

The gastric caecum of larval *Aedes aegypti*: stimulation of epithelial ion transport by 5-hydroxytryptamine and cAMP

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Abstract

We report measurements of ion transport across the gastric caecum of larvae of *Aedes aegypti*, a vector of yellow fever that inhabits a variety of aquatic habitats ranging from freshwater to brackish water. We provide the first measurements of the effect of 5-hydroxytryptamine (5-HT) on transepithelial potential (TEP), luminal ion concentrations and electrochemical potentials, as well as basolateral membrane potential and H^+ , Na^+ and K^+ fluxes. TEP, basolateral membrane potential, and H^+ , K^+ , and Na^+ fluxes across the gastric caeca declined within 3-6 mins after isolation of the entire midgut from the larva. 5-HT restored both the TEP and active accumulation of H^+ , K^+ , and Na^+ in the lumen. Additionally, 5-HT restored H^+ , K^+ , and Na^+ fluxes across the distal caecum of freshwater larvae, and restored H^+ fluxes across the distal caecum of brackish water larvae. There was no effect of 5-HT on ion fluxes across the proximal caecum. We have also shown that 5-HT restores the basolateral membrane potential in cells of the distal, but not proximal, caecum. Effects of 5-HT on TEP and basolateral membrane potential were mimicked by application of cAMP but not by a phorbol ester. We provide a working model which proposes that 5-HT and cAMP stimulate the vacuolar H^+ -ATPase of the distal caecum. Our results provide evidence that the gastric caecum is functionally distinct from the adjacent anterior midgut and we discuss possible roles of the gastric caecum in osmoregulation. We also describe similarities in the arrangement of ion transporters in the caecum with those of the Malpighian tubules.

1. Introduction

The larvae of *Aedes aegypti* possess gastric caeca, which are eight blind sacs that are situated immediately posterior to the cardia and open into the anterior region of the midgut (Volkman and Peters, 1989a). These gastric caeca are hypothesized to be important for digestion, nutrient and fluid reabsorption, and ion homeostasis (Jones and Zeve, 1968; Ramsay, 1950; Volkman and Peters, 1989b; Wigglesworth, 1933; Wigglesworth, 1942).

Larvae of *A. aegypti* inhabit a variety of aquatic habitats ranging from freshwater to brackish water. It has been reported that although the preferred medium for larvae is freshwater, 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). *A. aegypti* is responsible for the spread of dengue, Zika fever, chikungunya and yellow fever, and current mosquito population control measures are focused on freshwater larvae (Surendran et al., 2012). Understanding how larvae adapt to different rearing salinities can provide the foundation for development of novel larvicides.

In freshwater larvae there is a striking regionalization of vacuolar-type H⁺-ATPase (VA) and Na⁺/K⁺-ATPase (NKA) along the gastric caeca (D'Silva et al., 2017; Patrick et al., 2006). In larvae reared in brackish water this regionalisation is lost, and VA and NKA expression on the basal membrane appears as a mosaic pattern along the length of the caeca, with VA-rich cells being significantly smaller than the NKA-rich cells (D'Silva et al., 2017). It is unclear as to why these morphological changes occur, however, it was proposed that the ion transporting cells and digestive cells observed in previous studies (Volkman and Peters, 1989a; Volkman and Peters, 1989b) correspond

to VA-rich and NKA-rich cells, respectively (D'Silva et al., 2017). Since brackish water (30% sea water) is isosmotic to the larval haemolymph (Clark et al., 2004), the larvae do not need to hyper-regulate as freshwater larvae do. Cell size, and the length and diameter of apical microvilli in the ion transporting cells decrease with increases in water salinity (Volkman and Peters, 1989b). In conjunction with changes in VA regionalization in the caecum in freshwater versus brackish water larvae, these changes implicate the gastric caecum in osmoregulation (Patrick et al., 2006; Volkman and Peters, 1989b).

Ion transport and transepithelial potentials of the gastric caecum decreased precipitously within minutes of removal of the gut from the larva and bathing it in physiological saline (D'Silva et al., 2017). Previous studies showed that the transepithelial potential of the anterior midgut and posterior midgut of *A. aegypti* declined within the first few minutes, but recovered in response to serotonin (5-hydroxytryptamine; hereafter 5-HT) (Clark et al., 1999). 5-HT is a biogenic amine, which acts as a neurotransmitter in both vertebrates and invertebrates. In contrast to extensive studies on the effects of 5-HT on the physiological functions of the anterior and posterior midgut of invertebrates (Clark et al., 1999; Onken and Moffett, 2009; Onken et al., 2004a), its effects on the gastric caecum have yet to be studied.

This study is the first to examine the effects of 5-HT on the H^+ , Na^+ and K^+ transport rates along both the distal and proximal regions of the gastric caecum of *A. aegypti*, in addition to examining its effects on caeca from larvae reared in either freshwater or brackish water. We have also identified the putative intracellular second messenger which mediates the action of 5-HT. Whether 5-HT alters passive or active ion transport across the gastric caeca of freshwater and brackish water larvae has

been determined by calculation of electrochemical potentials for H^+ , Na^+ and K^+ from measurements of transepithelial potential (TEP), luminal pH, Na^+ concentration and K^+ concentration. Our results indicate that 5-HT's effects depend upon whether larvae are reared in freshwater or brackish water, and that 5-HT may be involved in controlling osmoregulation in the gastric caeca.

2. Materials and methods

2.1 Raising larvae

Aedes aegypti (Linnaeus) larvae were reared in freshwater (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:yeast mixture *ad libitum*. Larvae were raised at room temperature on a 12 h:12 h light:dark cycle. Fourth instar larvae were used for all experiments.

2.2 Dissections and Physiological Saline

The entire gut from each fourth instar larva, reared in freshwater or brackish water, was dissected out in *A. aegypti* 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 (70-150 kDa, Sigma-Aldrich Canada, Ltd.) to aid tissue adherence to the bottom of the dish, as described by (Naikkhwah and O'Donnell, 2011). All experiments were done at room temperature (23° C).

2.3 Measurement of transepithelial potential (TEP) and basolateral membrane potentials

Transepithelial potential (TEP) and basolateral membrane potentials were measured in the gastric caecum of *A. aegypti* larvae using double-barrelled theta-glass microelectrodes (TST150, WPI, New Haven, CT, USA); the natural bevel resulting from the prominent spear-like projection of the central septum facilitates impalement, allowing multiple impalements using a single electrode. After pulling, both barrels were filled with 150 mmol l⁻¹ KCl.

TEP measurements were recorded after impaling the gastric caecum and advancing the electrode until it rested in the caecal lumen. Basolateral membrane potentials were recorded after impaling the basolateral membrane of a distal gastric caecal cell or a proximal gastric caecal cell. Measurements for the initial TEP and basolateral membrane potential were made in larval *A. aegypti* saline at 3 time points: within 3 minutes of dissection (labelled 'Initial' in the Figures); 10 minutes after the initial measurement, before the addition of pharmacological agents (labelled 'Before' in the figures); 15 minutes after addition of the agent (labelled 'After' in the figures). The agents added to the bathing saline were: 5-HT (1 μM); ketanserin (5-HT antagonist, specific to 5-HT type 2 receptor ((Van Nueten et al., 1983), 10 μM); 8-bromo-cAMP (8-Br-cAMP, a cell permeable analogue of cAMP ((Sandberg et al., 1991); 100 μM); phorbol 12, 13-diacetate (PE, a protein kinase C (PKC) activator (Castagna et al., 1982), 22.3 μM); or dimethyl sulfoxide (DMSO, the carrier for ketanserin and PE; 0.005 %).

The effects of 5-HT, ketanserin, 8-Br-cAMP, or PE on basolateral membrane potential were also investigated.

2.4 Measurement of TEP and lumenal H⁺, Na⁺ and K⁺ concentrations

Lumenal H⁺, Na⁺ or K⁺ concentration and transepithelial potential were measured simultaneously in the gastric caecum of *A. aegypti* larvae using ion-selective double-barrelled microelectrodes as described previously (D'Silva et al., 2017). One impalement was made per gastric caecum, and voltages were measured at three time points: within 3 mins of dissection, after a further 10 minutes in saline, and finally 15 minutes after addition of 5-HT.

Micropipettes were pulled from theta-glass borosilicate capillaries (TST150, WPI, New Haven, CT, USA). 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 NaH₂PO₄, 0.1 Na₂HPO₄, 1.8 CaCl₂, 1.0 MgCl₂, 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 upper part of the barrel. 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 viewing the tip region under a stereo microscope and 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 ($>10^9 \Omega$) of the ion-selective barrel. V_{ref} and the difference ($V_i - V_{\text{ref}}$) were recorded using an AD converter and data acquisition system (PowerLab and LabChart software; ADInstruments Inc., CO, USA). Ion concentrations in the gastric lumen were calculated from the measured voltage voltages, as described previously (Ianowski et al., 2002).

2.5 Calculation of electrochemical potentials

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

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

where R is the gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$), T is the temperature in degrees Kelvin, F is the Faraday constant ($9.648 \times 10^4 \text{ C mol}^{-1}$), $[\text{ion}]_{\text{lumen}}$ 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 (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.

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

H^+ , Na^+ and K^+ scans were carried out in *A. aegypti* larval saline. Since protons may diffuse freely or in association with buffers in the saline, 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). Rates of ion secretion (from bath to lumen) or absorption (from lumen to bath) were then estimated from the measured concentration gradients using Fick's equation. Measurements were made at three sites, 50 μm apart, along the distal end or proximal end of gastric caecum.

All initial measurements were carried out within 3 minutes of dissection since rates of ion secretion and absorption decayed steadily after the first 3-6 minutes. 1 μM 5-HT was added to the bathing saline after the initial measurement, and the consequent effect on H^+ , Na^+ and K^+ fluxes were measured at 3 min intervals up to 30 min using SIET. Saline was used as a control for all data sets. 5-HT did not alter the slope of the H^+ , Na^+ or K^+ - selective electrodes.

