anal papillae of diptera larvae. *J Exp Biol* **15**, 152-160.

Linser, P. J., Smith, K. E., Seron, T. J. and Neira Oviedo, M. (2009). Carbonic anhydrases and anion transport in mosquito midgut pH regulation. *J Exp Biol* **212**, 1662-71.

Manouchehri, A. V., Javadian, E., Eshighy, N. and Motabar, M. (1976). Ecology of *Anopheles stephensi* Liston in southern Iran. *Trop Geogr Med* **28**, 228-32.

Messerli, M. A., Robinson, K. R. and Smith, P. J. S. (2006). Electrochemical sensor applications to the study of molecular physiology and analyte flux in plants. In *Plant Electrophysiology: Theory and Methods*, (ed. A. G. Volkov), pp. 73-107. Berlin, Heidelberg: Springer Berlin Heidelberg.

Naikkhwah, W. and O'Donnell, M. J. (2011). Salt stress alters fluid and ion transport by Malpighian tubules of *Drosophila melanogaster*: evidence for phenotypic plasticity. *Journal of Experimental Biology* **214**, 3443-54.

Onken, H. and Moffett, D. F. (2009). Revisiting the cellular mechanisms of strong luminal alkalinization in the anterior midgut of larval mosquitoes. *J Exp Biol* **212**, 373-7.

Onken, H., Moffett, S. B. and Moffett, D. F. (2008). Alkalinization in the isolated and perfused anterior midgut of the larval mosquito, *Aedes aegypti*. *J Insect Sci* **8**, 1-20.

Pacey, E. K. and O'Donnell, M. J. (2014). Transport of H^+, Na^+ and K^+ across the posterior midgut of blood-fed mosquitoes (*Aedes aegypti*). *J Insect Physiol* **61**, 42-50.

Patrick, M. L., Aimanova, K., Sanders, H. R. and Gill, S. S. (2006). P-type Na^+/K^+-ATPase and V-type H^+-ATPase expression patterns in the osmoregulatory organs of larval and adult mosquito *Aedes aegypti*. *Journal of Experimental Biology* **209**, 4638-51.

Pullikuth, A. K., Aimanova, K., Kang'ethe, W., Sanders, H. R. and Gill, S. S. (2006). Molecular characterization of sodium/proton exchanger 3 (NHE3) from the yellow fever vector, *Aedes aegypti*. *J Exp Biol* **209**, 3529-3544.

Ramasamy, R., Surendran, S. N., Jude, P. J., Dharshini, S. and Vinobaba, M. (2011). Larval development of *Aedes aegypti* and *Aedes albopictus* in peri-urban brackish water and its implications for transmission of arboviral diseases. *PLoS Negl Trop Dis* **5**, e1369.

Ramsay, J. A. (1950). Osmotic regulation in mosquito larvae. *J Exp Biol* **27**, 145-57.

Seron, T. J., Hill, J. and Linser, P. J. (2004). A GPI-linked carbonic anhydrase expressed in the larval mosquito midgut. *J Exp Biol* **207**, 4559-72.

Stobbart, R. H. (1971). Evidence for Na^+/H^+ and Cl^-/HCO_3^- exchanges during independent sodium and chloride uptake by the larva of the mosquito *Aedes aegypti* (L.). *J Exp Biol* **54**, 19-27.

Stobbart, R. H. (1974). Electrical potential differences and ionic transport in the larva of the mosquito *Aedes aegypti* (L.). *J Exp Biol* **60**, 493-533.

Surendran, S. N., Jude, P. J., Thabothiny, V., Raveendran, S. and Ramasamy, R. (2012). Pre-imaginal development of *Aedes aegypti* in brackish and fresh water urban domestic wells in Sri Lanka. *J Vector Ecol* **37**, 471-3.

Takeyasu, K., Tamkun, M. M., Renaud, K. J. and Fambrough, D. M. (1988). Ouabain-sensitive Na^+/K^+-ATPase activity expressed in mouse L cells by transfection with DNA encoding the alpha-subunit of an avian sodium pump. *J Biol Chem* **263**, 4347-4354.

Tripathi, S., Morgunov, N. and Boulpaep, E. L. (1985). Submicron tip breakage and silanization control improve ion-selective microelectrodes. *Am J Physiol* **249**, C514-21.

