THE ROLE OF ION-MOTIVE ATPASES IN THE INSECT GUT

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BY

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LAY ABSTRACT

This thesis focuses on investigating the roles of two enzymes, vacuolar-type H^+-ATPase (VA) and Na^+/K^+ ATPase (NKA), which utilize energy to transport electrically charged atoms (ions) across the cells of the insect gut. Although VA was considered the more important of the two enzymes until the early 2000s, I have demonstrated that NKA also plays a role in maintaining insect gut function in fruit flies and mosquito larvae. Furthermore, the activities of both enzymes are dependent on the salinity of the medium in which mosquito larvae are reared, suggesting that they play a role in maintaining the ionic composition of the gut fluids in freshwater larvae. Additionally, I have also demonstrated that a neurochemical, serotonin, can modulate the activity of gut cells in mosquito larvae. Overall, this thesis provides novel information on the actions of VA and NKA in the insect gut, and presents a number of new avenues for future research.

ABSTRACT

The present set of studies examines the roles of two ion-motive enzymes, vacuolar-type H^+-ATPase (VA) and Na^+/K^+ ATPase (NKA), in energizing transepithelial ion transport across the larval caecum and midgut epithelia of *Drosophila melanogaster* and *Aedes aegypti*. Even though both VA and NKA are expressed in insect epithelia, VA was considered the more important enzyme until the early 2000 because the ion transport was unaffected by the NKA inhibitor ouabain in many insect epithelia, a phenomenon termed the 'ouabain paradox'. This paradox was resolved by the discovery of an organic anion transporter (OATP) that is colocalized with NKA and prevents the actions of ouabain on NKA. Since the resolution of the ouabain paradox, this is the first set of studies that investigates the role of NKA in energizing ion transport across the caeca and midgut of insects. First, I show that both VA and NKA are expressed in the caecum and the midgut. Moreover, the ATPase enzyme activities of VA and NKA are quantitatively similar within each region of the gut that was studied, suggesting that both ATPases may be important for establishing favourable electrochemical gradients for transport of ions across the gut. I used ATPase inhibitors to demonstrate that cation transport is dependent on the actions of both VA and NKA. Furthermore, this is the first set of studies that provides an insight into the ion transport mechanisms of the gastric caecum, an organ that is understudied in insects. In *Aedes aegypti*, I show that 5-hydroxytryptamine regulates the VA-rich cells of the gastric caecum, and therefore the rates of ion transport of these cells. Additionally, I also show that rearing salinity conditions for *Aedes aegypti* larvae alters the expression patterns of VA and NKA in the

gastric caecum. In freshwater, increased activity of VA and NKA energizes transport of ions into the lumen of the caecum that likely maintains fluid volumes and ionic composition at levels appropriate for digestion and absorption. Overall, these studies provide novel information for caeca and midgut-specific actions of VA and NKA in insects, and present a number of new avenues for future research.

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I would like to thank my supervisor, Dr. Michael J. O'Donnell, for guiding me through this journey. Thank you for giving me an opportunity to be a part of your lab, and for helping me grow as an academic. I have greatly benefited from your expert guidance and professional mentorship.

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THESIS DEDICATION

To my Three Front Teeth:

At the outset of my PhD journey

I knew that I would lose blood, sweat, tears, and sleep

I was however unprepared for your demise

Rest in pieces, amigos.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
5-HT-R	5-HT receptor
8-Br-cAMP	8-bromo-cAMP
AC	adenylyl cyclase
ADB	antibody dilution buffer
ADP	adenosine diphosphate
AMG	anterior midgut
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
BW	brackish-water
CA	carbonic anhydrase
cAMP	cyclic adenosine monophosphate
cAMP	cyclic adenosine monophosphate
CC	copper cells
CCC	cation chloride cotransporter
CPA2	cation-proton antiporter
DAPI	4′,6-diamidino-2-phenylindole
DIDS	4,4-diisothiocyanatostilbene-2,2'-disulfonic acid
DMSO	dimethyl sulphoxide
DSHB	Developmental Studies Hybridoma Bank
EDTA	ethylenediaminetetraacetic acid
FW	freshwater
GC	gastric caecum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LFC	large flat cell
MMG	middle midgut
MT	Malpighian tubules
mV	millivolts
NADH	reduced nicotinamide adenine dinucleotide
NAT	nutrient amino acid transporter
NEM	N-ethylmaleimide
NGS	normal goat serum
NHE	Na^+/H^+ exchanger
NKA	Na^+/K^+ ATPase
OATP	organic anion transporter
PBS	Phosphate Buffered Saline
PBT	PBS/0.1% Triton X-100

PE	phorbol 12, 13-diacetate
PFA	paraformaldehyde
PKA	protein kinase A
PKC	protein kinase C
PMG	posterior midgut
RNA	ribonucleic acid
RNAi	RNA interference
SEI	sucrose + EDTA + imidazole
SEID	SEI + sodium deoxycholate
SIET	scanning ion-selective electrode technique
TEP	transepithelial potential
VA	vacuolar-type H^+-ATPase
V_b	basolateral membrane potential
V_i	voltage of the ion-selective barrel in a double-barrelled electrode
V_{ref}	voltage of the reference barrel in a double-barrelled electrode

DECLARATION OF ACADEMIC CONTRIBUTION

Thesis organization and format

This thesis is organized in the "sandwich thesis" format approved by McMaster University. Chapter 1 provides a general introduction of the thesis research. Chapters 2 through 5 integrate the experimental work that I have carried out. The corresponding manuscripts for Chapters 2 through 4 have been published in peer reviewed scientific journals. Chapter 5 is written up as a manuscript for submission to a peer reviewed scientific journal. Chapter 6 discusses the findings of the preceding chapters as well as the overall implications of the thesis work.

Chapter 2: The roles of V-type H^+-ATPase and Na^+/K^+-ATPase in energizing K^+ and H^+ transport in larval Drosophila gut epithelia

Authors: Natalie M. D'Silva, Andrew Donini and Michael J. O'Donnell

Journal: Journal of Insect Physiology 98:284-290 (2017).

Comments: NMD wrote this chapter with valuable guidance and editorial support from AD and MJO. NMD generated data for all the figures.

Chapter 3: Effects of rearing salinity on expression and function of ion motive ATPases and ion transport across the gastric caecum of *Aedes aegypti* larvae

Authors: Natalie M. D'Silva, Marjorie L. Patrick and Michael J. O'Donnell

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Chapter 4: The gastric caecum of larval *Aedes aegypti*: stimulation of epithelial ion transport by 5-hydroxytryptamine and cAMP

Authors: Natalie M. D'Silva and Michael J. O'Donnell

Journal: Journal of Experimental Biology 221 (2018)

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Chapter 5: The role of V-type H^+-ATPase in energizing the distal gastric caecum of larval *Aedes aegypti*

Authors: Natalie M. D'Silva and Michael J. O'Donnell

Manuscript in preparation

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Chapter 1

General introduction

An epithelium is a layer of cells that forms a barrier between the external and internal environments. Examples of transporting epithelia include the gills of fish and crustaceans, the salivary glands, and the gastrointestinal tract. In the gastrointestinal tract of an insect, the lumen of the gut containing the ingested food is in contact with the external environment, and the haemolymph bathing the gut constitutes the internal environment. The gut epithelium is comprised of a sheet of polarized cells surrounding the lumen, with an apical membrane that faces the lumen, and a basolateral membrane and basement membrane on the opposing face that is bathed in haemolymph (Figure 1). Each surface, apical and basolateral, has different sets of transporter proteins, thus allowing each membrane to perform specialized functions. It is the asymmetrical distribution of transporter proteins on the apical and basolateral surfaces that confers polarity on the epithelium, and determines the direction of transport of water, ions and solutes through the epithelium.. Septate junctions are proteins in insect epithelia that appear as ladder-like structures between the apical membranes of adjacent cells in transmission electron micrographs. Septate junctions maintain the polarity of epithelial tissues by acting as a barrier to lateral diffusion, thus preventing mixing of apical and basal proteins (Green and Bergquist, 1982). An additional function of septate junctions is the regulation of diffusion of molecules through the space between cells (Jonusaite et al., 2016). The transport of ions or water across an epithelium can occur via two routes: between the epithelial cells by the paracellular route, or through the cells by the transcellular route (Fig. 1). Whereas paracellular transport is based on diffusion can either be selective or nonspecific,

transcellular transport involves selective translocation of water, ions or nutrients via membrane-bound transporter proteins in the apical or basolateral membranes.

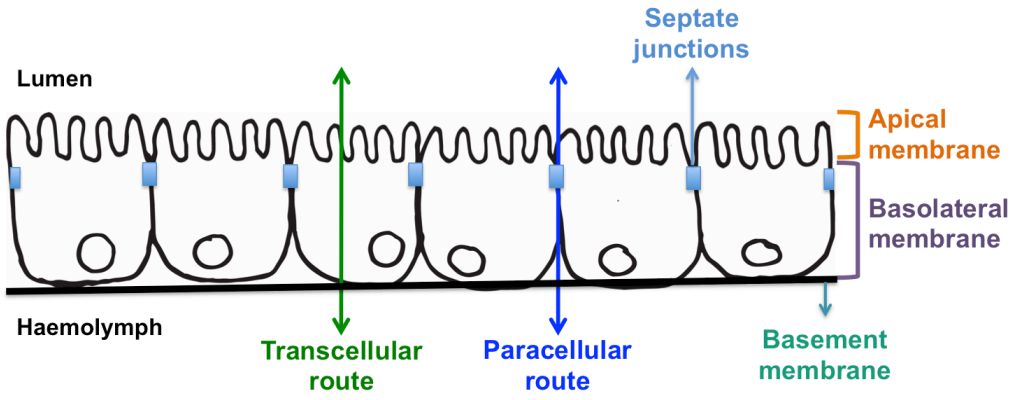

Figure 1: Insect gut epithelial transport. The transcellular (through the cells; green arrow) and paracellular (between the cells; blue arrow) routes control the passage of substances between the gut lumen and haemolymph.

1. Insect gut epithelial structure and function

The insect gut epithelium acts as a selective barrier between the gut lumen and insect tissues. It mediates the selective movement of water, ions and nutrients via transporters present in the epithelial membranes. The gut therefore plays an important role in immunity, digestion, absorption of nutrients and osmoregulation.

The insect gut epithelium is essentially a tube of cells surrounding the lumen. Ingested material is enveloped by the peritrophic membrane, a layer of chitin and proteins, of which peritrophins are the most important (Richards and Richards, 1977; Terra, 2001). In dipteran larvae, the peritrophic membrane is secreted by a few rows of cells called cardia that are present at the entrance of the midgut (Eisemann et al., 2001; Terra, 2001). The space between the epithelial

cells and the peritrophic membrane is called the ectoperitrophic space (Peters and Wiese, 1986).