2.7 Statistical analysis

Data were plotted using Graphpad InStat (Graphpad Software Inc., La Jolla, CA). TEP, basolateral membrane potential, luminal H^+ , Na^+ and K^+ concentrations and electrochemical potentials are expressed as mean \pm sem (N). The parameters were plotted with respect to treatment (saline, 5-HT, ketanserin, DMSO, 8-Br-cAMP, or PE) and analyzed by one-way ANOVA followed by Tukey's post-hoc test. A two-tailed paired t-test was used to analyze TEP for treatments (before and after 5-HT) within each concentration group. The rates of H^+ , Na^+ and K^+ transport are expressed as mean \pm sem for a number of caeca (N). The value for each caecum was based on the mean of measurements at 3 sites along the distal or proximal region of the caecum, and at each site a mean was calculated for 3 replicate measurements. H^+ ,

Na⁺ and K⁺ transport rates were plotted against treatment (saline or 5-HT) and time and analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. In all statistical tests, differences were considered significant at P<0.05.

3. Results

3.1 Transepithelial potential (TEP)

The lumen of the gastric caecum was at a positive potential with respect to the bathing saline (Fig. 1A, B), consistent with previous findings (D'Silva et al., 2017). TEP declined significantly over time when left unstimulated in physiological saline, in both freshwater (Fig. 1A, B) and brackish water reared larvae. A representative time-course of the lumen positive TEP of a preparation of the gastric caeca of larval *A. aegypti* shows the decline in TEP measured for multiple gastric caeca within the same larval preparation (Fig. 1A). The decline in TEP was consistent for the different caeca, numbered 1-5, for a single larval preparation, over time (Fig. 1A). Measurements of TEP in gastric caeca from multiple larval preparations over a time course of 0-8 mins indicated a one-phase exponential decay (Fig. 1B).

In larvae reared in freshwater, TEP recovered to its initial voltage within 12 - 15 minutes following addition of 1 μ M 5-HT (Fig. 2A), a concentration used previously on the larval midgut (Clark et al., 1999; Clark et al., 2000). After re-establishing a steady TEP, 5-HT was washed out and replaced by bathing saline. Washout of 5-HT resulted in a decline of TEP within 5 – 8 minutes to values not significantly different from the 'Before 5-HT' values (Fig. 2A). Addition of 0.1 and 10 μ M 5-HT also caused significant increases in TEP within 15 minutes (Figure

2B). In larvae reared in brackish water, TEP recovered to its initial voltage within 15 – 20 minutes of addition of 1 μ M 5-HT (Fig. 2C). Although we did not complete a detailed pharmacological analysis of the type of 5-HT receptor that mediates these responses, the 5-HT type 2 receptor antagonist ketanserin had no effect on TEP when added after or before the addition of 5-HT (Fig. S1A, B).

3.2. Intracellular signaling molecules

Having established the effects of 5-HT on TEP recovery, we wanted to determine the intracellular signaling molecules acting downstream of 5-HT signaling. To this end, we tested the effects of 8-bromo-cAMP (8-Br-cAMP), a cell permeable analogue of cAMP that mimics the effects of cAMP activity by activating cAMP-dependent protein kinase (Sandberg et al., 1991), and a phorbol ester (PE), phorbol 12, 13-diacetate, a protein kinase C (PKC) activator (Castagna et al., 1982). As seen earlier for 5-HT, addition of 8-Br-cAMP also caused the TEP to recover to its initial voltage (Fig. 3A). By contrast, addition of PE had no stimulatory effect on TEP (Fig. 3B)

3.3. Basolateral membrane potential

In addition to the decline in TEP, the basolateral membrane potential of the gastric caecal cells also declined significantly over 3 – 6 minutes when preparations isolated from freshwater reared larvae were bathed in saline alone (Fig. 4A-F). Basolateral membrane potential recovered to its initial voltage after addition of 1 μ M 5-HT for cells of the distal gastric caecum (Fig. 4A), but not for the proximal gastric caecum (Fig. 4B). At the distal gastric caecum, basolateral membrane potential

recovered after addition of 8-Br-cAMP, which mimics the effect of 5-HT in this region (Fig. 4A, C), whereas PE had no stimulatory effect on membrane potential, which continued to decrease after the addition of PE (Fig. 4E). Neither 8-Br-cAMP nor PE had any effect on membrane potential of the proximal gastric caecum (Fig. 4D, F).

Although we have not measured apical membrane voltage (V_a) directly, we can estimate changes in V_a in from measurements of TEP and basal membrane voltage (V_b), since $TEP = V_b + V_a$. If V_b depolarized after the initial measurement, and V_a had remained constant, TEP would hyperpolarize. However, since TEP depolarizes, we can conclude that V_a must also depolarize as well in the absence of 5-HT or cAMP. Similarly, if V_a did not change when V_b shifted negative in response to 5-HT or cAMP, then TEP would depolarize. The hyperpolarization of TEP indicates that V_a becomes more lumen-positive in response to cAMP or 5-HT.

3.4. Luminal concentrations and transepithelial electrochemical potentials for H^+ , Na^+ and K^+

Since 5-HT affects TEP, which is the difference in electrical potential between the lumen and the bath, we wanted to measure the luminal concentrations of H^+ , Na^+ and K^+ , and determine how transport of these ions into and out of the lumen may be affected in larvae reared in different conditions and by 5-HT. Luminal concentrations for H^+ , Na^+ and K^+ were measured using double-barrelled ion-selective microelectrode. The electrochemical potentials ($\Delta\mu/F$) for H^+ , Na^+ and K^+ were then 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.

There was no change in luminal pH between the initial pH and ‘Before 5-HT’ pH measurements in freshwater reared larvae (Fig. 5A). However, luminal pH was significantly lower after addition of 1 μ M 5-HT, relative to the value before the addition of 5-HT (Fig 5A). The electrochemical potential for H⁺ changed from active transport initially, to near electrochemical equilibrium in the absence of 5-HT (Fig. 5B). Addition of 5-HT was associated with recovery of the electrochemical potential for H⁺ to a value similar to the initial lumen-positive value (Fig. 5B). In brackish water reared larvae, there was no change in luminal pH between the initial pH and ‘Before 5-HT’ pH measurements (Fig 5C). Addition of 1 μ M 5-HT led to a significant reduction in luminal pH in comparison to both the initial and ‘Before 5-HT’ pH measurements (Fig 5C). The electrochemical potential for H⁺ favoured passive secretion of H⁺ into the gastric caecum in the absence of 5-HT, but indicated active secretion into the lumen after stimulation by 5-HT (Fig. 5D).

In freshwater larvae, luminal Na⁺ was unchanged when comparing the initial luminal Na⁺ concentration to the ‘Before 5-HT’ Na⁺ concentration, but was significantly increased after the addition of 1 μ M 5-HT (Fig. 6A). Na⁺ was initially near electrochemical equilibrium, however, passive transport of Na⁺ was favoured when bathed in saline only, in the absence of 5-HT (Fig. 6B). Addition of 5-HT led to an increase in the electrochemical potential of Na⁺ to a value near equilibrium, significantly higher than the initial value and that measured before addition of 5-HT (Fig. 6B). In brackish water larvae, luminal Na⁺ was unchanged in the absence or presence of 1 μ M 5-HT when compared to the initial Na⁺ concentration (Fig. 6C). Na⁺ was

initially actively secreted into the caecal lumen; however, when bathed in saline only, and unstimulated by 5-HT, the electrochemical gradient for Na^+ decreased significantly to near electrochemical equilibrium (Fig. 6D). Addition of 5-HT increased the electrochemical potential for Na^+ to the initial potential, indicating active secretion into the lumen (Fig. 6D).

Luminal K^+ was unchanged in the absence or presence of $1\ \mu\text{M}$ 5-HT when compared to the initial luminal K^+ in freshwater larvae (Fig. 7A). K^+ was initially actively transported into the gastric caecal lumen; the electrochemical potential declined significantly before the addition of 5-HT but recovered to a value similar to the initial electrochemical potential in the presence of 5-HT (Fig. 7B). In brackish water larvae, luminal K^+ was unchanged when comparing the initial and 'Before 5-HT' K^+ concentrations, but was significantly increased after the addition of 5-HT (Fig. 7C). K^+ was actively secreted into the gastric caecal lumen, and the electrochemical potential increased significantly after the addition of 5-HT in comparison to the initial and 'Before 5-HT' values (Fig. 7D).

3.5. Effect of 5-HT on transport rates of H^+ , Na^+ and K^+ along the gastric caecum

The regionalized effects of 5-HT on recovery of basolateral membrane potential led us to examine the effects of 5-HT on transport rates of H^+ , Na^+ and K^+ along the distal and proximal regions of the gastric caeca. Additionally, since 5-HT led to the recovery of TEP in both freshwater and brackish water reared larvae, we tested the effect of 5-HT on rates of ion transport for both rearing conditions. Initial transport rates of H^+ (Fig. 8A-D), Na^+ (Fig. 9A-D) and K^+ (Fig. 10A-D) across the basolateral membrane were

measured along the haemolymph-facing surface of the gastric caecum of larval *A. aegypti*. The initial SIET measurements revealed that H^+ was absorbed (into the bath) at the distal gastric caecum (Fig. 8A, C) and secreted (from bath to cell) at the proximal region (Fig. 8B, D). Initial values for Na^+ and K^+ indicated secretion at both the distal and proximal regions of the gastric caecum (Fig. 9A, C; Fig. 10A, C), consistent with previous findings (D'Silva et al., 2017).

In control larvae reared in freshwater, the rate of absorption of H^+ and secretion rates of Na^+ and K^+ at the distal gastric caecum were significantly reduced within 3-6 mins when measured in physiological saline and compared to the initial recorded value (Fig. 8A, 9A, 10A). In larvae treated with $1\mu M$ 5-HT, the rate of absorption of H^+ and rates of secretion of Na^+ and K^+ remained near initial values, and were significantly higher than the control at the corresponding time points (Fig. 8A, 9A, 10A).

