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RESEARCH ARTICLE

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 freshwater 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 freshwater. 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 freshwater, 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 freshwater 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.

KEY WORDS: Mosquito, V-ATPase, Na^+/K^+-ATPase, Ion-selective microelectrodes, Ionoregulation, Gastric caecum

INTRODUCTION

Aedes aegypti is responsible for the spread of dengue, Zika fever, chikungunya and yellow fever, all of which infect millions of humans every year (Tolle, 2009). The *A. aegypti* larval stage inhabits a variety of aquatic habitats, such as marshes, drains and rock pools. Although the preferred medium for *A. aegypti* larvae is freshwater, it has been reported that the adults also lay eggs in brackish water (5–30% 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 freshwater habitats (Surendran et al., 2012); therefore, understanding larval physiology and how they adapt to different rearing salinities can provide the foundation for the development of novel larvicides.

Freshwater mosquito larvae face the challenge of dilution of body fluids through freshwater 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 freshwater maintain their haemolymph ion concentrations at levels above those in the external medium by hyper-regulation (Bradley, 1994; Wigglesworth, 1938). Hyper-regulation of the body fluids is achieved through a 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,b, 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).

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 channelled 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, 1942). The caecum carries out these functions using four types of cells: ion transporting cells, reabsorbing/secreting cells, imaginal cells and cells that 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 freshwater. 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

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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 and colleagues 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 freshwater 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 in Hasselquist 1762) larvae were reared in freshwater (dechlorinated tap water) or brackish water (30% seawater; 10.5 g Instant Ocean® Sea Salt/litre 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 *A. 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) and was a gift from S. S. Gill (University of California, Riverside). NKA was localized using a monoclonal antibody (mAb), 'a5', raised against the α-subunit of avian P-type NKA 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. The secondary antibodies used were Alexa-Fluor-488-labelled goat anti-mouse and Cy3-labelled goat anti-rabbit (Jackson ImmunoResearch, 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 previously (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 α5 (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 and 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, three VA-rich and three NKA-rich cells were measured for each of five tissue samples. Although the measurements were done on whole mounts, we assumed that the surfaces were planar. Because 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-sensitive (Sigma-Aldrich, Oakville, ON, Canada) or bafilomycin-sensitive (LC Laboratories, Woburn, MA, USA) 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 that had been pre-coated with poly-L-lysine (0–150 kDa, Sigma-Aldrich) 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. Because 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 min of dissection because rates of ion secretion and absorption decayed steadily after the first 3–6 min (N.M.D. and M.J.O., unpublished). Previous measurements have shown that 5-hydroxytryptamine (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 TEP and luminal H^+, Na^+ and K^+ concentrations

Luminal H^+, Na^+ and K^+ concentrations and TEP 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 hole in the plastic lid of a 3 ml glass vial containing ∼200 µl of dimethyldichlorosilane. After 15–20 s, the pipette was transferred onto the surface of a hot plate at 200°C for 10–15 s. A small volume (∼100 nl) of the appropriate ion exchanger cocktail was introduced into the shank of the silanized barrel.

pH microelectrodes were first backfilled 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 tenfold change in ion concentration or 1 pH unit were: 57–60, 52–54 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 resistor–capacitor 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 analog-to-digital converter and data acquisition system (PowerLab and LabChart software; ADInstruments Inc., Colorado Springs, CO, USA).

Calculation of electrochemical potentials

The electrochemical potential [the change in chemical potential (Δµ)/Faraday's constant (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, USA) and values are expressed as means±s.e.m. 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 means± s.e.m. for a number of caeca (N). Each mean value was based on three replicate scans at three 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 means±s.e.m. (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 freshwater, VA was expressed along the distal third of the caeca and NKA was expressed along the proximal two-thirds (Fig. 1A,B), as shown previously (Patrick et al., 2006). Clustering of VA-rich cells in the distal region of the gastric caeca of larvae reared in brackish water was not as apparent as in freshwater (Fig. 1D), and VA-rich cells were interspersed between the NKA-rich cells in the proximal gastric caeca of larvae reared in brackish