Veenstra, J. A. (1988). Effects of 5-hydroxytryptamine on the Malpighian tubules of *Aedes aegypti*. *J Insect Physiol* **34**, 299-304.

Volkmann, A. and Peters, W. (1989a). Investigations on the midgut caeca of mosquito larvae-I. Fine structure. *Tissue Cell* **21**, 243-51.

Volkmann, A. and Peters, W. (1989b). Investigations on the midgut caeca of mosquito larvae-II. Functional aspects. *Tissue Cell* **21**, 253-61.

Weng, X. H., Huss, M., Wieczorek, H. and Beyenbach, K. W. (2003). The V-type H^+ ATPase in Malpighian tubules of *Aedes aegypti*: localization and activity. *Journal of Experimental Biology* **206**, 2211-9.

Wigglesworth, V. B. (1933a). The adaptation of mosquito larvae to salt water. *J Exp Biol*, 27-36.

Wigglesworth, V. B. (1933b). The function of the anal gills of the mosquito larva. *J Exp Biol* **10**, 16-26.

Wigglesworth, V. B. (1938). The regulation of osmotic pressure and chloride concentration in the haemolymph of mosquito larvae. *J Exp Biol* **15**, 235-247.

Wigglesworth, V. B. (1942). The storage of protein, fat, glycogen and uric acid in the fat body and other tissues of mosquito larvae. *J Exp Biol* **1942**, 56-77.

Zhuang, Z., Linser, P. J. and Harvey, W. R. (1999). Antibody to H^+ V-ATPase subunit E colocalizes with portasomes in alkaline larval midgut of a freshwater mosquito (*Aedes aegypti*). *J Exp Biol* **202**, 2449-60.