The insect gut epithelium is comprised of a single layer of cells, made up primarily of two cell types: enterocytes and endocrine cells, with the majority of cells being enterocytes (Chapman et al., 2013; Lehane and Billingsley, 1996; Lemaitre and Miguel-Aliaga, 2013). Membrane foldings greatly expand the surface area of both apical and basal membranes of the enterocytes. The enterocytes typically have a brush border of microvilli on the apical membrane. On the opposite side, facing the haemolymph, are extensive infoldings of the basolateral membrane. Enterocytes are important for the production of digestive enzymes, the absorption of nutrients, and the movement of ions and water between the gut lumen and the haemolymph (Chapman et al., 2013; Lehane and Billingsley, 1996). Endocrine cells, on the other hand, do not have infoldings, and are scattered among the enterocytes; they are responsible for the production of various neuropeptides (Beehler-Evans and Micchelli, 2015; Reiher et al., 2011; Zoephel et al., 2012). Analysis of the insect gut has shown that a wide variety of neuropeptides and their receptors, are expressed both within the gut and in the surrounding tissues (Beehler-Evans and Micchelli, 2015; Veenstra, 2009; Veenstra et al., 2008; Veenstra and Costes, 1999; Veenstra and Ida, 2014; Veenstra et al., 1995; Veenstra et al., 1997). Endocrine modulators regulate homeostasis, and modulate the activity of ion transport via membrane-bound transporter proteins in the dipteran gut (Bendena et al., 1999; Clark et al., 1999; Clark et al., 2000; Gade et al., 1997; Nassel, 2002; Nassel and Winther, 2010; Onken et al., 2004; Veenstra, 2009; Veenstra et al., 2008).

Two of the primary membrane-bound transporter proteins are ion-motive ATPases. In insects, a vacuolar-type H^+-ATPase (VA) drives transepithelial H^+ transport in multiple epithelia, including the salivary glands, gut and Malpighian (renal) tubules. In dipterans and lepidopterans, an apical VA in the gut energizes the secondary transport of other ions across both membranes (Gomes et al., 2013; Onken and Moffett, 2009; Patrick et al., 2006; Shanbhag and Tripathi, 2005; Shanbhag and Tripathi, 2009; Wieczorek, 1992; Wieczorek et al., 1999; Wieczorek et al., 1991). Another ion-motive ATPase, the Na^+/K^+-ATPase (NKA), was shown to be expressed in the insect gut, using immunohistochemical staining (Dubreuil et al., 2000; Okech et al., 2008a; Patrick et al., 2006), but the role of this ATPase in energizing the insect midgut epithelium has not been explored.

2. Ion-motive ATPases

Ion-motive ATPases are enzymes that utilize the energy generated by the hydrolysis of ATP to drive movement of ions across cell membranes. There are three classes of ion-motive ATPases: F-type, P-type, and V-type ATPases (Pedersen and Carafoli, 1987a; Pedersen and Carafoli, 1987b). F-type ATPase, also known as the H^+ transporting ATPase, is an ATP synthase, wherein ATP is synthesised using a H^+ gradient. However, the F-type ATPase can also work in reverse to create an H^+ gradient by utilising ATP. F-type ATPases are usually found on membranes of bacteria, and cell organelles such as chloroplasts and mitochondria. The P-type ATPases are cation pumps that transport cations across a membrane using ATP hydrolysis for energy. The P-type ATPases are also

known as E1-E2 ATPases since the enzyme cycles between two conformations, E1 and E2 (described in section 2). An example of a P-type ATPase is NKA which is found in cell plasma membranes (Jorgensen, 1982). Lastly, VA, or vacuolar ATPases, transports H^+ across membranes, thereby providing an electrochemical gradient which can provide the driving force for the secondary transport of solutes across the membrane. VA was first found to be present in fungal and plant endomembranes (Bowman and Bowman, 1986), but was later found to energise cell plasma membranes in epithelia of both vertebrates (Nelson and Harvey, 1999) and insects (Wieczorek, 1992; Wieczorek et al., 1999; Wieczorek et al., 1991; Wieczorek et al., 1989).

The presence and localization of both ATPases, VA and NKA, have been established in epithelia of multiple insect species, where they are proposed to play an integral role in ion transport (Dadd, 1975; Dubreuil et al., 2000; Harvey et al., 2009; Okech et al., 2008a; Onken and Moffett, 2009; Patrick et al., 2006; Smith et al., 2008; Xiang et al., 2012; Zhang et al., 1994).

The mechanism by which VA energizes ion transport in insect epithelia can be illustrated using the model developed by Wieczorek et al. (Wieczorek, 1992; Wieczorek et al., 1999; Wieczorek et al., 1991; Wieczorek et al., 1989). According to the 'Wieczorek model' (Fig. 2), the VA on the apical membrane drives the secretion of H^+ into the lumen, thereby establishing an electrochemical gradient for H^+ across the apical membrane. This H^+ gradient energizes electrogenic cation:H^+ exchange across the apical membrane via a cation:nH^+-antiporter (where nH^+ is a number of protons). The cation:nH^+-antiporter uses the voltage generated by the VA to drive H^+ back into the

cells and K^+ (or Na^+) from the cell into the lumen (Harvey, 2009). Addition of bafilomycin, a VA inhibitor, dissipates the H^+ gradient (Bowman et al., 1988), which in turn prevents the transport of ions through the cation:nH^+-antiporter. Inhibition by bafilomycin thus established that VA was important for transepithelial transport of cations.

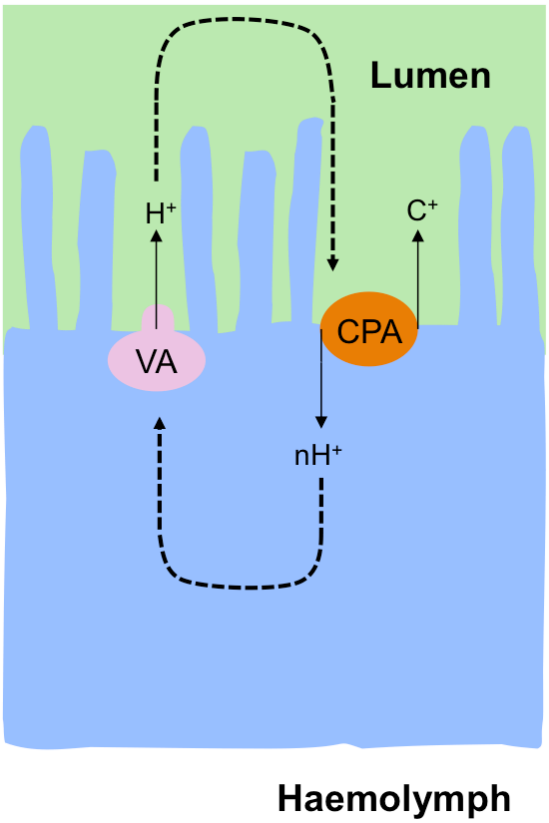

Figure 2: Wieczorek model. Proton secretion via V-type H^+-ATPase (VA) is coupled to cation secretion *via* electrogenic cation:nH^+-antiporter (CPA) in apical membranes of insect midgut cells.

The discovery of VA in insect midgut (Wieczorek, 1992; Wieczorek et al., 1999; Wieczorek et al., 1991; Wieczorek et al., 1989) prompted investigations into the role of VA in other insect epithelial tissues such as the Malpighian tubule (analogous to the human kidney). Similar to their action in

the gut, in Malpighian tubules the apically expressed VA and cation:nH^+-antiporter work in concert to transport K^+ or Na^+ into the lumen, while maintaining pH of the lumen by cycling of H^+ across the apical membrane (Bertram and Wessing, 1994; Dow et al., 1994; Maddrell and O'Donnell, 1992). The high rates of transport of K^+ and/or Na^+ from haemolymph to tubule lumen lead to high flow rates of osmotically obliged water (Beyenbach et al., 2000; Maddrell and O'Donnell, 1992; Wieczorek et al., 1991; Zeiske, 1992). Application of amiloride (an inhibitor of Na^+ channels and exchangers) reduces transepithelial ion transport and results in luminal acidification; this was interpreted as inhibition of the cation:nH^+-antiporter and a consequent unmasking of H^+ secretion by the apical VA (Dow et al., 1994; Maddrell and O'Donnell, 1992; Wessing et al., 1993).

Since VA coupled to secondary ion transport via the cation:nH^+-antiporter has proven to be very important in energizing insect epithelia, the 'Wieczorek model' is widely accepted as dogma. Papers published in the mid to late '90's, for example, often modelled the VA as the only ion-motive ATPase driving transepithelial secretion of ions and fluid in Malpighian tubules (Bertram and Wessing, 1994; Dow et al., 1994; Gill et al., 1998; Maddrell and O'Donnell, 1992; O'Donnell et al., 1996). However, in vertebrate epithelial cells, including those segments of the renal tubule with apical H^+-ATPases involved in renal acidification, a basolateral NKA acts to pump Na^+ out of the cell and K^+ in to the cell. Expulsion of Na^+ from the cell keeps cytosolic Na^+ concentration low, thus creating a Na^+ gradient across the basolateral membrane (Therien and Blostein, 2000). The tendency of Na^+ to leak back into

the cell can be utilized to drive secondary transport of other solutes across the basolateral membrane. Examples include excretion of H^+ via a Na^+/H^+ exchanger, Na^+ or K^+-dependent glucose uptake, and entry of K^+ and Cl^- via a $Na^+/K^+/2Cl^-$ cotransporter (Orlowski and Grinstein, 2004).

Since NKA is expressed in the basolateral membrane of insect Malpighian tubules (Lebovitz et al., 1989), it was predicted that NKA would play a role in fluid secretion. Additionally, the *Drosophila melanogaster* NKA α-subunit is highly sensitive to ouabain, a NKA inhibitor (Glynn, 1957), *in vitro* (Lebovitz et al., 1989). However, ouabain failed to inhibit Malpighian tubule fluid secretion in many insects, such as *Drosophila melanogaster* (Dow et al., 1994), the European red wood ant, *Formica polyctena* (Leyssens et al., 1994), the New Zealand Alpine weta, *Hemideina maori* (Leader and Neufeld, 1997) and the tsetse fly, *Glossina morsitans* (Gee, 1976). Ouabain inhibited Malpighian tubule fluid secretion in the yellow fever mosquito, *Aedes aegypti* (Hegarty et al., 1991). Conversely, ouabain stimulated fluid secretion in the Malpighian tubules of *Drosophila melanogaster* (Linton and O'Donnell, 1999), and in the bloodfeeding hemipteran *Rhodnius prolixus* (Maddrell and Overton, 1988). Despite the conflicting reports on ouabain action in insect Malpighian tubules, later dubbed the "ouabain paradox" (Torrie et al., 2004), the preponderance of evidence for the absence of ouabain sensitivity led to the view that NKA is unimportant in energizing ion transport across insect epithelia. Years later, the "ouabain paradox" was resolved by (Torrie et al., 2004). Insensitivity to ouabain is a consequence not of the absence of the enzyme or the ouabain binding site, but is the result of co-localization of NKA with an organic

anion transport system which actively transports ouabain when applied to the basolateral membrane, thus protecting the ATPase from the inhibitory effects of ouabain (Torrie et al., 2004).