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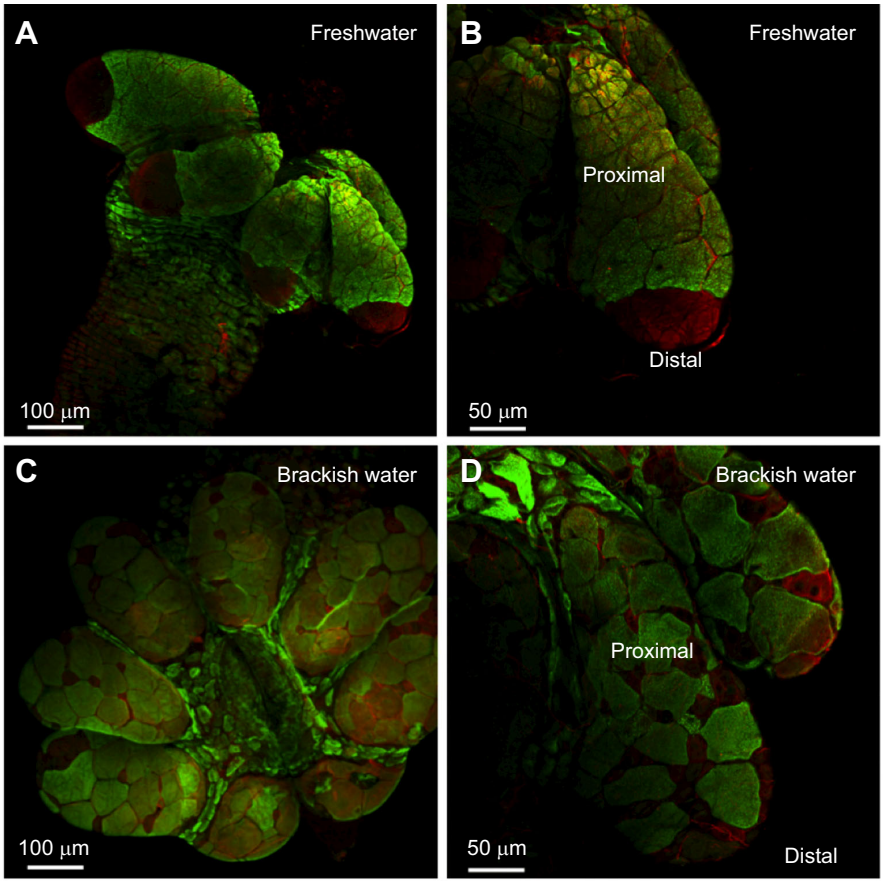

Fig. 1. Immunolocalization of ATPases in the gastric caeca in whole mounts of fourth instar *Aedes aegypti* larvae. In larvae reared in freshwater (A,B), the distal third expressed V-type H^+-ATPase (VA; red) and the proximal two-thirds expressed Na^+/K^+-ATPase (NKA; green). In larvae reared in brackish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

water (Fig. 1C,D). The basolateral surface area of the VA-rich cells was ~249 µm^2, approximately ninefold lower than the corresponding surface area of ~3102 µm^2 of the NKA-rich cells of larvae reared in brackish water (Fig. 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 freshwater (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 freshwater (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

Table 1. Basolateral surface area measurements for V-type H^+-ATPase (VA)-rich and Na^+/K^+-ATPase (NKA)-rich cells of the gastric caeca of fourth instar *Aedes aegypti* larvae

	Freshwater		Brackish water	
	Mean±s.e.m. (µm^2)	*N*	Mean±s.e.m. (µm^2)	*N*
VA	2178±273	15	249±25	15
NKA	3103±220	15	3102±326	15

Measurements are from images obtained after immunolocalization of whole mounts, using ImageJ. *N* is the total number of cells measured for each treatment.

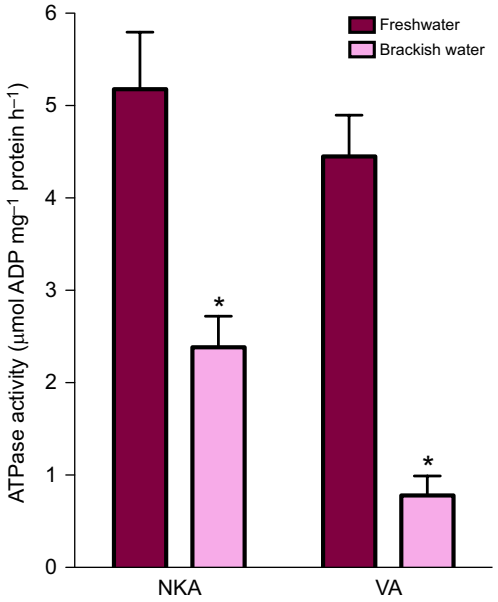

Fig. 2. NKA and VA ATPase activity for caeca of fourth instar *A. aegypti* larvae reared in freshwater or brackish water. Data are expressed as means±s.e.m. (*N*=8). *Significant differences for either NKA or VA in freshwater versus brackish water. Significance (P<0.05) was determined by two-way ANOVA followed by Tukey's *post hoc* test.