Figures

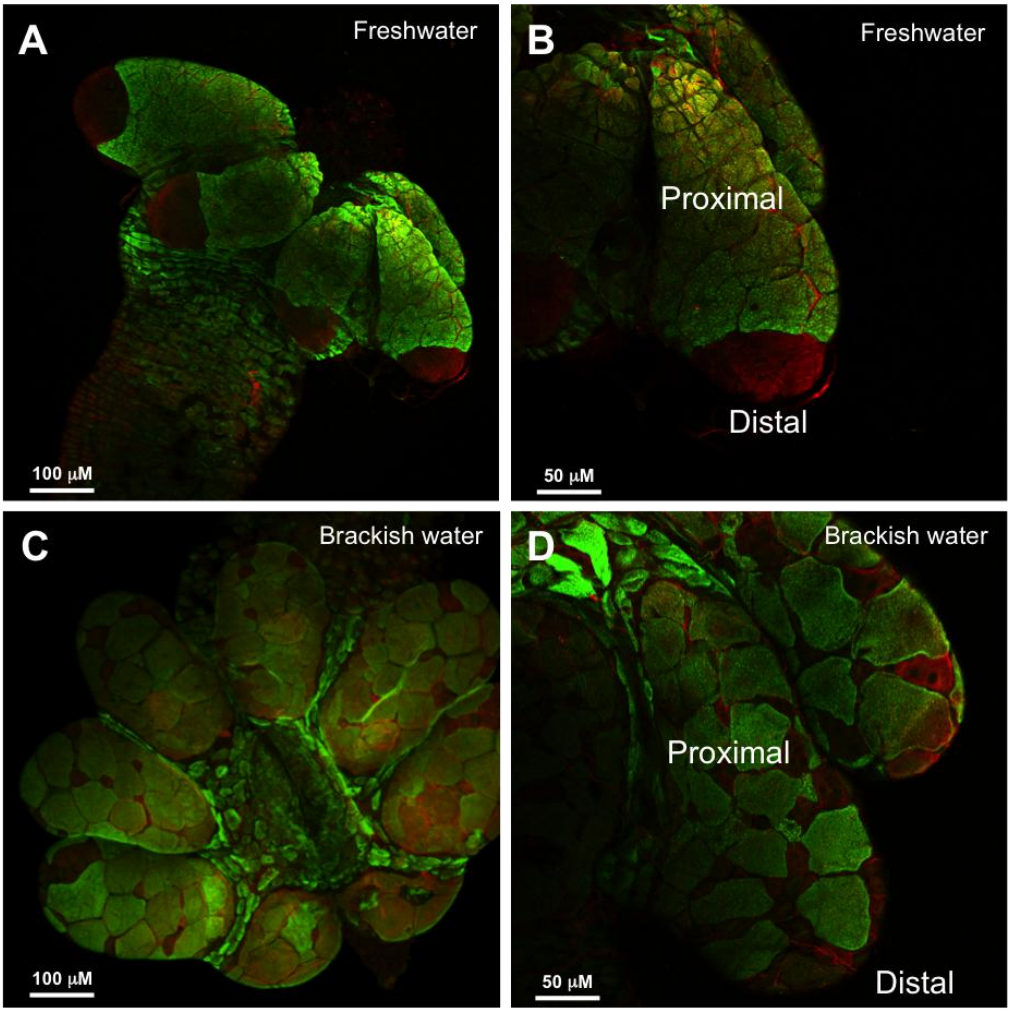

Figure 1: Immunolocalization of V-type H^+-ATPase (VA; red) and Na^+/K^+-ATPase (NKA; green) in the gastric caeca in whole mounts of fourth instar *Aedes aegypti* larvae. In larvae reared in fresh water (A-B), the distal third expressed VA, and the proximal two-thirds expressed NKA. In larvae reared in brackish water (C-D), there were smaller cells expressing VA interspersed between the NKA expressing cells. All images were overlays of both Alexa fluor 488 (green) and Cy3 (red) signals.

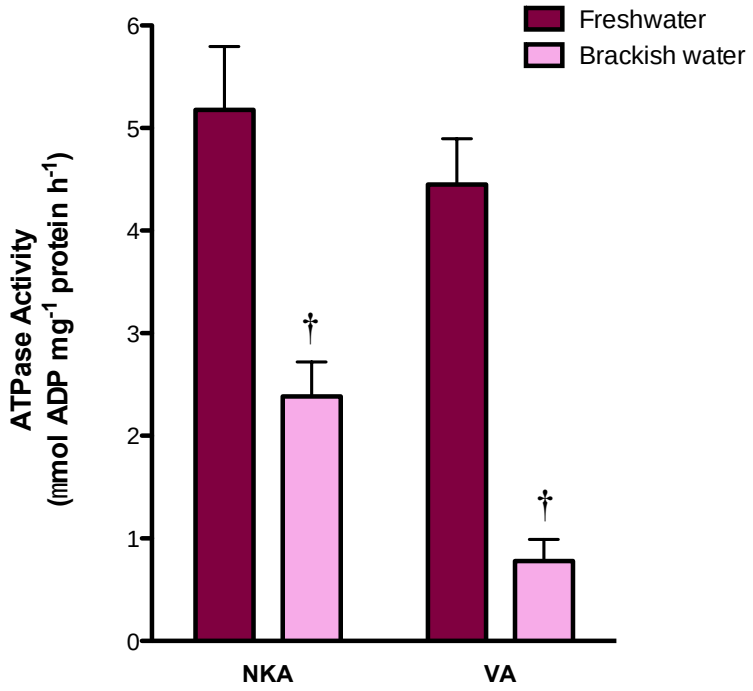

Figure 2: NKA and VA ATPase activity for caeca of fourth instar Aedes aegypti larvae reared in fresh water or brackish water. Data are expressed as mean ± sem (N= 8). Dagger symbols indicate significant differences for either NKA or VA in freshwater versus brackish water. Significance ($p < 0.05$) was determined by two-way ANOVA followed by Tukey's post-hoc test.