At the proximal gastric caecum of larvae reared in freshwater, the rates of secretion of H^+ and Na^+ were significantly reduced within 3-6 minutes when measured in physiological saline (Fig. 8B, 9B). The rate of secretion of K^+ did not diminish over time (Fig. 10B). The addition of $1\mu M$ 5-HT had no effect on the rates of secretion of H^+ , Na^+ and K^+ in the proximal region of the caecum when compared to the rates of secretion at the corresponding time points (Fig. 8B, 9B, 10B).

In larvae reared in brackish water, the rate of absorption of H^+ and secretion rates of Na^+ and K^+ at the distal gastric caecum were significantly reduced within 3 – 6 minutes when measured in physiological saline (Fig. 8C, 9C, 10C). On addition of $1\mu M$ 5-HT, the rate of absorption of H^+ recovered to and maintained the initial

value, and was significantly higher than rates of H^+ absorption measured at the corresponding time points in the unstimulated caecum (Fig. 8C). Rates of transport of Na^+ when treated with 5-HT were similar to the control rates at the corresponding time points (Fig. 9C). Addition of 5-HT led to a significant decline in K^+ secretion and a transient switch to K^+ absorption between 3 and 9 minutes of incubation with 5-HT, after which it returned to secretion at rates similar to control values measured at the corresponding time points (Fig. 10C).

At the proximal gastric caecum of larvae reared in brackish water, the rate of secretion of H^+ , Na^+ and K^+ were significantly reduced over time when measured in physiological saline (Fig. 8D, 9D, 10D). The addition of $1\mu M$ 5-HT had no effect on the rates of secretion of H^+ , Na^+ and K^+ in the proximal region when compared to the control rates of secretion at the corresponding time points (Fig. 98D, 9D, 10D).

4. Discussion

This study of the *A. aegypti* larval gastric caecum reports the first measurements of the effects of the biogenic amine 5-HT on luminal pH, and luminal concentrations of Na^+ , and K^+ across the caecum, as well as corresponding transepithelial electrochemical potentials. We have also shown that 5-HT has a regionalized effect on the basolateral membrane potential, and measured fluxes of H^+ , K^+ , and Na^+ , which differ in the proximal and distal regions of the caecum. Moreover, we have shown that the effects of 5-HT are altered depending upon rearing salinity. This study is also the first to investigate the effects of intracellular

signaling molecules on TEP and basolateral membrane potentials of the larval gastric caecum.

The TEP, basolateral membrane potential, and H^+ , K^+ , and Na^+ fluxes of the gastric caeca declined within 3-6 mins after isolation of the entire midgut (including the anterior region, posterior region and hindgut). The decay of initial TEP cannot be explained by tissue deterioration as the TEP was restored to initial TEP on stimulation by 5-HT. This effect is similar to that observed in the anterior and posterior regions of the *A. aegypti* larval midgut in response to 5-HT (Clark et al., 1999). The loss of the initial TEP and subsequent revival by 5-HT shows that the gastric caecum is stimulated by 5-HT *in vitro*. Taken together, our measurements of TEP, basolateral membrane potential, luminal ion concentrations and net electrochemical potentials for H^+ , Na^+ and K^+ suggest that endogenous 5-HT signaling is critical for gastric caeca ion regulation *in vivo*, and that this signaling is lost when the tissue is isolated from the hemolymph and bathed with artificial saline.

4.1 Mechanisms of action of 5-HT

In brackish water larvae, there was a decline in TEP when allowed to remain in saline, which recovered to initial TEP values when 5-HT was added (Fig. 2C). We also demonstrated that the hyperpolarization of TEP caused by the addition of 5-HT, in freshwater larvae, declines when 5-HT is washed out and replaced by physiological saline (Fig. 2A). These results therefore establish that 5-HT is critical to maintaining TEP in the gastric caecum. Significant effects of 5-HT on TEP of the gastric caecum were evident at concentrations between 0.1 μ M and 10 μ M 5-HT (Fig.

2B). (Clark et al., 1999) reported maximal stimulation of TEP in the posterior midgut of the larval *A. aegypti* at 0.1 to 1 μ M.

Previous work showed that ketanserin, a 5-HT type 2 receptor antagonist (Van Nueten et al., 1983), inhibited fluid secretion of the Malpighian tubules by 28%, suggesting that 5-HT type 2 receptor regulates ion transport/fluid secretion in MTs (Clark and Bradley, 1997). We speculated that a 5-HT type 2 receptor was also responsible for the effects of 5-HT on gastric caeca. However, we observed no effects on the TEP of the gastric caeca when ketanserin was added either after or before the addition of 5-HT (Fig. S1A, B). Hence, additional investigations using other 5-HT receptor agonists and/or antagonists are needed to determine the type of 5-HT receptor expressed in the caeca.

The mechanism of 5-HT action via intracellular signaling molecules on TEP was investigated in the gastric caeca of larvae reared in freshwater. 5-HT can act on cells and regulate its activity via intracellular second messengers such as cAMP, or intracellular effectors like PKC. We show that 8-Br-cAMP, a cAMP analogue, stimulated the recovery of TEP, while PE, a PKC activator, did not stimulate TEP (Fig. 3A, B). This is in contrast to the adjacent anterior midgut, where PE stimulates TEP, suggesting that the actions of 5-HT in the anterior midgut region are dependent on PKC activation (Clark et al., 1999). Stimulation of TEP in the *A. aegypti* gastric caecum on the other hand is similar to the Malpighian tubules, where stimulation of diuretic activity by 5-HT in larval tubules is mediated through cAMP (Clark and Bradley, 1996; Clark and Bradley, 1997; Clark and Bradley, 1998; Donini et al., 2006).

The mechanism of 5-HT action on the basolateral membrane potential of larvae reared in freshwater was also investigated through use of these putative intracellular signaling molecules. Freshwater larvae were chosen for this part of the study since there is a distinct regionalization of VA-rich and NKA-rich cells along the gastric caeca, with VA-rich cells occurring on the distal third and NKA-rich cells occurring on the proximal two-thirds (D'Silva et al., 2017; Patrick et al., 2006). It was therefore easy to identify which types of cells (VA-rich distal cells or NKA-rich proximal cells) were being impaled when measuring basolateral membrane potentials. Basolateral membrane potentials were not measured for larvae reared in brackish water as VA and NKA expression is not regionalized. VA and NKA are expressed in a mosaic pattern along the length of the brackish water larval caecum (D'Silva et al., 2017), hence making it difficult to identify the cell type under a dissection microscope.

The actions of 5-HT and 8-Br-cAMP on the basolateral membrane potentials of the distal (VA-rich) cells and proximal (NKA-rich) cells of the gastric caecum were markedly different for the two regions (Fig. 4A, B, C, D). Immediately after isolation, basolateral membrane potential was approximately -40 mV for both types of cells. This potential decreased within 3-6 minutes of dissection for both types of cells, just as TEP decreased over the same time period. Application of 5-HT after the decline of the basal membrane potential caused a hyperpolarization of the VA-rich cells but not the NKA-rich cells (Fig. 4A, B). Similarly, 8-Br-cAMP had a hyperpolarizing effect on the VA-rich cells, with no effect on the NKA-rich cells (Fig. 4C, D). The VA-rich and NKA-rich cells thus appear to be two electrically distinct cell types. Additionally, TEP recovers fully in the presence of 5-HT or 8-Br-cAMP (Fig. 2A, 3A). Since TEP reflects the activity of the

two cell types, VA-rich and NKA-rich cells, this finding suggests that VA-rich cells have a more pronounced role in maintaining the TEP of the gastric caeca.

Both 8-Br-cAMP and 5-HT had similar effects on the basolateral membrane potential and TEP, consistent with 5-HT activation of adenylyl cyclase and a consequent rise in intracellular cAMP. Our data also show that 5-HT caused an increase in H^+ transport as indicated by increased H^+ absorption at the distal gastric caecum (Fig. 8A, C), decreased luminal pH (Fig. 5A, C), and a shift in the electrochemical gradient towards active H^+ secretion into the lumen (Fig. 5B, D). These data, along with the effects of 5-HT or 8-Br-cAMP at the distal gastric caecum (Fig. 4A, C), imply that the VA present on both the apical and basolateral membrane of the distal gastric caecum (Patrick et al., 2006) may be regulated by 5-HT through cAMP. Figure 11 (modified from (D'Silva et al., 2017) summarizes the proposed action of 5-HT on the VA present on the apical and basal membranes of the distal gastric caecum. The action of cAMP on VA is similar to previous studies in other species which showed that an increase in intracellular cAMP levels led to an increase in VA activity and H^+ transport (Baumann and Walz, 2012; Coast et al., 2001; Dames et al., 2006; O'Donnell et al., 1996; Voss et al., 2010; Wiczorek et al., 1999).