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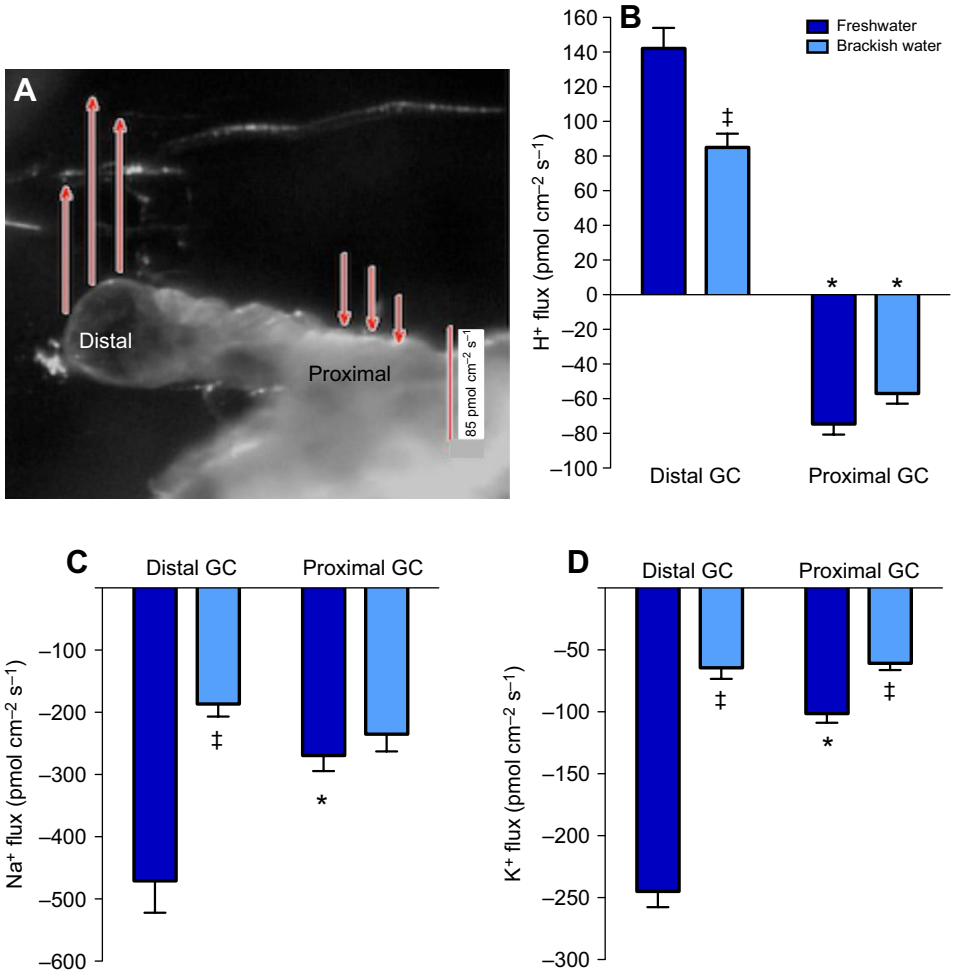

Fig. 3. Scanning ion-selective microelectrode technique (SIET) measurements of fourth instar *A. aegypti* larvae reared in freshwater or brackish water. (A) Representative example of *in vivo* H^+ transport along the surface of the gastric caecum of larvae reared in freshwater. (B) H^+, (C) Na^+ and (D) K^+ transport rates along the distal and proximal gastric caecum (GC). 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 means± s.e.m. (*N*=12–20). *Significant difference between the rates of transport across the distal versus proximal regions for larvae reared in the same rearing medium (freshwater or brackish water). ‡Significant differences between ion transport rates for caeca of freshwater 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.

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 freshwater 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 freshwater compared to larvae reared in brackish water (Fig. 3B).