Thus, for about thirty years, apically expressed VA was considered the dominant electromotive force energizing insect epithelia, which led to it becoming the focus of ion transport studies (Dow et al., 1997; Harvey and Wieczorek, 1997; Wieczorek, 1992; Wieczorek et al., 1999). Since the resolution of the "ouabain paradox" for the Malpighian tubules of *Drosophila melanogaster* in 2004, and prior to the studies included in this thesis, the importance of NKA in energizing insect epithelial ion transport has been extended by a few studies, demonstrating a role for NKA in ion transport by the larval *Aedes aegypti* midgut (Linser et al., 2009; Onken and Moffett, 2009), ionoregulation by the rectum of larval *Chironomus riparius* (Jonusaite et al., 2013) and the Malpighian tubules of adult *Aedes aegypti* (Hine et al., 2014). Furthermore, NKA gene expression was found in larval *Drosophila melanogaster* (Chintapalli et al., 2013), and NKA localization was shown along the *Aedes aegypti* gut and Malpighian tubules (Patrick et al., 2006). The role of NKA in energizing ion transport across the caeca and midgut epithelia of larval *Drosophila melanogaster*, and the gastric caecum of larval *Aedes aegypti* has largely been unexplored. Thus, a prime objective of this thesis is to elucidate the roles of both NKA and VA in energizing ion transport across the gut and gastric caecum of these two dipterans, *Drosophila melanogaster* and *Aedes aegypti*.

2.1. Structure and regulation of VA

VA is a heteromultimeric protein comprised of two functional complexes, a peripherally associated catalytic V_1 complex and a membrane-bound, proton translocating V_0 complex (Fig. 3). The V_1 complex consists of eight different subunits, A to H, whereas the V_0 complex consists of four different subunits a, c, d and e (Merzendorfer et al., 2000). VA activity is regulated *in vivo* through reversible dissociation and reassociation of the two complexes, V_1 and V_0 (Kane, 1995; Sumner et al., 1995). In an active VA, the V_1 and V_0 complexes are associated, with the V_1 complex being the site of ATP hydrolysis, and the V_0 complex translocating H^+.

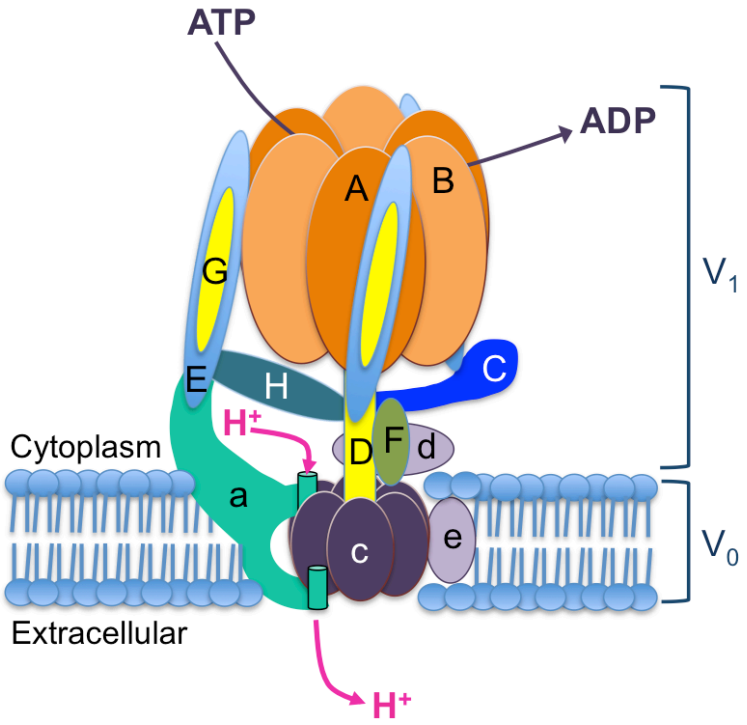

Figure 3: Eukaryotic vacuolar type H^+-ATPase structure. The peripheral V_1 complex consists of eight different subunits identified with capital letters A–H. The membrane-bound V_O complex consists of four different subunits

identified with lowercase letters a, c, d, and e. It uses the energy from ATP hydrolysis to transport H^+ out of the cell.

The V_1 complex consists of a hexamer of alternating catalytic A and B subunits, a central rotor D, three copies of the peripheral stators G and E, and one copy of regulatory subunits C and H (Kitagawa et al., 2008) (Fig. 3). When associated with the V_0 complex, V_1 is active and hydrolyses ATP; when dissociated from V_0, it is inactive. The C subunit is a flexible stator that plays a key role in maintaining the assembly of VA, by holding together the two complexes (Drory et al., 2004). Release of subunit C results in dissociation of the two complexes therefore making the VA inactive.

In the V_1 complex, hydrolysis of ATP drives a conformational change in the catalytic A|B hexamer along with the rotation of the central rotor D. Rotation of the central stalk subunits D, F and c results in proton translocation via proton conducting channels in the V_0 complex (Huss and Wieczorek, 2009).

Neuropeptides and 5-hydroxytryptamine (5-HT) have been shown to regulate the activity of VA *in vivo* (Berridge and Patel, 1968; Coast et al., 2001), and the effect of the intracellular second messenger cAMP has been extensively studied in the salivary gland of the blowfly *Calliphora vicina* (Baumann and Walz, 2012). In salivary glands of the blowfly, 5-HT induces activation of adenylyl cyclase and production of intracellular cAMP which acts to promote the reversible assembly of the V_1 and V_0 complexes resulting in formation of a functional VA (Baumann and Walz, 2012).

The activity of VA can be inhibited by the plecomacrolide antibiotics bafilomycin and concanamycin, which were found to be highly potent

inhibitors with IC50 values in the nanomolar range (Bowman et al., 1988; Drose et al., 1993; Huss et al., 2002). The plecomacrolide binding site resides in subunit c of the membrane-associated V_0 complex (Bowman and Bowman, 2002; Huss et al., 2002; Rautiala et al., 1993), specifically in the luminal half of subunit c (Bowman et al., 2006). In addition to plecomacrolides, VA is also sensitive to sulfhydryl reagents such as N-ethylmaleimide (NEM) (Feng and Forgac, 1992; Mellman et al., 1986). The cysteinyl residue(s) responsible for NEM sensitivity is housed in subunit A of the V_1 complex (Arai et al., 1987; Bowman and Bowman, 1986; Mandala and Taiz, 1986; Moriyama and Nelson, 1987). Inhibitors of VA have been used in biochemical assays to characterize the activity of VA, as well as to test for functioning of VA in whole cells or tissues.

2.2. Structure and regulation of NKA

The NKA is a membrane-bound enzyme that hydrolyses one molecule of ATP, and extrudes three Na^+ out in exchange for the uptake of two K^+ ions. In vertebrates, NKA helps maintain the resting membrane potential, in part through its own electrogenicity and primarily through maintenance of high intracellular K^+ concentrations. Low intracellular concentrations of Na^+ maintained by NKA establish the Na^+ gradient that drives Na-coupled transporters (Therien and Blostein, 2000).

Insect NKA is a heterodimer comprised of two polypeptide subunits, α and β, (Fig. 4) (Emery et al., 1998). The α-subunit is a catalytic subunit

containing the binding site for Na^+, K^+, and ATP. The site for phosphorylation by ATP is on the cytoplasmic side of the α-subunit (Skou and Esmann, 1992). The glycosylated β-subunit is important for the insertion of the αβ complex into the cell membrane (Skou and Esmann, 1992). Dissociation of the αβ complex leads to loss of enzymatic activity (Jorgensen and Andersen, 1988).

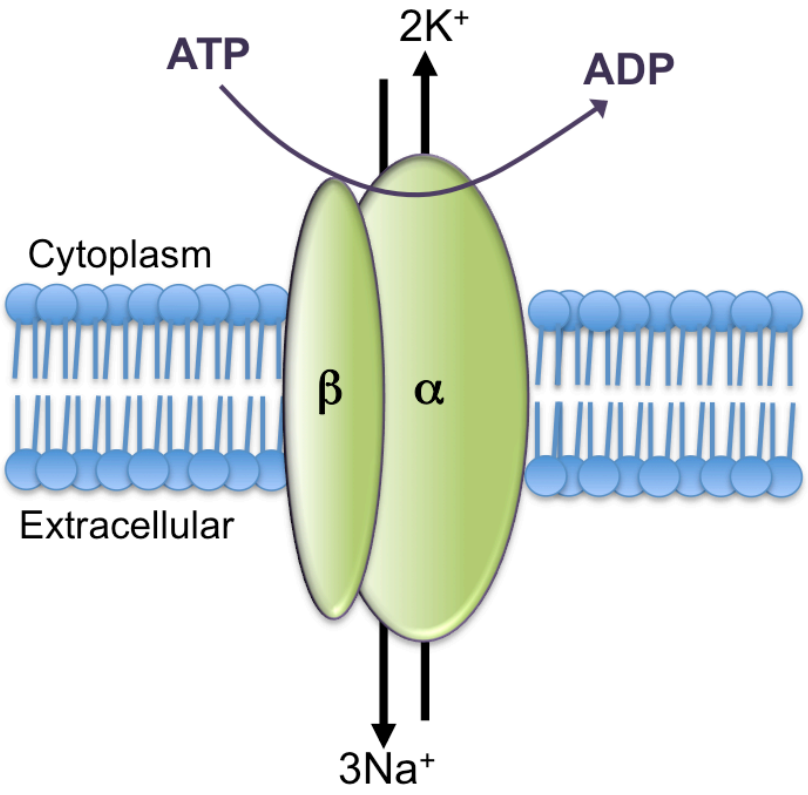

Figure 4: Insect Na^+/K^+ ATPase structure. This heterodimer is made up of two membrane-bound polypeptide subunits, α and β. It uses the energy from ATP hydrolysis to transport $3Na^+$ out of the cell in exchange for $2K^+$ into the cell.

During the catalytic cycle, NKA cycles through two different cation-dependent conformations called E1 and E2, also known as the Albers-Post

scheme (Repke and Schon, 1992; Sachs and Munson, 1991; Skou and Esmann, 1992). Whereas hydrolysis of ATP drives a conformational change in the catalytic A|B hexamer of VA to cause rotation of the central rotor and associated proton translocation, the conformational changes and the accompanying electrogenic active transport of NKA are facilitated by the phosphorylation-dephosphorylation of an aspartate residue in the cytoplasmic α-subunit. In the Albers-Post scheme (Fig. 5), the E1 conformation faces the cytoplasmic surface. E1 is phosphorylated (E1P) using the energy from ATP hydrolysis, and in the E1P state binds three Na^+ from the cytosol and exports them to the extracellular side. Following the release of Na^+, the enzyme enters the phosphorylated E2 (E2P) conformation on the extracellular side. The pump is then dephosphorylated and enters the E2 conformation which binds two extracellular K^+, following which it imports the two K^+ into the cytosol. The pump then returns to the E1 conformation on the cytosolic side after the binding of ATP. Binding and release of three Na^+ and two K^+ ions occurs sequentially, and when bound, the enzyme is in the occluded state preventing the binding of other cations. (Goldshleger and Karlish, 1999; Goldshleger et al., 2001; Jorgensen, 2001; Jorgensen and Andersen, 1988; Jorgensen et al., 2001).