4.2 Effects of 5-HT in freshwater or brackish water reared larvae

The effects of 5-HT on the basolateral membrane potentials of the distal and proximal gastric caecum suggests that 5-HT is responsible for the regulation of ion transport in the VA-rich cells of the gastric caeca, and consequently ionoregulation by the gastric caeca. We therefore tested the effects of 5-HT on H^+ , Na^+ and K^+ transport rates

along the distal and proximal regions, for both freshwater or brackish water reared larvae. The rates of transport for H^+ , Na^+ and K^+ were consistent with previously reported rates of ion transport for larvae reared in freshwater or brackish water (D'Silva et al., 2017). In freshwater larvae, and in the absence of 5-HT, the rates of transport of all three ions decreased at the distal region, and rates of H^+ and Na^+ secretion at the proximal region also decreased. 5-HT led to the recovery of H^+ , Na^+ and K^+ transport at the distal but not the proximal gastric caecum, corresponding to the stimulation of the basolateral membrane potential by 5-HT for the distal cells but not the proximal cells. The site of action of 5-HT thus coincides with the location of the ion transporting cells in the distal third of the caecum (Volkman and Peters, 1989a), which express VA on both the apical and basolateral membranes (Patrick et al., 2006). 5-HT acts via cAMP to increase VA activity in these cells, thus stimulating increased VA activity on both membranes (Fig. 11). We propose that increased activity of the basolateral VA accounts for the increased H^+ absorption across the basolateral membrane, thus contributing to a negative basolateral membrane potential (Fig. 11). Additionally, increases in the magnitude of the lumen-positive TEP, the positive transepithelial electrochemical potential for H^+ (Fig. 5B) and the decrease in luminal pH in response to 5-HT are consistent with stimulation of apically expressed VA (Fig. 11). Increased secretion of H^+ into the lumen can then drive the secretion of Na^+ or K^+ into the lumen via an apically expressed NHE (Fig. 11). This is similar to the action of 5-HT on the Malpighian tubules of *Rhodnius*, where 5-HT stimulates an apically expressed VA that in turn drives the secretion of Na^+ or K^+ into the lumen via an apically expressed Na^+/H^+ antiporter. In turn, the reduction in intracellular levels of Na^+ and K^+ may enhance the gradient favouring uptake of Na^+ , K^+ and Cl^- into

the cell through a basolateral cation chloride cotransporter (CCC; Figure 11), consistent with the increase in Na^+ and K^+ fluxes at the distal GC of freshwater larvae. Again, there is similarity to the role of CCC in uptake of ions into Malpighian tubule cells (Ianowski et al., 2002; Ianowski et al., 2004). Taken together, our data indicate suggest that 5-HT is responsible for maintenance of ion transport in the gastric caeca, specifically at the distal gastric caecum (VA-rich cells) of freshwater larvae.

In brackish water larvae, a large amount of NaCl is ingested along with the food, leading to an increased Na^+ concentration in the lumen of the caecum (33 mM; Fig. 6C) in comparison to larvae reared in freshwater (15 mM; Fig. 6A). On the other hand, luminal K^+ concentration is lower in the caecum of larvae reared in brackish water (19 mM; Fig. 7C) compared to freshwater (47 mM; Fig. 7A). The shift to higher luminal Na^+ in the gastric caecum of brackish water larvae presumably reflects the ready availability of Na^+ in the food and ingested water. The shift in electrochemical potentials for Na^+ and K^+ to positive or more positive values in response to 5-HT is consistent with stimulation of transporters that actively accumulate both ions in the lumen of the gastric caecum of brackish water larvae as well (Fig. 11).

While the transport rates of H^+ , Na^+ and K^+ were reduced in the absence of 5-HT at both the distal and proximal region of the brackish water caecum, only H^+ absorption at the distal caecum recovered in the presence of 5-HT. This is in stark contrast to freshwater larvae, where 5-HT had an effect on transport rates of all three ions. The differences in response to 5-HT in the two rearing conditions may be a consequence of the mosaic pattern of VA-rich and NKA-rich cells observed in brackish water larvae, wherein small VA-rich cells are dispersed amongst the larger NKA-rich cells (D'Silva et

al., 2017), thus masking the effect of 5-HT on the VA-rich cells when measured using SIET. Alternatively, since larvae reared in brackish water experience much smaller ionic gradients for passive movements of ions and water when compared to freshwater larvae, they do not need to hyper-regulate since brackish water larvae are able to tolerate a salinity of up to 30% seawater, which is roughly isosmotic to the larval haemolymph (Clark et al., 2004). It is also worth noting that although the secretion rates of H^+ , Na^+ and K^+ across the proximal caecum of larvae reared in either freshwater or brackish water were of similar magnitudes, the rate of absorption of H^+ and rates of secretion of Na^+ and K^+ at the distal gastric caecum were much higher in freshwater larvae when compared to brackish water larvae. Freshwater larvae ingest large amounts of dilute media thus osmotically favouring movement of water from the midgut into the haemolymph thereby reducing the volume of luminal fluid and diluting the haemolymph. The larvae therefore compensate by increasing rates of secretion of Na^+ and K^+ across the distal caecum into the lumen, leading to increased fluid secretion from haemolymph to lumen. Maintaining adequate fluid secretion into the gut lumen is important to regulate haemolymph volume and ion concentrations, as well as to support digestion and luminal fluid circulation. This provides further evidence for the importance of the VA-rich cells and 5-HT in ionoregulation, where 5-HT drives the action of VA and consequently secondary ion transporters to maintain fluid secretion into the caecal lumen despite intake of dilute medium by freshwater larvae.

It is worth noting that previous electrophysiological studies of the isolated midgut of larval mosquitoes have perfused the lumen (e.g. Onken et al., 2004b), thus allowing precise control of the luminal ion composition. We did not perfuse the lumen of the

gastric caecum since each caecum is a blind-ended sac, making exchange of luminal contents difficult, and because the opening into each caecum is covered by the caecal membrane (Volkman and Peters, 1989a). Our results may thus differ from the caecum *in vivo*, when the composition of the caecal contents may be influenced by ingestion of food and bathing medium.

Although we do not have sufficient information to describe the processes that lead to differences in electrical properties of the two types of gastric caecal cells, VA-rich and NKA-rich cells, we can conclude that 5-HT and its second messenger, cAMP, play an important role in stimulation of the ionoregulatory cells (VA-rich cells), and subsequently TEP. Inability of 5-HT to aid in recovery from the decline of the basolateral membrane potential or ion transport rates of H⁺, Na⁺ or K⁺ of the NKA-rich cells of the proximal GC indicates that other neuromodulators or hormones may be responsible for sustaining the transport activity of the proximal gastric caeca *in vivo*.

Conclusion

Together, our results suggest that the function of the gastric caecum is partially controlled and maintained by the effects of 5-HT and that there are spatial differences in the 5-HT-induced recovery of ion transport across the distal and proximal regions of the gastric caecum. These observations are consistent with the extensive serotonergic input of the gut from central neurons (Moffett and Moffett, 2005; Petrova and Moffett, 2016) and provide functional correlates of the regionalization of VA and NKA 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 also provide further evidence that the caecum is functionally distinct from the adjacent anterior midgut, and may have an osmoregulatory function. Moreover, there are intriguing similarities in the arrangement of basolateral and apical transporters in the gastric caecum and the Malpighian tubule. Our results also suggest that additional neuromodulators or hormones may contribute to functional control of the gastric caecum, particularly the proximal region.

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Figures

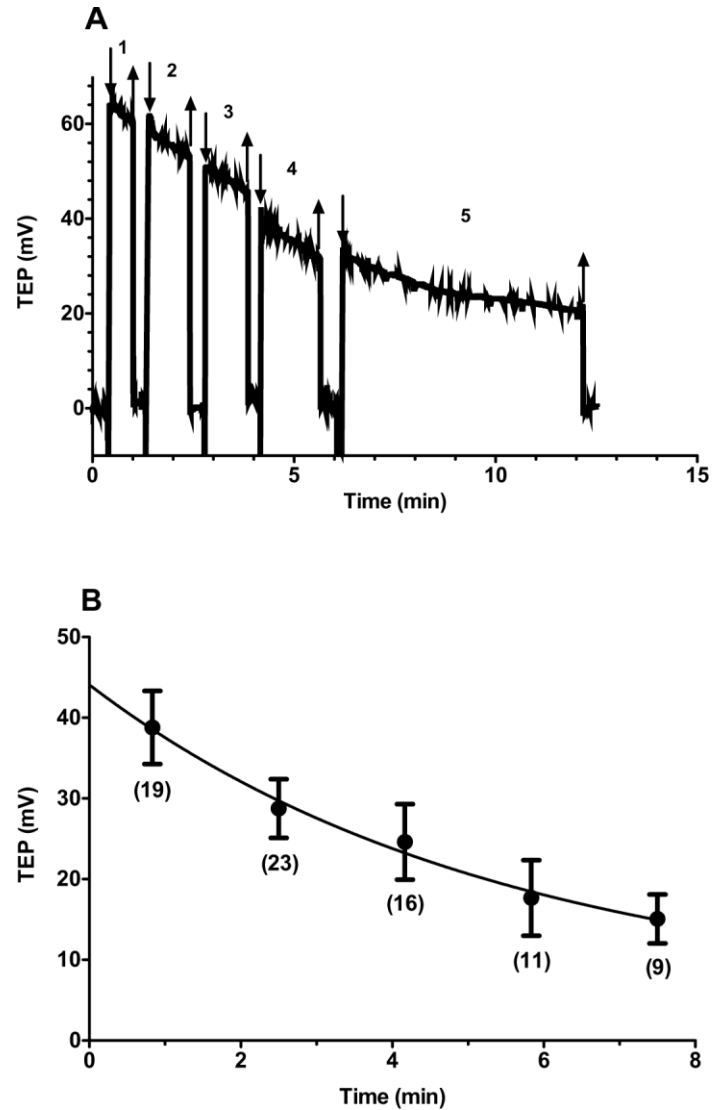


Figure 1: Decline of transepithelial potential (TEP) of the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. (A) TEP was measured for 5 gastric caeca within a single animal that are numbered 1-5 in order of impalement.

Downward arrows indicate impalement of the gastric caecum, and upward arrows

indicate withdrawal of the electrode from the gastric caecal lumen. (B) TEP was measured over time since dissection in gastric caeca of multiple animals. Data are expressed as mean \pm sem. The data were fit to a one phase decay equation ($r^2 = 0.99$): $TEP = (Y_0 - \text{Plateau}) \cdot \exp(-K \cdot \text{time}) + \text{Plateau}$, where $Y_0 = 44.09$, $K = 0.1841$, $\text{Plateau} = 5.155$.