Na^+ secretion rates at the distal gastric caecum were higher in larvae reared in freshwater in comparison to larvae reared in brackish water (Fig. 3C). At the proximal gastric caecum, the rate of secretion of Na^+ in larvae reared in freshwater was similar to larvae reared in brackish water (Fig. 3C). In larvae reared in freshwater, 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 freshwater than the corresponding rates for larvae reared in brackish water (Fig. 3D). In larvae reared in freshwater, 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).

TEP 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 freshwater-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 negative TEP of the lumen of the adjacent anterior midgut measured within 10 min of impalement (−11.6±5.3 mV, *N*=10). Previous studies in which the anterior midgut was perfused with symmetrical saline also showed a negative lumen TEP, of −66 mV, which decayed to −10 mV within 10–15 min (Clark et al., 1999).

Luminal pH of the gastric caecum of freshwater-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 freshwater (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 freshwater (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

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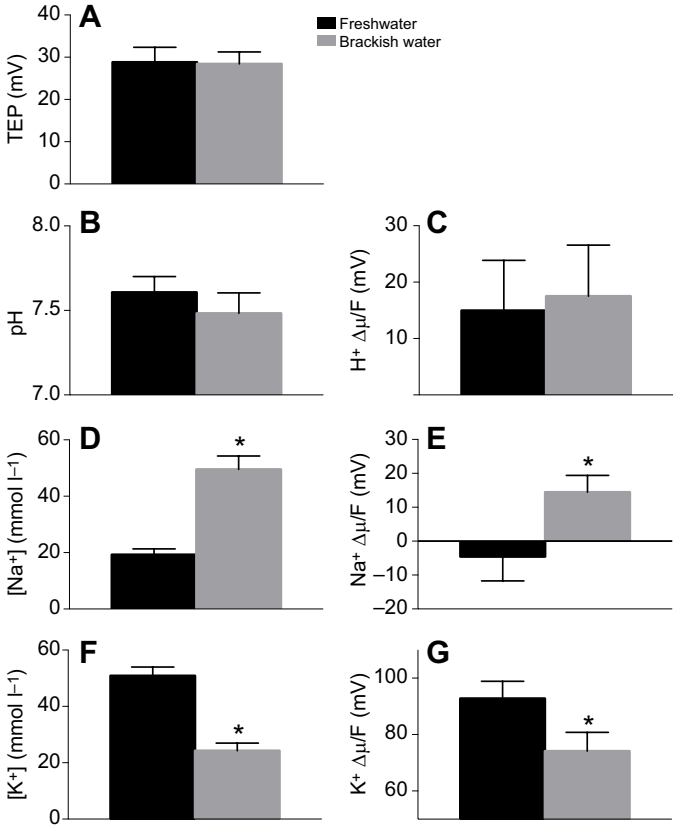

Fig. 4. TEP and transepithelial electrochemical potential for H^+, Na^+ and K^+ of fourth instar *A. aegypti* larvae reared in freshwater or brackish water. (A) Transepithelial potential difference (TEP) measurements. Luminal pH (B), and luminal (D) Na^+ and (F) K^+ concentrations, and the electrochemical potentials [$\Delta\mu/F$; the change in chemical potential ($\Delta\mu$)/Faraday's constant (F)] for (C) pH, (E) Na^+ and (G) K^+ of the gastric caecum of fourth instar *A. aegypti* larvae reared in freshwater or brackish water. Data are expressed as means±s.e.m. (N=8). *Significant difference between freshwater or brackish water treatments. Significance of differences between freshwater and brackish water means of each parameter (P<0.05) were determined by Student's t-tests.

compared with freshwater 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 freshwater (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 because the TEP is declining. A parallel study (N.M.D. and M.J.O., 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-HT (serotonin; 1 μmol l^{-1}). The directions of transport across the caeca are the same in the presence or absence of 5-HT (N.M.D. and M.J.O., 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; Filippov et al., 2003). 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 freshwater and brackish water also implicate the caecum in ionoregulation.

Freshwater

Our study confirms the regionalization of VA and NKA along the basal membrane of the gastric caeca of larvae reared in freshwater (Fig. 1A,B), consistent with the findings of Patrick et al. (2006). In addition, our measurements of ATPase activity show that VA and NKA are functional ATPases in the caeca, and that ATPase activity is higher in the caeca of larvae reared in freshwater relative to those reared in brackish water (Fig. 2). Fig. 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 in the sections below of H^+, Na^+ and K^+ transport rates across the distal and proximal regions of the caecum.