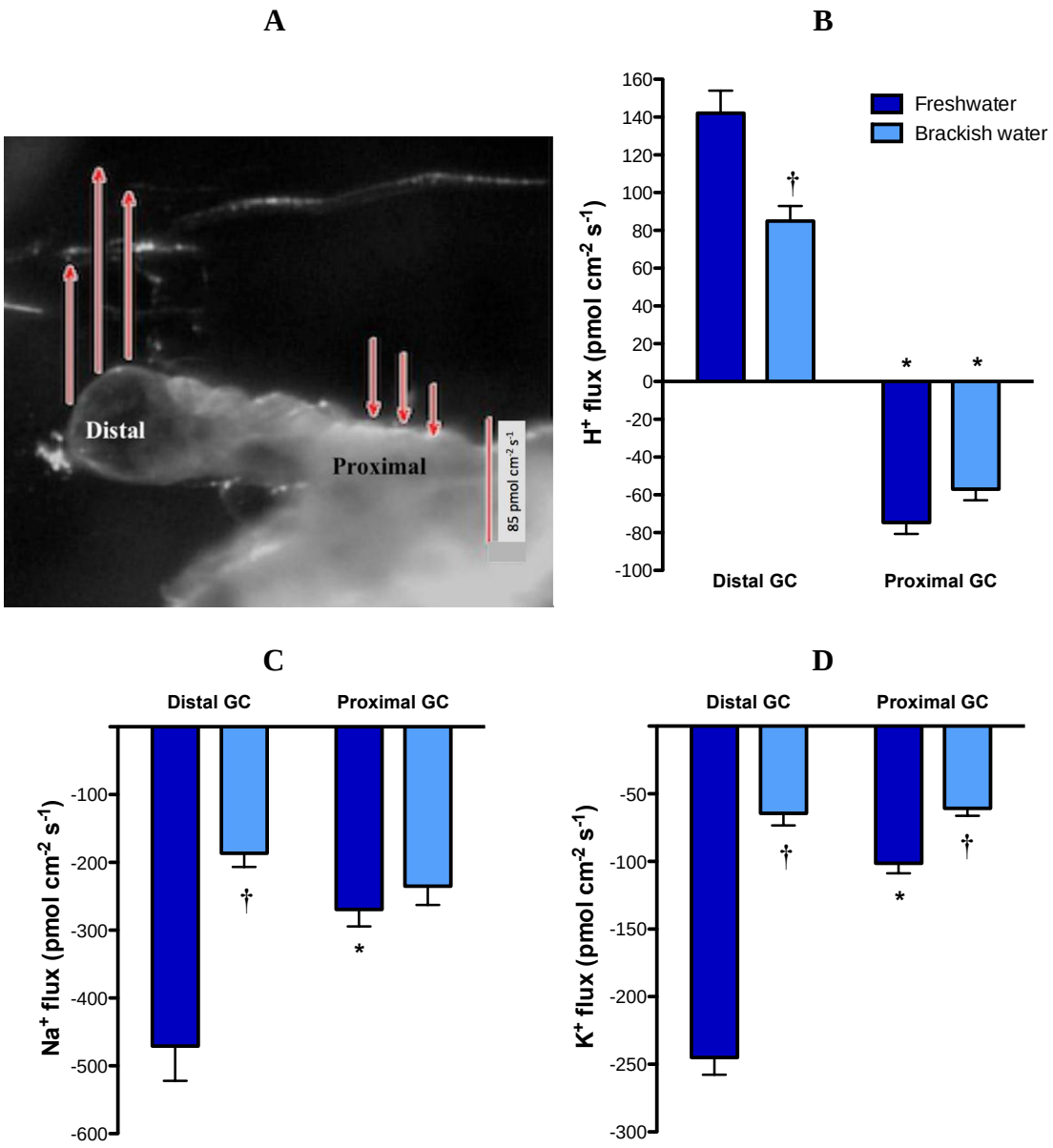

Figure 3: Scanning Ion-Selective Microelectrode Technique (SIET) measurements of (A) representative example of *in vivo* H^+ transport along the surface of the gastric

caecum of larvae reared in fresh water (B) H^+, (C) Na^+, and (D) K^+ transport rates along the distal and proximal gastric caecum (GC) of fourth instar *Aedes aegypti* larvae reared in fresh water or brackish water. Positive values denote absorption of the ion (i.e. from lumen to bath); negative values denote ion secretion from the bath towards the lumen. Data are expressed as mean ± sem (N= 12-20). Asterisks indicate significant difference between the rates of transport across the distal versus proximal regions for larvae reared in the same rearing medium (fresh water or brackish water). Dagger symbols indicate significant differences between ion transport rates for caeca of fresh water versus brackish water larvae within a region (distal or proximal). Significance ($p < 0.05$) was determined by two-way ANOVA followed by Tukey's post-hoc test.