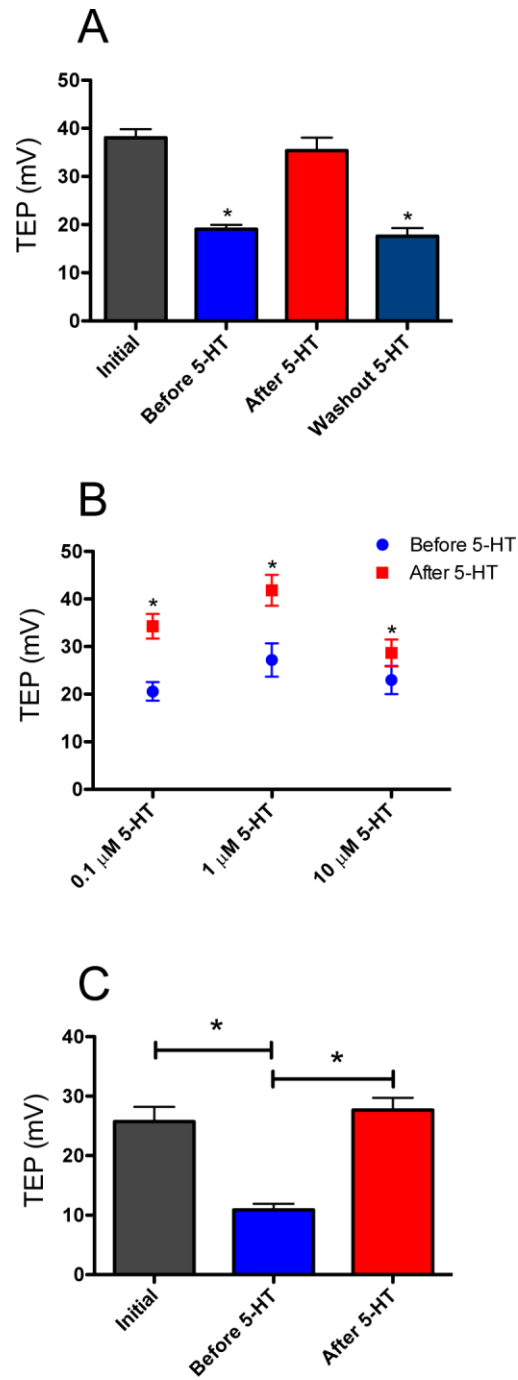


Figure 2: Effects of 5-HT on transepithelial potential (TEP) of the gastric caecum of fourth instar *Aedes aegypti* larvae. (A) TEP was measured in the absence and presence of 5-hydroxytryptamine (5-HT) in larvae reared in freshwater. (B) Effects

of different doses of 5-HT on the TEP of larvae reared in freshwater. (C) TEP was measured in the absence and presence of 5-hydroxytryptamine (5-HT) in larvae reared in brackish water. Data are expressed as mean \pm sem (N= 24 for A, 4-6 for B and 24 for C). Asterisks denote significant differences between treatments; significance ($p < 0.05$) was determined by one-way ANOVA followed by Tukey's post-hoc test (A, C) or paired t-test (B).

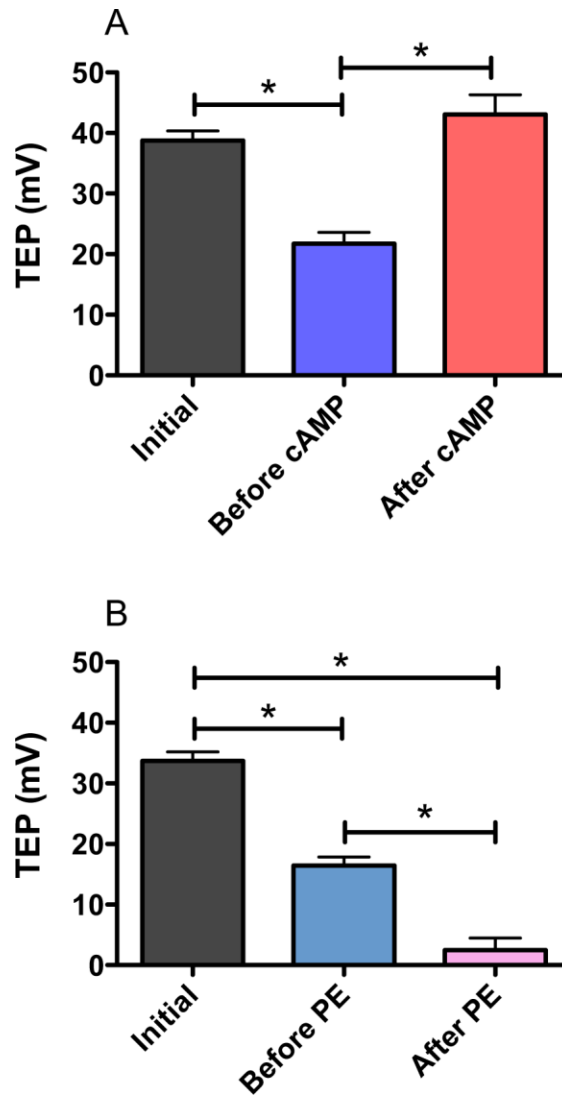


Figure 3: Transepithelial potential (TEP) of the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. TEP was measured in the (A) absence and presence of cAMP, (B) absence and presence of PE. Data are expressed as mean \pm sem (N= 5-6). Asterisks denote significant differences between treatments; significance ($p < 0.05$) was determined by one-way ANOVA followed by Tukey's post-hoc test. 5-HT: 5-hydroxytryptamine; cAMP: cyclic adenosine monophosphate; PE: phorbol ester.

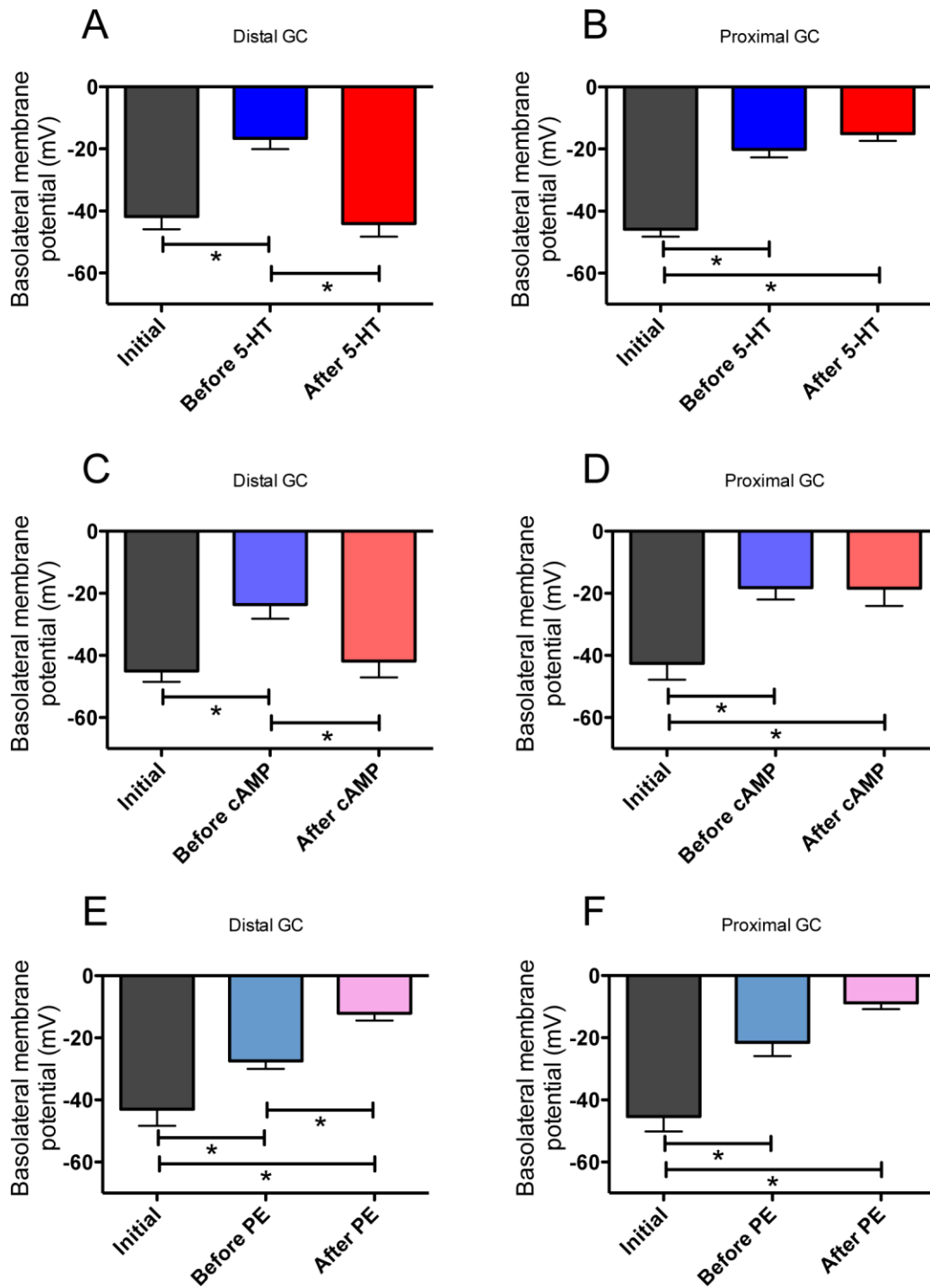


Figure 4: Basolateral membrane potential of the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. Basolateral membrane potential was measured at the distal (A, C, E) and proximal (B, D, F) gastric caecum (GC) in the

absence and presence of 5-HT (A, B), absence and presence of cAMP (C, D), and absence and presence of PE (E, F). Data are expressed as mean \pm sem (N= 5-7). Asterisks denote significant difference between treatments; significance ($p < 0.05$) was determined by one-way ANOVA followed by Tukey's post-hoc test. 5-HT: 5-hydroxytryptamine; cAMP: cyclic adenosine monophosphate; PE: phorbol ester.