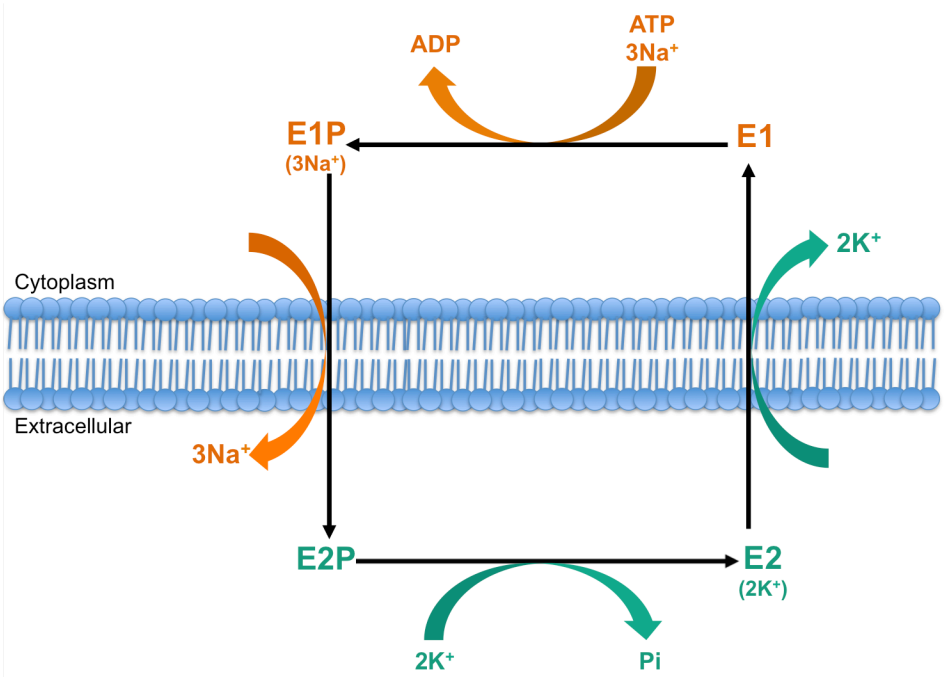

Figure 5: Albers-Post scheme for the NKA reaction cycle. The enzyme can assume two major conformations: E1 (orange) with ion binding sites on the cytoplasmic face, and E2 (green) with ion binding sites on the extracellular side. Hydrolysis of ATP takes place on the cytoplasmic side.

In vertebrates, NKA is primarily regulated by the availability of Na^+, K^+ and ATP. Since ATP is usually present in sufficient concentration in most cell types, it is not a limiting factor for NKA activity. Na^+ is the major regulating factor (Haber et al., 1987); increased intracellular Na^+ concentrations result in stimulation of NKA to extrude Na^+ to maintain low levels of intracellular Na^+. There is also evidence that indicates that a low intracellular K^+ concentration also stimulates NKA (Zhou et al., 2003). In vertebrates, NKA can also be regulated by peptide hormones, protein kinase A (PKA) and protein kinase C (PKC) (Aperia, 2001; Bertorello et al., 1991; Brismar et al., 2000; Ewart and Klip, 1995; Gomes and Soares-da-Silva,

2002; Hubbard and Henderson, 1995; Hussain and Lokhandwala, 1998; Schwartz et al., 1988).

A cardiac glycoside, ouabain, also regulates NKA activity by binding on the extracellular side of the α-subunit (Hootman and Ernst, 1988; Skou and Esmann, 1992) to cause a change in conformational state resulting in an inactive enzyme (Lingrel et al., 1990). The adrenal cortical cells of humans, rodents, and bovines secrete ouabain, which then enters blood circulation; this endogenous ouabain inhibits cardiac NKA (Hamlyn and Blaustein, 2016). In insects, ouabain-sensitive NKA was reported in Malpighian tubules (Hegarty et al., 1991; Kolosov et al., 2018; Linton and O'Donnell, 1999; Maddrell and Overton, 1988). Additionally, there is immunohistochemical evidence for NKA in the midgut of dipterans (Dubreuil et al., 2000; Patrick et al., 2006).

3. Choice of insect models

For this thesis I have chosen to study two dipterans, *Drosophila melanogaster* and *Aedes aegypti*. A recent upsurge of interest in the digestive system of *Drosophila melanogaster* is based on its potential applications in studying the development of the human gastrointestinal system and its metabolism (Baker and Thummel, 2007; Schlegel and Stainier, 2007). *Drosophila melanogaster* has long been used as a model system in fields such as genetics, microbial pathogenesis, disease and physiology (Reiter, 2001), and was one of the very first animals to have a completely sequenced

genome (Adams et al., 2000). In this thesis I have focused on the larvae of *Drosophila melanogaster* as they feed constantly, leading to continuous and robust ion transport across the gut, thus making it easier to manipulate and track electrophysiological parameters over an extended period of time.

In contrast to the use of *Drosophila melanogaster* as a model for human disease and physiology, the mosquito, *Aedes aegypti*, is a vector for diseases such as yellow fever, chikungunya, dengue, and Zika fever, all of which infect millions of humans every year (Tolle, 2009). *Aedes aegypti* originated in Africa (Mousson et al., 2005), but now inhabits tropical, subtropical and temperate regions throughout the world (Leta et al., 2018). Although *Aedes aegypti* prefer to lay their eggs in freshwater, they also lay eggs in brackish water (5–30% seawater) and the larvae survive to form adults (Jude et al., 2012; Ramasamy et al., 2011; Surendran et al., 2012). Understanding larval physiology, and how they adapt to different rearing salinities, can provide the foundation for the development of novel larvicides and control measures.

Furthermore, both organisms have a short life cycle, and are easy to rear in the laboratory, yielding a high number of experimental organisms in a very short period of time.

3.1. *Drosophila melanogaster*

The *Drosophila* gut serves as an excellent tool to help in understanding the structure, development and function of the gastrointestinal tract and related epithelia due to its simple organization and low

heterogeneity of cell types (Shanbhag and Tripathi, 2009). The gut is a single layered tubular epithelium reinforced by musculature and supplied with a tracheal network (Dimitriadis and Kastritsis, 1984). In addition to carrying out the vital functions of digestion and absorption, the gut also serves as the first line of defence against ingested pathogens and toxins.

The digestive and excretory systems of *Drosophila* are similar to their respective mammalian counterparts. The *Drosophila* midgut is analogous to the gastrointestinal tract in vertebrates, wherein digestion and absorption of nutrients occur, whereas the Malpighian tubules and the hindgut form the functional kidney. The larval midgut is compartmentalized (Fig. 6) into three segments based on differences in luminal pH; the neutral to mildly alkaline anterior segment, a short acid-secreting middle segment (middle midgut; MMG) and a long base-secreting posterior segment (posterior midgut; PMG) (Shanbhag and Tripathi, 2009). The anterior segment of the midgut is absorptive, the middle segment has alternating absorptive and secretory cells, and the posterior segment is absorptive (Shanbhag and Tripathi, 2009). The epithelial cells are separated into two domains: the apical membrane is a brush border epithelium that faces the lumen of the gut, whereas the basal membrane is enveloped by visceral muscles which in turn are bathed by the haemolymph which circulates throughout the abdominal cavity.

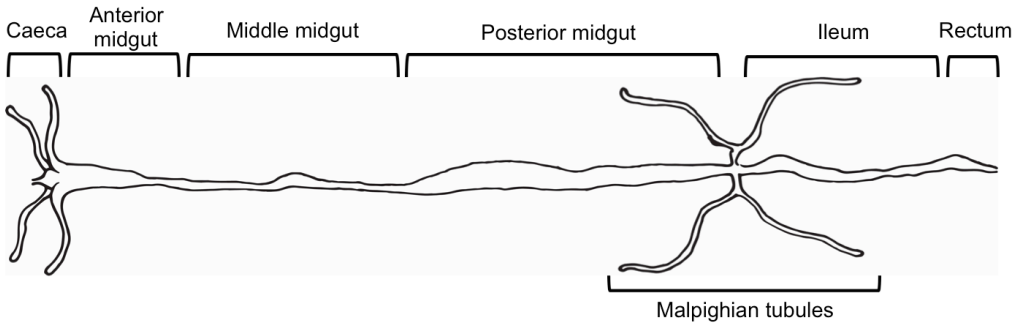

Figure 6: Schematic diagram of the *Drosophila melanogaster* larval gut. The dashed brackets indicate the regions of each gut segment, the caeca, regions of the midgut (anterior midgut, middle midgut, and posterior midgut), regions of the hindgut (ileum and rectum), and the Malpighian tubules.

In contrast to the Malpighian tubule (Dow et al., 1994), very few transporters have been functionally characterized in *Drosophila* gut epithelia. A bafilomycin-sensitive VA has been identified in *Drosophila* midgut (Shanbhag and Tripathi, 2005), however, the links between VA and the transport of other ions like K^+ and Na^+ were not explored. In the 'Wieczorek model' an apical VA and cation:nH^+ transporter function together to transport cations into the lumen (Wieczorek, 1992; Wieczorek et al., 1999; Wieczorek et al., 1991). Since adult *Drosophila* has a high level of gene-expression of VA along with Na^+/H^+ antiporter (NHA) genes in the midgut, hindgut and Malpighian tubules, it was hypothesized that VA works along with NHA in the larval midgut. In this thesis I determine if the 'Wieczorek model' proposed for the adult midgut (Day et al., 2008) holds true for the larval gut epithelia as well.

3.2. *Aedes aegypti*

Aedes aegypti, primarily occupy freshwater habitats, which are hypo-osmotic to the haemolymph of larval mosquitoes, leading to loss of ions from the body fluids into the surrounding water, as well as dilution of body fluids through freshwater uptake across the body surfaces or during feeding. The larvae maintain their haemolymph ion concentrations at levels higher than those of the external medium by hyper-regulation (Bradley, 1994; Wigglesworth, 1938). This is achieved by reduction in drinking, production of dilute urine by the Malpighian tubules, and active ion absorption by ionoregulatory tissues, including the anal papillae, midgut and rectum (Koch, 1938; Ramsay, 1950; Stobbart, 1974; Wigglesworth, 1933a; Wigglesworth, 1933b; Wigglesworth, 1938).

There is a wealth of literature on the mechanisms of 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, however, about ion transport mechanisms across the gastric caeca, which are outpocketings of the larval midgut. While previous studies showed immuolocalization of ion transporters like VA, NKA, cation:H^+ exhangers (AeNHE3, AeNHE8), cation chloride cotransporter (CCC) in the gastric caeca of *Aedes aegypti* (Filippov et al., 2003; Kang'ethe et al., 2007; Patrick et al., 2006;

Pullikuth et al., 2006), the contribution of these transporters to the overall ion transport of the caecum was not elucidated.

The gastric caeca are eight blind sacs that are situated immediately posterior to the cardia and open into the anterior region of the midgut (Fig. 7) (Volkmann and Peters, 1989a). Digestion and absorption of water, ions, and nutrients occurs as the ingested fluid flows through the lumen of the anterior midgut and then the posterior midgut, after which the luminal fluid enters into the ecto-peritrophic space and moves anteriorly towards the caeca via antidromic peristalsis (Ramsay, 1950; Volkmann and Peters, 1989b; Wigglesworth, 1933a; Wigglesworth, 1933b). This countercurrent flow, posteriorly through the anterior and posterior midgut and anteriorly through the ecto-peritrophic space, allows for better utilization of ingested nutrients (Volkmann and Peters, 1989b).