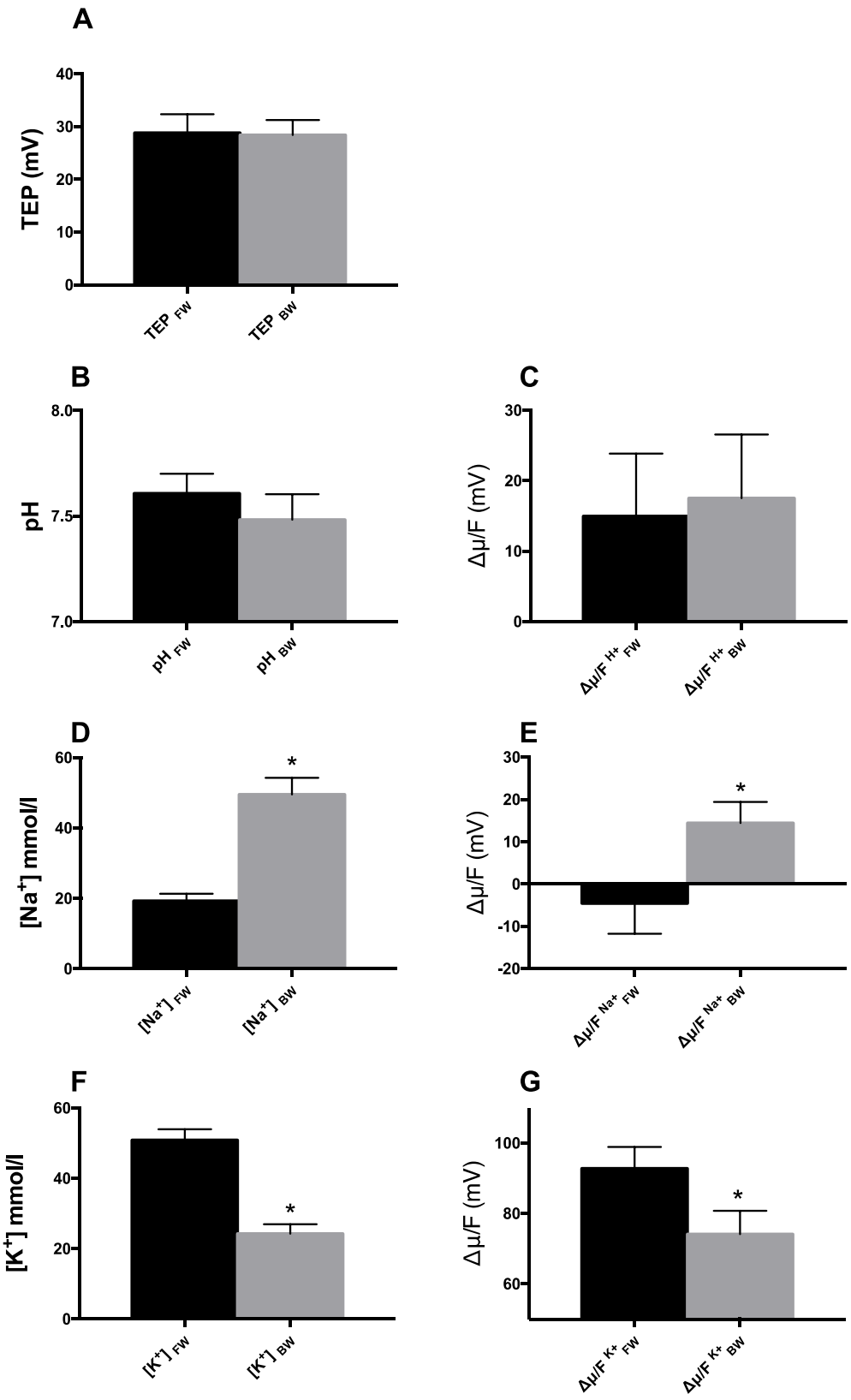

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Figure 4: (A) Transepithelial potential difference (TEP) measurements, luminal (B) pH (D) Na^+ (F) K^+ concentrations, and the electrochemical potentials for (C) pH (E) Na^+ (G) K^+ of the gastric caecum of fourth instar *Aedes aegypti* larvae reared in fresh water or brackish water. Data are expressed as mean ± sem (N= 8). Asterisks indicate significant difference between fresh water or brackish water treatments. Significance of differences between FW and BW means of each parameter ($p < 0.05$) were determined by Students *t*-tests.