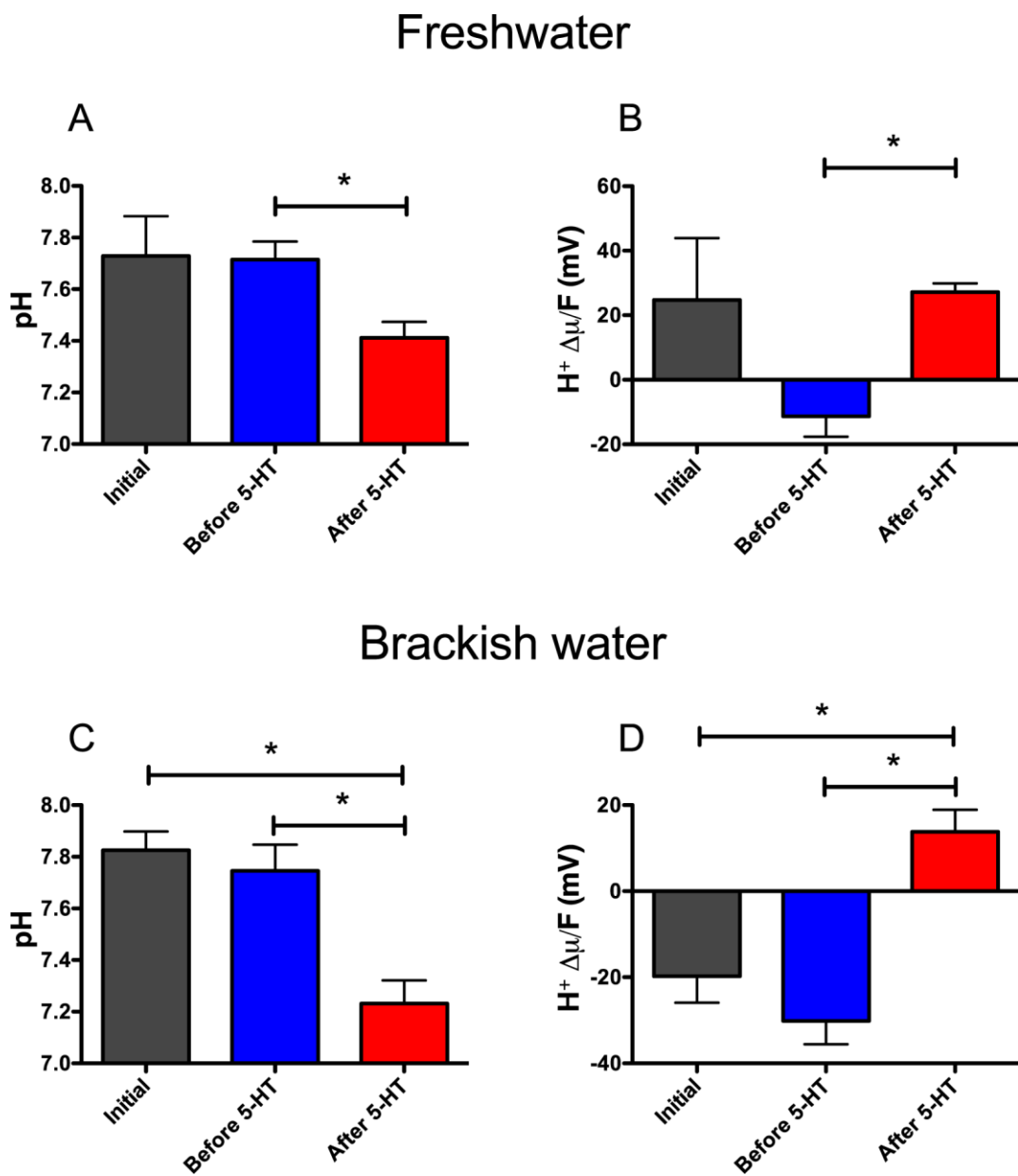


Figure 5: (A, C) Luminal pH and (B, D) electrochemical potentials for H⁺ in the gastric caecum of fourth instar *Aedes aegypti* larvae reared in (A, B) freshwater or (C, D) brackish water. Data are expressed as mean ± sem (N= 7-8). Asterisks denote significant difference between treatments; significance ($p < 0.05$) was determined by one-way ANOVA followed by Tukey's post-hoc test.

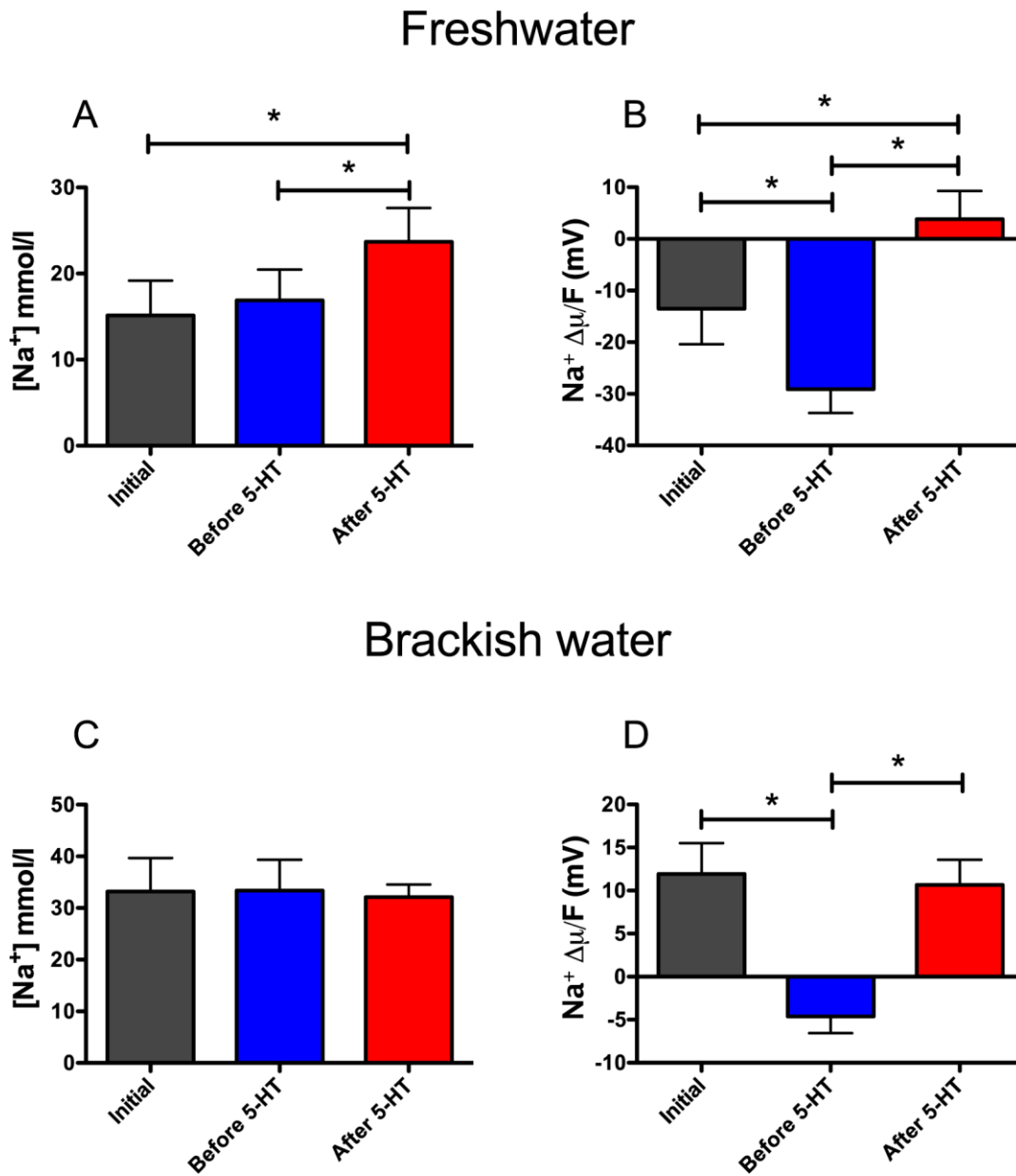


Figure 6: (A, C) Luminal [Na⁺] and (B, D) electrochemical potentials for Na⁺ in the gastric caecum of fourth instar *Aedes aegypti* larvae reared in (A, B) freshwater or (C, D) brackish water. Data are expressed as mean ± sem (N= 8-9). Asterisks denote significant difference between treatments; significance ($p < 0.05$) was determined by one-way ANOVA followed by Tukey's post-hoc test.