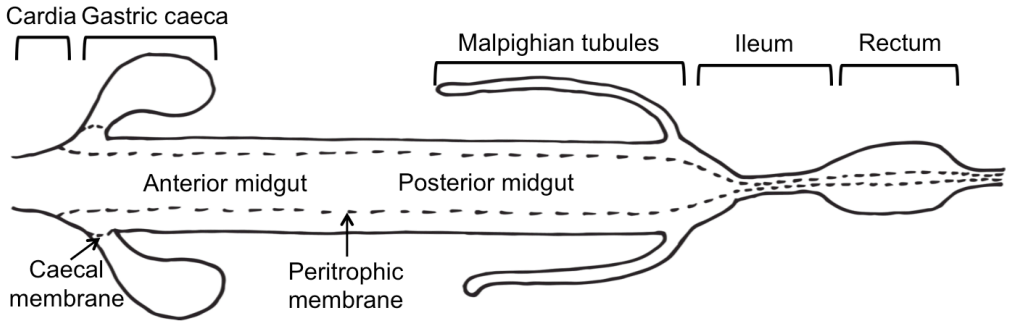

Figure 7: Schematic diagram of the *Aedes aegypti* larval gut. The brackets indicate the regions of the cardia, gastric caeca, ileum, and rectum, which are gut segments, and the Malpighian tubules. The two regions, anterior midgut and posterior midgut, comprise the middle midgut. Each gastric caecum has a caecal membrane at its opening secreted by cells of the caecum, whereas the contents of the gut are bound by a peritrophic membrane which is secreted by the cardia.

The caecum comprises four types of cells: ion transporting cells, reabsorbing/secreting cells, imaginal cells and cells that secrete the caecal membrane (Volkmann and Peters, 1989b). The caecal membrane is present at the junction of the caecum and the anterior midgut, separating the caecal lumen from the lumen of the midgut. The caecal membrane has a smaller pore size in comparison to the peritrophic membrane of the anterior and posterior midgut (Volkmann and Peters, 1989b). Additionally, specialized cells in the gastric caecum secrete the caecal membrane (Volkmann and Peters, 1989a), whereas the cells of the cardia secrete the peritrophic membrane (Eisemann et al., 2001; Terra, 2001).

Of the four types of cells in the gastric caecum, the ion transporting cells, and the reabsorbing/secreting cells are the most abundant (Volkmann and Peters, 1989b). The reabsorbing/secreting cells described by (Volkmann and Peters, 1989b) are hereafter referred to as digestive cells to avoid confusion with cells implicated in secretion and absorption of ions. Based on the morphology of cells present in the gastric caecum, particularly the ion transporting cells and digestive cells, it was hypothesized that the caecum is important in digestion, resorption/storage of nutrients, fluid reabsorption and ion homeostasis (Jones and Zeve, 1968; Ramsay, 1950; Volkmann and Peters, 1989b; Wigglesworth, 1933a; Wigglesworth, 1933b; Wigglesworth, 1942). In cockroaches, for example, amylase and trypsin are released by both merocrine and apocrine secretion primarily from the gastric caeca (Tamaki et al., 2014). In the alimentary canal of *Anopheles gambiae*, a microarray-based analysis of transcriptional compartmentalization indicates that metabolism

and absorption of proteins and carbohydrates takes place mainly in the gastric caeca and posterior midgut, whereas the anterior midgut specializes in the metabolism and absorption of lipids (Neira Oviedo et al., 2008). Two Na^+-aromatic amino acid symporters are also strongly expressed in the gastric caeca of mosquito larvae, consistent with a role in amino acid absorption (Okech et al., 2008b). A L-amino acid transporter with specificity for neutral amino acids such as leucine is also strongly expressed in the gastric caeca of *Aedes aegypti* larvae and leucine transport is enhanced in the presence of Na^+ (Jin et al., 2003). Taken together, these studies suggest that an important role for ion transport across the gastric caecum in mosquito larvae is the maintenance of the appropriate milieu (e.g. luminal pH and Na^+ concentration), for digestion and absorption of amino acids and carbohydrates.

An important advantage of studying the gastric caecum of larval *Aedes aegypti* is the pronounced regionalization of VA and NKA, which raises the possibility that fluxes of ions may differ in the two regions. VA is expressed along the apical membrane throughout the length of the caecum, and on the basal membrane of the distal third. NKA is expressed only on the basal membrane of the proximal two-thirds of the caecum (Patrick et al., 2006). This regionalization provides an important advantage for application of the scanning ion-selective electrode technique (described in Chapters 2-5), which allows non-invasive measurement of ion transport rates across particular regions of an epithelium. Ultrastructural studies also show that there is regional distribution of ion transporting and digestive cells along the

caecum, with the ion transporting cells making up the distal end, and the digestive cells present only in the proximal end (Volkmann and Peters, 1989b). Barring a single study (Boudko et al., 2001), which measured H^+ gradients at the gastric caecum, the ion transport profile of the gastric caecum has not been explored yet. In particular, the latter authors did not take into account the regionalization of cell types reported by Volkmann and Peters (1989a). It was not until a few years after the study by Boudko et al. (2001), that the regionalization of VA and NKA along the gastric caecum was discovered (Patrick et al., 2006). Since there is regionalization of cell type as well as ATPase expression along the gastric caecum, my hypothesis is that ion absorption across the two regions of the caecum are energized by the activity of VA and NKA and that there will be regional differences in transport of H^+, K^+ and Na^+ across the caecum.

4. Thesis objectives and hypotheses

Given the established role of VA in insect epithelia, more recent evidence for an ancillary role for NKA in Malpighian tubules, as well as immunohistochemical evidence for the presence of NKA in the insect midgut, the main objective of this thesis is to explore the roles of these two ion-motive ATPases in promoting ion transport across *Drosophila melanogaster* larval gut epithelia and *Aedes aegypti* larval gastric caecum. In addition to VA, I propose that NKA plays a role in driving epithelial ion transport in insect gut epithelia. Additionally, since larvae of *Aedes aegypti*, a freshwater mosquito, are able to survive in brackish water conditions as well, I investigated the

effects of rearing salinity on the expression and activity of the ATPases in the gastric caecum

In **Chapter 2**, I hypothesized that VA and NKA are important drivers of ion transport along the larval gut epithelium. I show that both VA and NKA enzymes are expressed in the gut, and have similar enzyme activity when measured *in vitro*. I also use the VA-specific inhibitor, bafilomycin, and the NKA-specific inhibitor, ouabain, in conjunction with the Scanning Ion-selective Electrode Technique (SIET) to demonstrate that both ATPases, VA and NKA, drive ion transport along the *Drosophila melanogaster* larval gut epithelium.

In **Chapter 3**, since there is a striking regionalization of VA and NKA along the caecum of freshwater *Aedes aegypti* larvae, I hypothesized that VA and NKA are important drivers of ion transport across the gastric caecum. Furthermore, since larvae are also able to survive in brackish water, I investigated the effects of rearing salinity on VA and NKA expression patterns and activity.

I show that the activity and expression of VA and NKA are vastly different in the two rearing conditions. Additionally, I use SIET to measure ion concentration gradients along the distal and proximal caecum, showing that the two regions transport the same ions at different rates. The ion transport rates are significantly lower in the corresponding regions of larvae reared in brackish water, corresponding to lower activity of VA and NKA in gastric caecum. Overall, I show that the activity and expression of VA and NKA are dependent on rearing conditions. I also observed that the ion transport rates declined within 3 - 6 mins of isolation of the gut from the animal and bathing it in artificial saline.

In **Chapter 4**, I hypothesized that the decline in ion transport observed during the study presented in Chapter 3 could be prevented by the action of a biogenic amine, 5-HT, since 5-HT prevented the decline of transepithelial potential (TEP) of the anterior and posterior regions of the larval midgut (Clark et al, 1999). I showed that 5-hydroxytryptamine (5-HT) is critical for maintaining TEP of the gastric caecum and the modulation of ion transport across the gastric caecum of *Aedes aegypti* larvae reared in freshwater or brackish water. In this chapter I discuss the role 5-HT, and its second messenger, cAMP, in stimulation of transepithelial ion transport and maintenance of TEP across the gastric caecum. Particularly, 5-HT prevented the decline of ion transport, and basolateral membrane potential across the distal gastric caecum, but not the proximal caecum of freshwater larvae.

In **Chapter 5**, since 5-HT stimulates the distal gastric caecum of *Aedes aegypti* larvae, I elucidate the mechanisms by which transepithelial transport occurs in this region in larvae reared in freshwater. I use SIET and ion transport inhibitors in the presence of 5-HT to demonstrate that VA is responsible for H^+ transport as well as secondary cation transport. To achieve this I use a VA inhibitors, N-ethylmaleimide (NEM) and concanamycin, a cation chloride co-transporter inhibitor (bumetanide), and Na^+- exchanger or Na^+-channel blocker (amiloride) and analyzed the effects of each of these on ion transport across the caecum. Additionally, I show that VA is responsible for maintaining TEP, and basolateral membrane potential of the cells of the distal caecum.

Finally, in **Chapter 6**, I integrate the results of chapters 2 - 5. Overall, in this thesis I have demonstrated that NKA, along with VA, are important in

energizing ion transport across the gut epithelium. Additionally, I have suggested a model for ion transport mechanisms of the gastric caecum, and provide suggestions for further experiments to aid in elucidating the regulation of the gastric caecum.

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Chapter 2

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The roles of V-type H^+-ATPase and Na^+/K^+-ATPase in energizing K^+ and H^+ transport in larval *Drosophila* gut epithelia

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ABSTRACT

We analyzed V-type H^+-ATPase (VA) and Na^+/K^+-ATPase (NKA) along the caeca and midgut of third instar *Drosophila* larvae using immunohistochemistry and ATPase activity assays. Corresponding H^+ and K^+ fluxes were characterized using the Scanning Ion-Selective Electrode Technique (SIET), and the roles of transport ATPases in energizing ion transport across the larval gut were investigated by basal application of bafilomycin, a VA inhibitor, and ouabain, a NKA inhibitor. Addition of bafilomycin led to a decrease in H^+ absorption along the caeca and midgut except at the copper cells and large flat cell zone of the middle midgut. H^+ absorption was decreased by acetazolamide, consistent with carbonic anhydrase activity in all regions except at the large flat cell zone of the middle midgut. Bafilomycin or acetazolamide also led to decreased K^+ absorption across the caeca and the anterior midgut. Our data show the dependence of K^+ transport on H^+ gradients established by the VA in the latter regions, consistent with the presence of a Cation-Proton Antiporter (CPA2) identified in other insect epithelia. Addition of ouabain led to the increase of K^+ absorption along the anterior midgut and the large flat cell zone of the middle midgut, suggesting a role for the NKA in these regions. This study shows the importance of both ATPases in driving ion transport across the gut of larval *Drosophila*.

1. Introduction

The *Drosophila* gut carries out the vital functions of digestion and absorption and also serves as the first line of defense against ingested pathogens (Apidianakis and Rahme, 2011; Overend et al., 2016). Based on luminal pH, the larval midgut is compartmentalized into three segments – an absorptive anterior segment (neutral to mildly alkaline), a middle segment (acid-secreting) which has alternating absorptive and secretory cells, and an absorptive posterior segment (base-secreting) (Shanbhag and Tripathi, 2009). Each epithelial cell is separated into two domains: the apical membrane is comprised of a brush border that faces the lumen of the gut, whereas the basal membrane is bathed by the haemolymph. The midgut epithelium is characterized by long junctional complexes with little or no intercellular volume; hence most absorption is predicted to be via transcellular transport (Shanbhag and Tripathi, 2009).