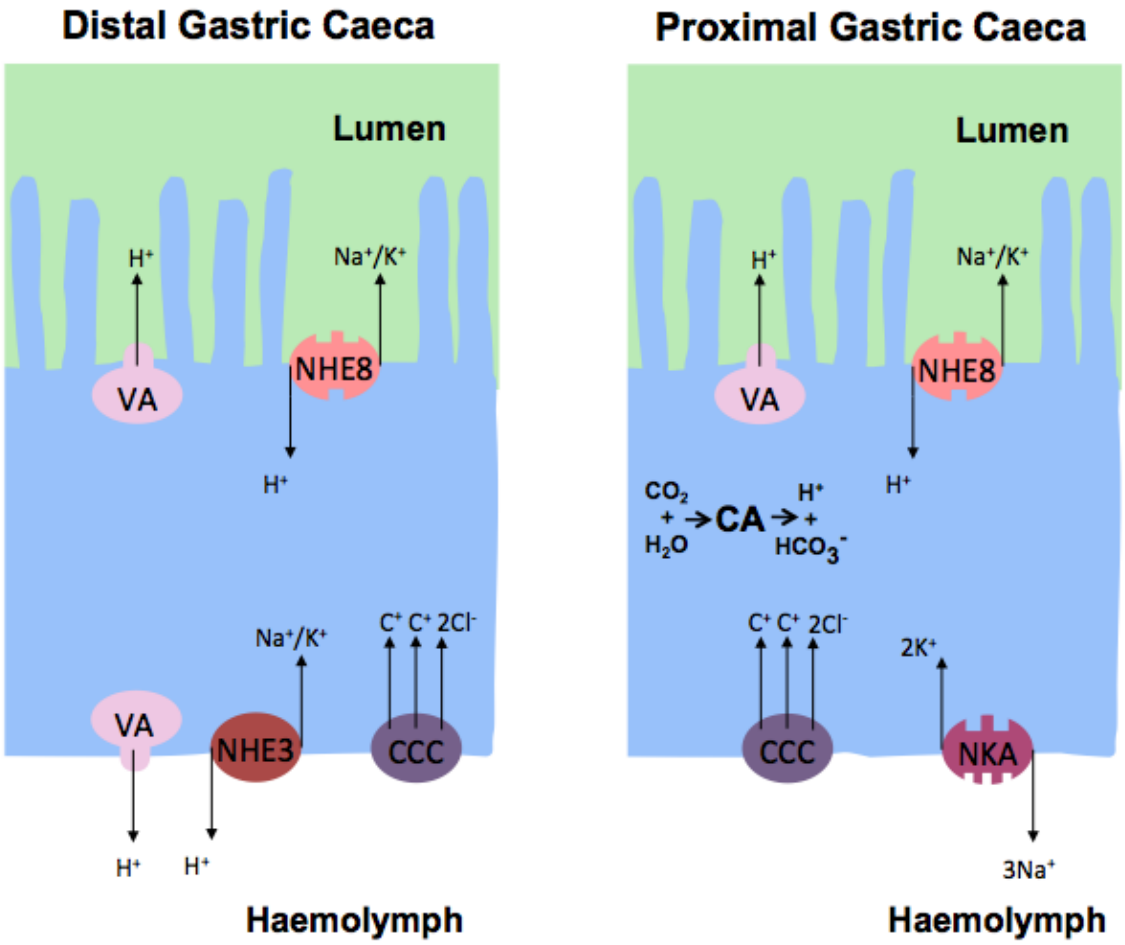

Figure 5: Schematic diagram illustrating the role of membrane transport proteins in H^+, Na^+ and K^+ transport at the distal and proximal gastric caecum. VA, V-type H^+ ATPase; NKA, Na^+/K^+ ATPase; NHE3, *A. aegypti* Na^+/H^+ Exchanger (AeNHE3); NHE8, *A. aegypti* Na^+/H^+ Exchanger (AeNHE8); C^+, cation (Na^+ or K^+); CCC, Cation Chloride Cotransporter; CA, Carbonic Anhydrase.

Table 1: Basolateral surface area (μm^2) measurements for V-type H^+-ATPase-rich (VA) and Na^+/K^+-ATPase-rich (NKA) cells of the gastric caeca of fourth instar *Aedes aegypti* larvae, measured on images obtained after immunolocalization of whole mounts, using ImageJ. N is the total number of cells measured for each treatment.

	Fresh water			Brackish water		
	Mean (μm^2)	sem	N	Mean (μm^2)	sem	N
VA	2178	273	15	249	25	15
NKA	3103	220	15	3102	326	15