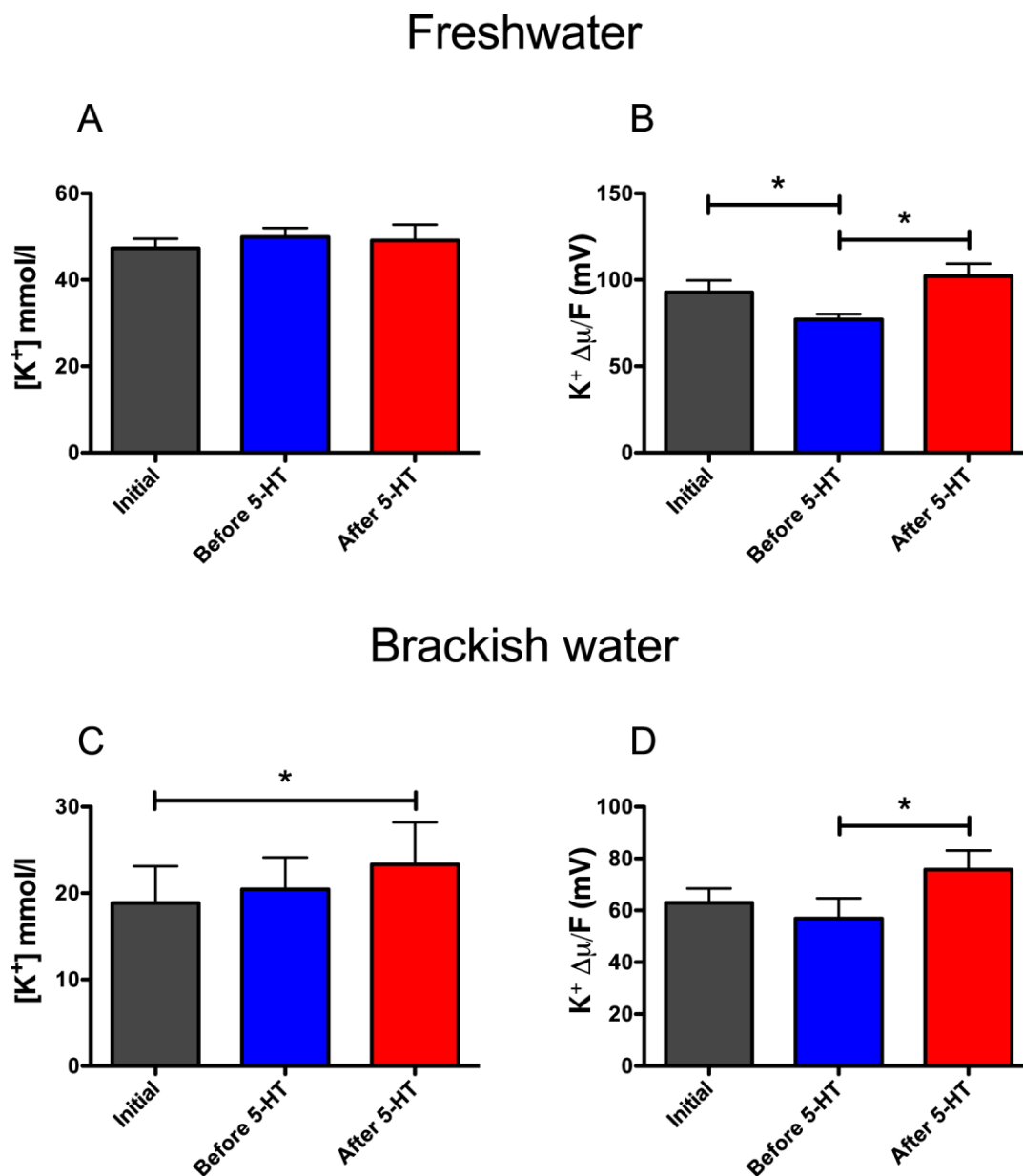
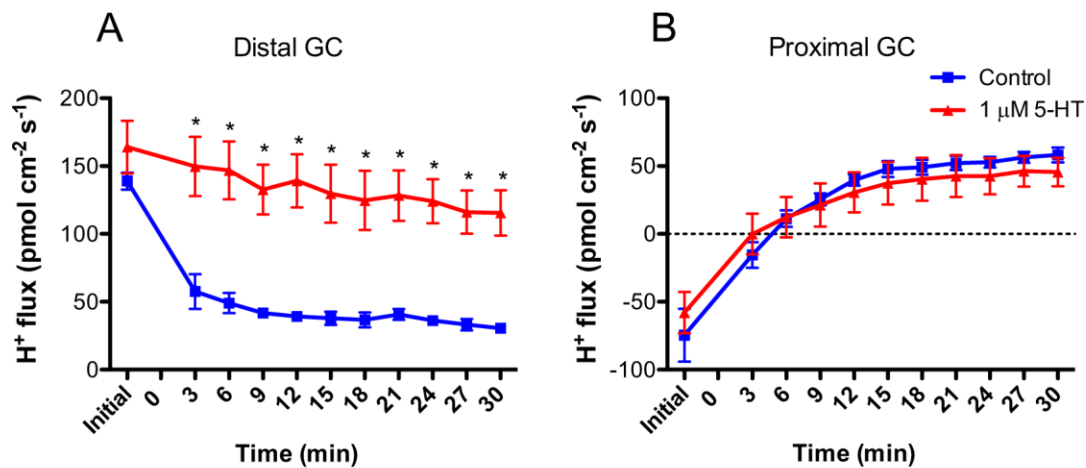


Figure 7: (A, C) Luminal [K⁺] and (B, D) electrochemical potentials for K⁺ in the gastric caecum of fourth instar *Aedes aegypti* larvae reared in (A, B) freshwater or (C, D) brackish water. Data are expressed as mean ± sem (N= 8). Asterisks denote significant difference between treatments; significance ($p < 0.05$) was determined by one-way ANOVA followed by Tukey's post-hoc test.

Freshwater



Brackish water

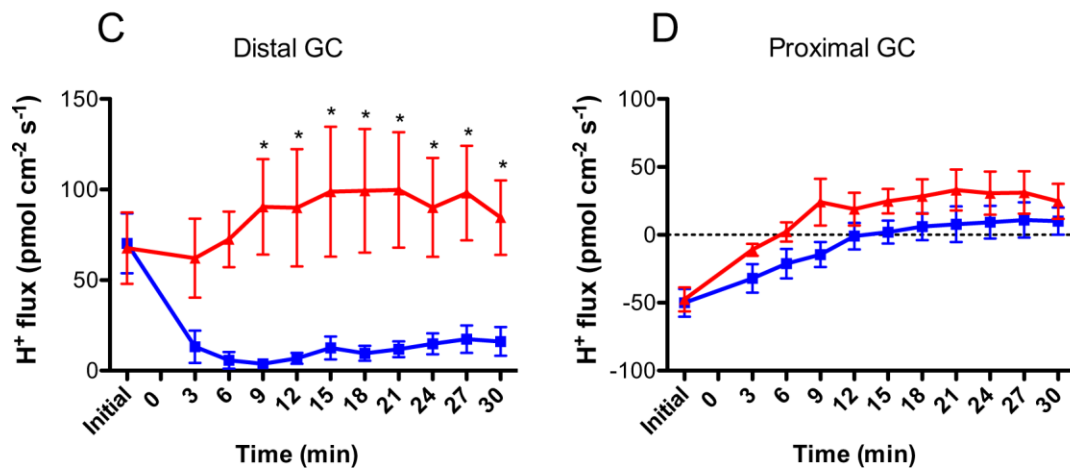
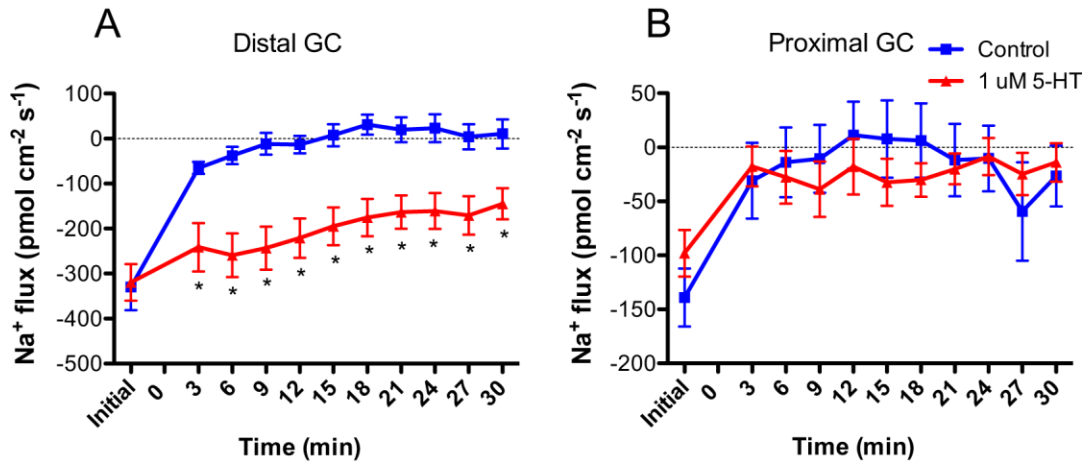


Figure 8: Scanning Ion-Selective Microelectrode Technique (SIET) measurements of *in vitro* H^+ transport rates along the surface of the (A, C) distal and (B, D) proximal gastric caecum of fourth instar *Aedes aegypti* larvae reared in (A, B) freshwater or (C, D) brackish water. Positive values denote 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 \pm sem (N= 5-17). Asterisks denote significant difference between treatments; significance ($p < 0.05$) was determined by two-way ANOVA followed by Bonferroni's post-hoc test.