While the function of the V-type H^+-ATPase (VA) and Na^+/K^+ ATPase (NKA) transporters in the *Drosophila* Malpighian tubule has been extensively studied (Dow et al., 1994; Ianowski and O'Donnell, 2004; Linton and O'Donnell, 1999; Maddrell and O'Donnell, 1992; O'Donnell et al., 1996; Torrie et al., 2004), fewer studies have focused on the gut epithelia. The effects of bafilomycin on transepithelial potential, intracellular pH and short circuit current across the posterior midgut of larval *Drosophila* led to the hypothesis that a bafilomycin-sensitive VA is present in the basal membrane (Shanbhag and Tripathi, 2005). The effects of bafilomycin on H^+ concentrations at the basal membrane suggested that VA was responsible for transport of H^+ from cell to bath (Shanbhag and Tripathi, 2005). Other regions of the gut (besides the posterior midgut), or the effects of bafilomycin on transport of other ions such as K^+ have not been examined; however a recent study has shown that VA is highly expressed in the acidic region of the middle midgut and that knockdown of VA by RNAi is correlated with an increase in luminal pH of this region (Overend et al., 2016).

In other insects, the VA is responsible for establishing ion gradients that drive the secondary transport of other ions and nutrients

Abbreviations: VA, V-type H+-ATPase; NKA, Na+/K+ ATPase; SIET, Scanning Ion-Selective Electrode Technique.

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into the cell. For example, the apical K^+ pump (Harvey et al., 1983) in the highly alkaline midgut of larval *Manduca sexta* (lepidopteran) has two components – the VA and a $K^+/2H^+$ antiporter (Wieczorek, 1992; Wieczorek et al., 1999, 1991b). The VA creates a high lumen positive transepithelial voltage and drives secondary K^+ secretion into the lumen through a $K^+/2H^+$ antiporter; luminal alkalinization is sustained by concurrent secretion of bicarbonate/-carbonate (Dow, 1984; Moffett and Cummings, 1994; Moffett, 1994; Wieczorek et al., 1991b). The high transapical voltage across the brush border membrane of the adjacent columnar cells drives K^+-dependent secondary active transport of nutrients.

In addition to VA, ouabain-sensitive, basally located NKA was reported in Malpighian tubules of *Rhodnius prolixus* (Maddrell and Overton, 1990) and *Drosophila* (Linton and O'Donnell, 1999). The role of NKA as a driver of inorganic ion transport in insect Malpighian tubules of *Drosophila* has been underestimated due to the effects of an active ouabain transport system, which is co-localized with NKA and prevents ouabain from reaching concentrations sufficient to block NKA activity (Torrie et al., 2004). Although there is immunohistochemical evidence for NKA along the middle midgut of *Drosophila* (Dubreuil et al., 2000) and the caeca of *Aedes aegypti* (Patrick et al., 2006), the pump has not been functionally characterized in any region of the dipteran gut.

In this study, we have evaluated the roles of both VA and NKA in transport of H^+ and K^+ across multiple regions of the gut of larval *Drosophila*. We have used immunohistochemistry and ATPase activity assays to determine the presence and activities of both transporters. We have also used the Scanning Ion-Selective Electrode Technique to measure H^+ and K^+ fluxes along the gut. The contributions of the two ion-motive ATPases to transepithelial H^+ and K^+ transport have been assessed through measurement of the effects of specific inhibitors.

2. Materials and methods

2.1. Larvae

Third instar larvae of *Drosophila melanogaster* Oregon R strain (Bloomington #2376) were used for all experiments. Larvae were raised on diet modified from (Roberts and Stander, 1998). Solution A consisted of 800 ml distilled water, 100 g sucrose, 18 g agar, 8 g KNa tartrate, 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgCl_2$ and 0.5 g $CaCl_2$. Solution B consisted of 200 ml distilled water and 50 g dry active yeast. The two solutions were autoclaved, combined and stirred. After cooling to 55 °C, 10 ml of an acid mix (11 parts distilled water, 10 parts propionic acid and one part 85% *o*-phosphoric acid) was added to the mixture. Flies were allowed to lay eggs on dishes containing fly food for 3 h and then removed and incubated at 25 °C.

2.2. Whole mount immunolocalization

NKA was immunolocalized using a monoclonal mouse anti-chicken antibody raised against the cytoplasmic domain of the catalytic a-subunit (a5; Douglas Fambrough, Developmental Studies Hybridoma Bank, IA, USA) (Takeyasu et al., 1988). This antibody has previously been shown to immunolocalize *Drosophila* NKA in Malpighian tubules (Lebovitz et al. 1989). Immunolocalization of VA was performed using a guinea pig anti-hornworm (*Manduca sexta)* antibody which is directed against the V_1 complex of the VA (antibody 353-2; gifted by H. Wieczorek, Universität Osnabrück) (Weng et al., 2003).

Larvae were dissected in Phosphate Buffered Saline (PBS; NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, made up to 1000 mL with distilled water) to remove the entire gut, and fixed in 2% paraformaldehyde/PBS (PFA/PBS) for 2 h at room temperature. Tissues were washed 3 times in PBS at room temperature to remove all PFA/PBS fixative. Tissues were then incubated in Antibody Dilution Buffer (ADB; 10 g goat serum, 3 g bovine serum albumin, 50 µL Triton-X, made up to 100 mL with PBS). The tissues were then incubated with primary antibodies diluted in ADB (1:10 NKA and 1:2000 VA) for 48 h at 4 °C. An ADB solution lacking the primary antibodies served as a negative control. Following incubation in primary antibody, tissues were washed 4 times in PBS at room temperature, and then incubated with Alexa Fluor® 594 (red) anti-mouse (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and Alexa Fluor® 488 (green) anti-guinea pig (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 2 h at room temperature in dark. Tissues were then washed 4 times in PBS at room temperature in the dark, and mounted on slides using a mounting medium (Molecular Probes ProLong Antifade, Invitrogen Canada Inc., Burlington, ON, Canada) containing 5 µg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada).

2.3. VA and NKA activity assay

VA and NKA activities were determined according to a method previously outlined (Jonusaite et al., 2011). The method relies on the enzymatic coupling of ouabain-sensitive or bafilomycin-sensitive hydrolysis of adenosine triphosphate (ATP) to the oxidation of reduced nicotinamide adenine dinucleotide (NADH). Disappearance of NADH is directly measured in a microplate spectrophotometer. *Drosophila* guts or gut segments were collected into 2 ml microcentrifuge tubes (10–20 guts or 60–100 gut segments per tube) containing an ice-cold solution of 150 mM sucrose, 10 mM EDTA and 50 mM imidazole (SEI; pH 7.3). Tissue was then homogenized in SEID (0.5% sodium deoxycholic acid in SEI) on ice using a PRO 250 homogenizer with a 5 × 75 mm generator (PRO Scientific Inc.,) for 10 s. Homogenates were then centrifuged in a 5810R centrifuge (Eppendorf, Canada) at 10,000*g* for 10 min and 4 °C. Supernatants from each tube were divided into four aliquots which were transferred to 0.6 ml microtubes and stored at −85 °C.

Three assay solutions A, B and C were prepared and kept on ice. Solution A was prepared using 4 units/mL lactate dehydrogenase, 5 units/mL pyruvate kinase, 2.8 mM phophoenolpyruvate, 3.5 mM ATP, 0.22 mM NADH, 50 mM imidazole, 189 mM NaCl, 1.07 mM $MgCl_2$, 42 mM KCl, 1.70 mM Imidazole. Solution B was prepared by the addition of ouabain (5 mM; SigmaAldrich Canada, Ltd.) to solution A, and solution C was prepared by the addition of bafilomycin (10 µM; LC Laboratories, Woburn, MA, USA) to solution A.

The supernatants of experimental samples were thawed and added to the microplate in six replicates of 10 µl per well, on ice. Two wells per sample were filled with 200 µl of solution A, two with solution B, and two with solution C. The absorbance was then read in a Multiskan Spectrum microplate spectrophotometer (Thermo Fisher Scientific, Burlington, ON) set at 25 °C, at 340 nm every minute for 30 min to track the decrease in NADH absorbance. The slopes for each set containing solutions A, B and C were measured, and activities (µmol ADP mg^{-1} protein h^{-1}) were calculated using the following equations

$$\text{NKA activity} = ((\text{Slope of A} - \text{Slope of B})/S * 60)/[P]$$

$$\text{VA activity} = ((\text{Slope of A} - \text{Slope of C})/S * 60)/[P]$$

where:
S: slope of ADP standard curve
[P]: protein concentration of the sample

An adenosine diphosphate (ADP) standard curve was run using ADP (Sigma-Aldrich Canada, Ltd.) standards (in nM) of 0, 5, 10, 20 and 40, prepared in imidazole buffer (50 mM l^{-1} imidazole; pH 7.5). The sample protein content was quantified using the Bradford assay (Sigma-Aldrich Canada, Ltd.) using bovine serum albumin (Bioshop Canada Inc., Burlington, ON, Canada) as a standard. The final activity was expressed as micromoles of ADP per milligram of protein per hour.

2.4. Dissection and physiological saline

The entire guts from larva were isolated in *Drosophila* saline (in mM: 10 glutamine, 20 glucose, 8.6 HEPES, 4.3 NaH_2PO_4, 10.2 $NaHCO_3$, 8.5 $MgCl_2$, 2 $CaCl_2$, 20 KCl, 117.5 NaCl; titrated to pH 7) and transferred to 35 mm Petri dishes with saline. Dishes were pre-coated with poly-L-lysine (70–150 kDa, Sigma-Aldrich Canada, Ltd.) to promote adherence of the isolated gut to the bottom of the dish, as described by Naikkhwah and O'Donnell (2011). K^+ scans were carried out in *Drosophila* saline. To facilitate measurement of H^+ gradients in the unstirred layer, SIET measurements were carried out in *Drosophila* saline with reduced buffering (in mM: 10 glutamine, 20 glucose, 1 HEPES, 0.43 NaH_2PO_4, 1.02 $NaHCO_3$, 8.5 $MgCl_2$, 2 $CaCl_2$, 20 KCl, 140 NaCl).

2.5. Ion-selective microelectrodes and the scanning ion-selective microelectrode technique

Methods for fabrication and calibration of K^+-selective and H^+-selective microelectrodes and measurement of ion fluxes by SIET have been described previously (Pacey and O'Donnell, 2014).

SIET measurements of H^+ and K^+ fluxes were carried out along the caeca and the midgut of *Drosophila* third instar larvae. The midgut was divided into 3 major segments – the anterior midgut, middle midgut and the posterior midgut. The middle midgut was further subdivided based on its cell types into the copper cell region and the large flat cell region, the latter named on the basis of their flattened appearance and cell length which is ~3-fold that of cells in the copper cell region (Poulson and Waterhouse, 1960). The posterior midgut was further subdivided based on its luminal pH into the neutral region and the alkaline region. Prior to scanning, each region was identified based on morphological features (Naikkhwah and O'Donnell, 2012b). Fluxes of K^+ or H^+ were measured at 5 sites 250 μm apart along each region of the midgut, and at 5 sites 150 μm apart along the caeca.