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 freshwater (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 owing to the cycling of H^+ across the apical membrane and 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^+

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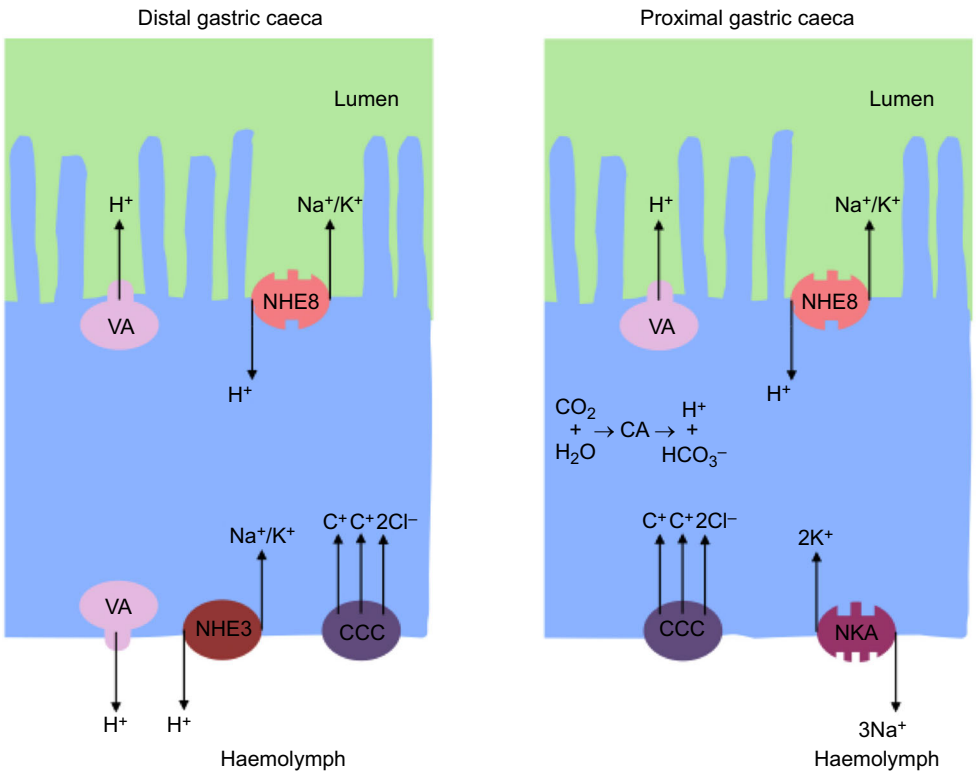

Fig. 5. Schematic diagram illustrating the role of membrane transport proteins in H^+, Na^+ and K^+ transport at the distal and proximal gastric caecum. VA, V-type H^+-ATPase; NKA, Na^+/K^+-ATPase; NHE3, *A. aegypti* Na^+/H^+ exchanger (AeNHE3); NHE8, *A. aegypti* Na^+/H^+ exchanger (AeNHE8); C^+, cation (Na^+ or K^+); CCC, cation chloride cotransporter; CA, carbonic anhydrase.

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-barrelled 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 (Figs 3C,D and 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 freshwater 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 summary, H^+ and K^+ are actively secreted into the lumen of the gastric caecum, 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 freshwater (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 freshwater 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 freshwater. 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) because the larvae do not face an osmotic challenge (Clark et al., 2004). In contrast, in larvae reared in freshwater, a higher mitochondrial density and higher ATPase activity may be necessary for the larvae to hyper-regulate (Bradley, 1994; Wigglesworth, 1938). When freshwater 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 freshwater 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,b) correspond to VA-rich and NKA-rich cells, respectively (Fig. 1C,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 freshwater (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 freshwater, 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 freshwater, 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,b). 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 freshwater. In contrast, luminal K^+ concentration is significantly lower in the caecum of larvae reared in brackish water compared with freshwater (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 ($\sim$70 mmol l^{-1}) in the lumen of larvae reared in either fresh or 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 freshwater and brackish water.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.M.D., M.J.O.; Methodology: N.M.D., M.L.P., M.J.O.; Formal analysis: N.M.D., M.L.P., M.J.O.; Investigation: N.M.D., M.L.P., M.J.O.; Writing - original draft: N.M.D., M.J.O.; Writing - review & editing: N.M.D., M.L.P., M.J.O.; Supervision: M.J.O.; Funding acquisition: M.J.O.

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