Freshwater



Brackish water

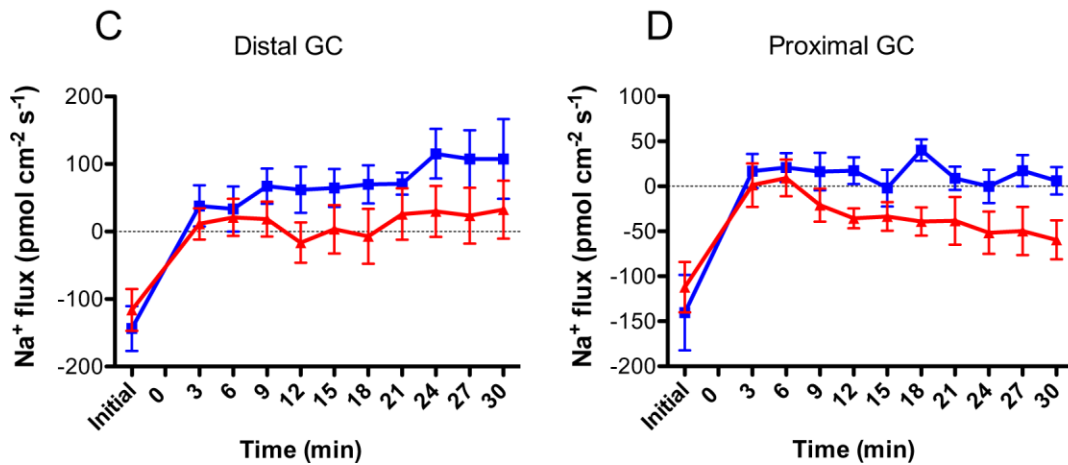
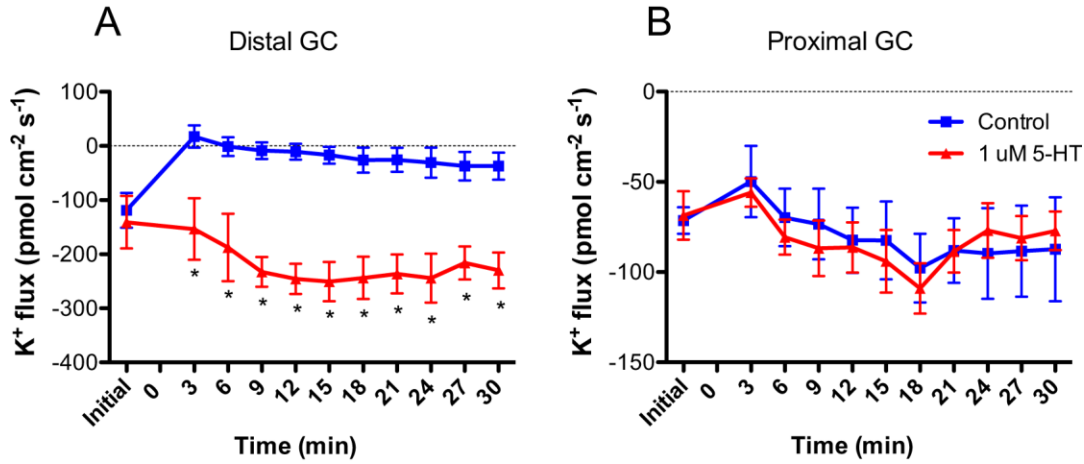


Figure 9: Scanning Ion-Selective Microelectrode Technique (SIET) measurements of *in vitro* Na⁺ transport rates along the surface of the (A, C) distal and (B, D) proximal gastric caecum of fourth instar *Aedes aegypti* larvae reared in (A, B) freshwater or (C, D) brackish water. Positive values denote 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 \pm sem (N= 5-12). Asterisks denote

significant difference between treatments; significance ($p < 0.05$) was determined by two-way ANOVA followed by Bonferroni's post-hoc test.

Freshwater



Brackish water

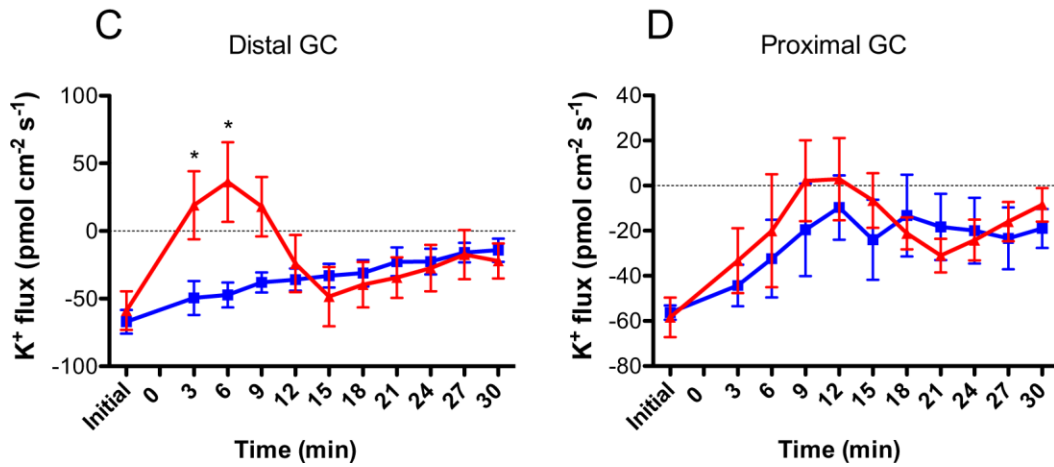


Figure 10: Scanning Ion-Selective Microelectrode Technique (SIET) measurements of *in vitro* K⁺ transport rates along the surface of the (A, C) distal and (B, D) proximal gastric caecum of fourth instar *Aedes aegypti* larvae reared in (A, B) freshwater or (C, D) brackish water. Positive values denote 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= 6-12). Asterisks denote

significant difference between treatments; significance ($p < 0.05$) was determined by two-way ANOVA followed by Bonferroni's post-hoc test.

Distal Gastric Caecum

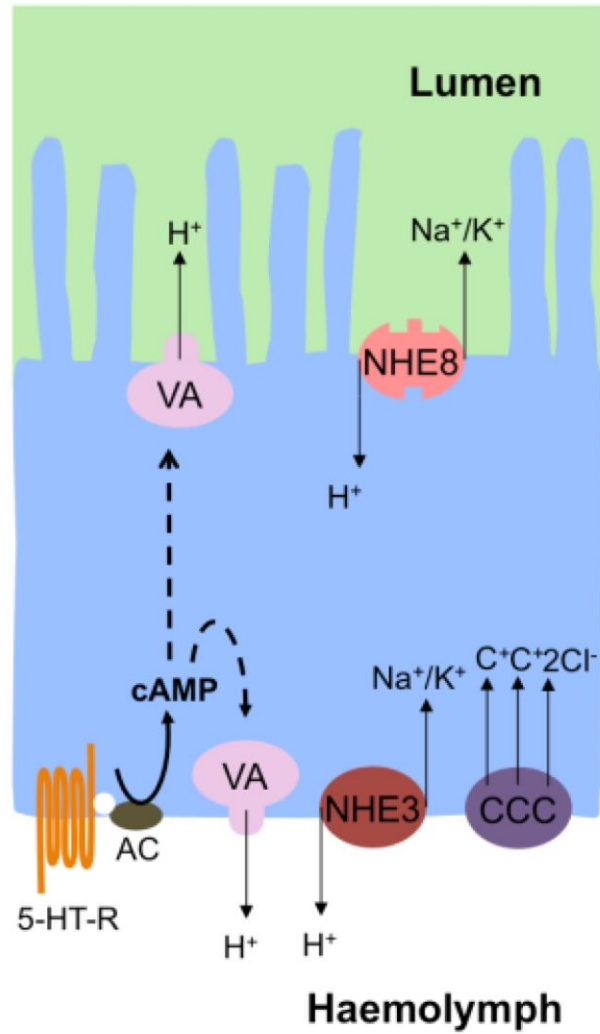


Figure 11: Schematic diagram illustrating the role of 5-HT on activation on VA in the distal gastric caecum. VA: V-type H⁺ ATPase; *A. aegypti* NHE: Na⁺/H⁺ Exchanger (AeNHE8; AeNHE3); CCC: Cation Chloride Cotransporter

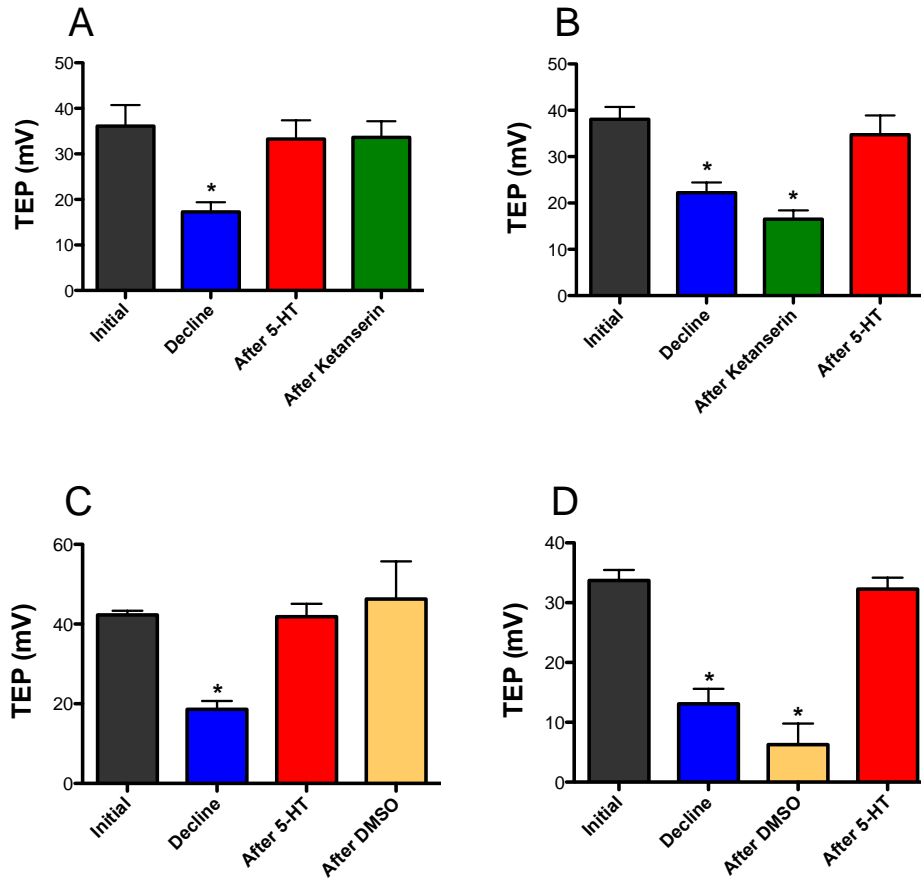


Figure S1: Transepithelial potential (TEP) of the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. An initial TEP was measured, followed after 10 mins by a measurement during the decline in TEP. TEP was then measured (A) after the addition of 5-HT followed by ketanserin, (B) after the addition of ketanserin followed by 5-HT, (C) after the addition of 5-HT followed by DMSO (dimethyl sulfoxide; carrier for ketanserin), (D) after the addition of DMSO followed by 5-HT. Data are expressed as mean \pm sem (N= 4-8). Asterisks denote significant difference between treatments; significance ($p < 0.05$) was determined by one-way ANOVA followed by Tukey's post-hoc test.