Bafilomycin (VA blocker; 5 μM) or acetazolamide (carbonic anhydrase blocker; 1 mM) were added to *Drosophila* saline, and the consequent effect on H^+ and K^+ fluxes was measured after 5 min using SIET. Similarly, the effects on K^+ fluxes were measured after 5 min after addition of ouabain (NKA blocker; 1 mM). None of the drugs at the concentrations used in the experiments altered the slope of either the K^+-selective or H^+-selective electrodes.

2.6. Statistical analysis

Data were plotted using Graphpad InStat (Graphpad Software Inc., La Jolla, CA), and values are expressed as means ± sem. NKA and VA ATPase activity data for the combined caeca and midgut epithelia, and within each region, were analyzed using a paired Student's *t*-test.

H^+ and K^+ fluxes are expressed as mean ± sem for a number of guts (N). Each mean value was based on three replicate scans at each of the 5 sites along each region of the gut. Data in which H^+ and K^+ absorption is plotted against region were analyzed by one-way ANOVA with a post hoc test for linear trend. The effects of bafilomycin or acetazolamide on H^+ or K^+ absorption within each region were analyzed by one-way ANOVA followed by Tukey's comparison test. The effects of ouabain on K^+ absorption within each region were analyzed by Student's unpaired *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. VA and NKA expression in the epithelia of the gut

Immunohistochemical localization revealed VA and NKA expression in the gut of larval *D. melanogaster* (Fig. 1). VA was present throughout the gut, from the caeca to the neutral zone of the posterior midgut, with apparently less staining in the alkaline zone of the posterior midgut (Fig. 1A, F, G). VA staining was sporadic in the caeca (Fig. 1B) but uniform in the anterior and middle midgut (Fig. 1C, D, E). NKA was present in the caeca and midgut, with apparent maximal staining in the anterior midgut (Fig. 1A). Apical versus basal VA and NKA staining could not be readily discerned in optical sections of the whole mounts. Controls in which the NKA and VA antisera were omitted from the procedure showed no staining (not shown).

3.2. NKA and VA activity profiles

NKA and VA activities (μmol ADP mg^{-1} protein h^{-1}) were quantitatively similar for the combined tissue samples of caeca and midgut collected from third instar larvae (Student's *t*-test; Fig. 2A).

For analysis of ATPase activity in specific regions of the gut, the small mass of individual gut segments required pooling of 60–100 samples, and sample size was therefore reduced (N = 3–4). There were no significant differences between NKA and VA activities within each region (caeca, anterior midgut, copper cells of the middle midgut, large flat cells of the middle midgut, and neutral zone of the posterior midgut) (Fig. 2B). There were also no significant differences between the activities of either ATPase across the different regions, with the exception of the alkaline zone of the posterior midgut where there was no detectable NKA or VA activity (Fig. 2B).

3.3. H^+ and K^+ fluxes at the caeca and midgut: Effects of bath application of bafilomycin, ouabain and acetazolamide

H^+ and K^+ fluxes were measured along the basal surface of the caeca and midgut. H^+ was absorbed from tissue to the bathing saline at all sites measured, and the magnitudes of the fluxes were similar in all regions (Fig. 3A). K^+ was also absorbed in all regions with a trend towards decreasing K^+ absorption from caeca to posterior midgut (one-way ANOVA, post hoc test for linear trend). The K^+ fluxes at the caeca and anterior midgut were significantly higher than at the posterior midgut (Fig. 3B).

The contributions of VA and NKA to H^+ and K^+ fluxes in specific regions were assessed using bafilomycin (VA inhibitor), ouabain (NKA inhibitor), and acetazolamide (carbonic anhydrase inhibitor). The effect of each drug was analyzed after a 5-min period of treatment. Control tissues were treated with saline containing the vehicle (0.1% DMSO) for bafilomycin and acetazolamide.

H^+ fluxes in the caeca, anterior and posterior midgut decreased on exposure to bafilomycin, but there was no significant effect on the middle midgut (Fig. 4A). Bafilomycin reduced H^+ fluxes by 95% and 87% at the caeca and anterior midgut respectively (Fig. 4A). There was no significant effect of bafilomycin at the middle midgut, whereas fluxes were reversed from absorption to secretion at the neutral and alkaline zones of the posterior midgut (Fig. 4A). Acetazolamide reduced H^+ fluxes at the caeca by 47%, copper cells of the middle midgut (74%) and neutral zone of the posterior midgut (55%) (Fig. 4A). H^+ flux along the alkaline zone of the posterior

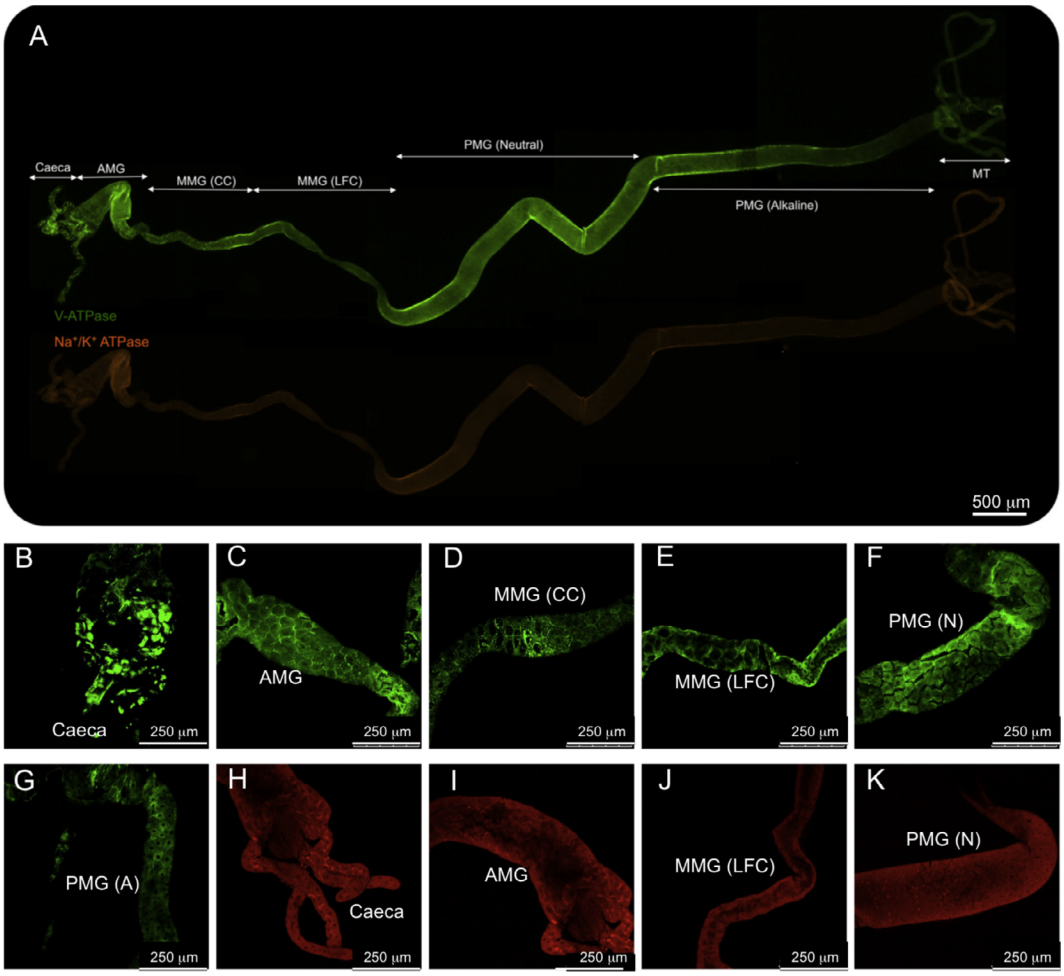

Fig. 1. Immunolocalization of V-type H^+-ATPase (green) and Na^+/K^+ ATPase (red) along the gastrointestinal tract of *Drosophila melanogaster* third instar larvae [A]. In caeca VA expression is sporadic [B] whereas NKA expression appears uniform [H]. Both VA and NKA are expressed in the anterior midgut, AMG [C, I]; large flat cell zone of the middle midgut, MMG (LFC) [E, J]; neutral zone of the posterior midgut, PMG (N) [F, K] and Malpighian tubules, MT. VA was also expressed in the copper cells of the middle midgut, MMG (CC) [D] and the alkaline zone of the posterior midgut, PMG (A) [G].

midgut was reversed from absorption to secretion by treatment with acetazolamide.

To determine if there was a link between VA-dependent H^+ transport and K^+ transport, the effects of bafilomycin and acetazolamide on K^+ fluxes were also measured. Bafilomycin reduced K^+ fluxes by 61% at the caeca and 66% at the anterior midgut (Fig. 4B). Acetazolamide also reduced K^+ fluxes, by 81% at the caeca and 72% at the AMG. There was no significant reduction in K^+ fluxes in response to either drug across the middle midgut and the posterior midgut (Fig. 4B).

Given our evidence of NKA immunolocalization and NKA activity along the gut, we examined if there was a change in K^+ fluxes in response to the NKA inhibitor ouabain. K^+ fluxes were significantly higher at the anterior midgut (177%) and large flat zone of the middle midgut (72%) after exposure to ouabain (Fig. 4C). Ouabain had no significant effect on K^+ fluxes at the caeca, copper cells of the middle midgut, or posterior midgut.

4. Discussion

This study is the first to use SIET to directly measure H^+ and K^+ concentration gradients along the larval gut. Corresponding ion fluxes can be estimated from the measured gradients using the Fick equation, as described previously (Naikkhwah and O'Donnell, 2012a; Pacey and O'Donnell, 2014). In the steady state, the fluxes are indicative of transepithelial transport, given the small volume of the cells relative to that of the lumen. This study is also the first to quantify ATPase activity of VA and NKA in regions of the gut, and has also used immunohistochemistry to confirm the expression of both enzymes in specific regions of the gut. Importantly, we have used SIET to show the contributions of VA and NKA to transepithelial transport of H^+ and K^+. At the caeca and anterior midgut the application of the VA inhibitor bafilomycin or the carbonic anhydrase inhibitor acetazolamide reduced the rates of H^+ and K^+ absorption, suggesting that K^+ transport is dependent on VA-driven H^+ transport. Treatment with the NKA inhibitor ouabain increased K^+ absorption rates at the anterior midgut and large flat cell zone of the middle midgut, indicating a role for NKA in K^+ transport across these regions. It is important to mention one caveat in the interpretation of SIET measurements based on the application of inhibitors to the entire isolated gut. Changes in ion fluxes across a specific region of the gut in response to an inhibitor may reflect not just inhibition of a specific ion transporter, but also changes in luminal ion composition or pH in that region or in upstream regions of the gut.

4.1. Caeca and anterior midgut

The presence of VA in the caeca and anterior midgut was shown by an ATPase activity assay (Fig. 2B) and immunostaining of whole

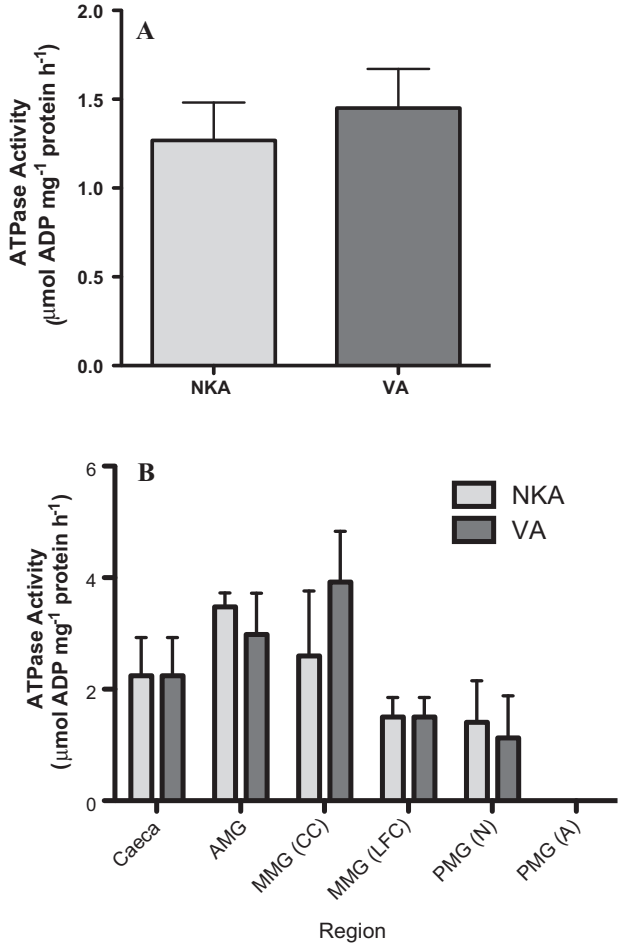

Fig. 2. [A] Total NKA and VA ATPase activity for the combined caeca and midgut epithelia (anterior midgut, AMG; copper cells of the middle midgut, MMG (CC); large flat cell zone of the middle midgut, MMG (LFC); neutral zone of the posterior midgut, PMG (N); and alkaline zone of the posterior midgut, PMG (A) of the third instar *Drosophila melanogaster* larvae. Data are expressed as mean ± sem (N = 11). [B] NKA and VA ATPase activity in specific regions of the gastrointestinal tract of third instar larvae. Data are expressed as mean ± sem (N = 3–4).

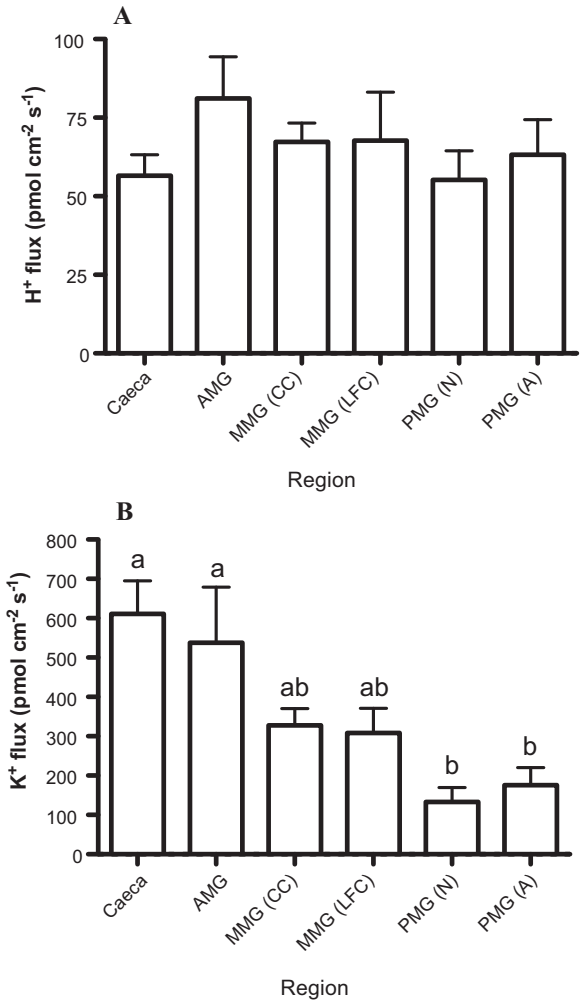

Fig. 3. SIET measurement of H^+ [A] and K^+ [B] absorptive fluxes along the caeca and anterior midgut AMG; copper cells of the middle midgut, MMG (CC); large flat cell zone of the middle midgut, MMG (LFC); neutral zone of the posterior midgut, PMG (N); and alkaline zone of the posterior midgut, PMG (A) of third instar *Drosophila melanogaster* larvae in control saline. Data are expressed as mean ± sem (N = 7–15). Bars labeled with different lowercase letters are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's comparison test.

mounts (Fig. 1A, B, C). However, apical versus basal staining could not be readily discerned in optical sections of these preparations. Since there is overall absorption of H^+ at both the caeca and anterior midgut (Fig. 3A), which is reduced on exposure to bafilomycin or acetazolamide (Fig. 4A), it is possible that VA may be present on the basal membrane. Bafilomycin reduces H^+ absorption directly by inhibiting VA, whereas acetazolamide inhibits VA activity indirectly by reducing production of cellular H^+ availability through inhibition of carbonic anhydrase.

Exposure to bafilomycin also led to a decrease in K^+ absorption at the caeca and anterior midgut (Fig. 4B). This dependence of K^+ transport on VA activity is consistent with the Harvey model of transepithelial ion transport in the mosquito gut, whereby an apically located VA drives the secretion of H^+ into the lumen, thereby leading to a H^+ gradient which drives the electrogenic exchange of $1K^+/2H^+$ across the apical membrane via a cation:proton antiporter (CPA2) (Harvey et al., 2009). Reabsorption of H^+ into the cell is thus coupled to secretion of K^+ into the lumen. The CPA2 antiporter also creates a K^+ gradient, which may drive uptake of nutrients into the cell along with K^+ via a nutrient amino acid transporter (NAT). The cellular K^+ may then exit the cell across the basal membrane via a NAT or a K^+ channel. We propose that addition of bafilomycin inhibits the apical VA, thereby reducing the H^+ gradient across the apical membrane, which in turn limits H^+/K^+ exchange through the CPA2, and the subsequent recycling of K^+ from lumen to cell through the NAT.

In addition to VA activity, NKA activity was also confirmed in the caeca and anterior midgut (Fig. 2B), and NKA was detected in immunostained whole mounts (Fig. 1A, H, I). SIET measurements revealed that K^+ transport at the anterior midgut increased when exposed to ouabain (Fig. 4C). This increased absorption is consistent with the inhibition of K^+ influx through a basal NKA, thus unmasking sustained cell to bath leakage through pathways such as basolateral K^+ channels or NATs. Our findings suggest that the absorption of Na^+ across the caeca reported in a previous study (Naikkhwah and O'Donnell, 2012b) may be driven in part by an ouabain-sensitive NKA.

4.2. Middle midgut

The presence of VA along the copper cells and large flat cells of the middle midgut was shown by immunostaining (Fig. 1A, D, E) and ATPase activity assays (Fig. 2B). SIET measurements indicated that H^+ was absorbed at the copper cells and large flat cells of

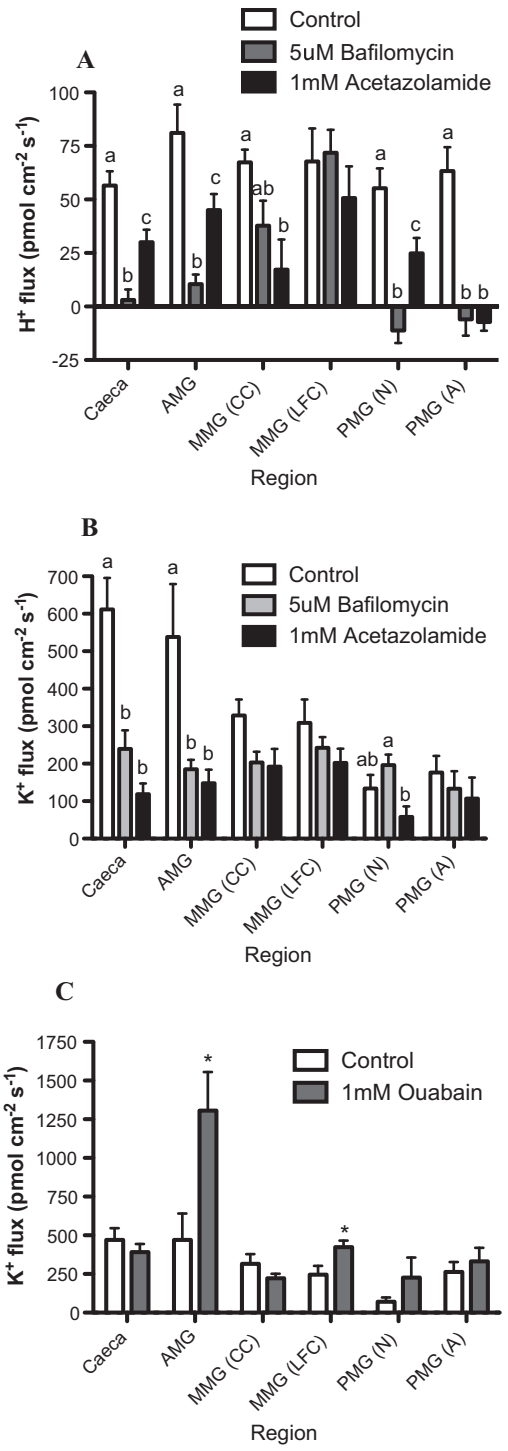

Fig. 4. SIET measurement of H^+ and K^+ fluxes along the caeca and anterior midgut, AMG; copper cells of the middle midgut, MMG (CC); large flat cell zone of the middle midgut, MMG (LFC); neutral zone of the posterior midgut, PMG (N); and alkaline zone of the posterior midgut, PMG (A) of third instar *Drosophila melanogaster* larvae treated with [A, B] 5 μM bafilomycin or 1 mM acetazolamide, and K^+ fluxes after treatment of 1 mM ouabain [C]. Data are expressed as mean ± sem (N = 5–10). For A and B, within each region bars labeled with different lowercase letters are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's comparison test. In C, within each region bars labeled with an * are significantly different from the corresponding control, as determined by an unpaired Student's *t*-test.

the middle midgut (Fig. 3A). Although bafilomycin had no significant effect on the measured H^+ fluxes, addition of acetazolamide resulted in alkalinization of the bathing saline along the copper cells of the middle midgut (Fig. 4A). Since VA is dependent on cellular H^+ derived from activity of carbonic anhydrase, inhibition of carbonic anhydrase activity by acetazolamide may lower cellular H^+ concentration sufficiently to reduce the rate of H^+ absorption from cell to bath. The absorptive H^+ flux in the middle midgut is somewhat unexpected, given that the lumen is acidic. One possibility is that metabolic CO_2 is the source of H^+ in the boundary layer near the surface of the middle midgut. For example, carbon dioxide excreted across fish gills is hydrated catalytically to form HCO_3^- and H^+ ions in water near the gill surface (Wright et al., 1989). Our findings that acetazolamide but not bafilomycin inhibited the absorptive H^+ flux in the middle midgut are consistent with a role for CO_2 as the source of protons in the unstirred layer near the midgut surface.

NKA was also visualized along the large flat cells of the middle midgut in immunostained whole mounts (Fig. 1A, J), and ATPase activity was detected in both the copper cells and large flat cells of the middle midgut (Fig. 2B). Immunohistochemical evidence for NKA along the middle midgut was also reported by (Dubreuil et al., 2000). SIET measurements showed an increase in K^+ absorption along the large flat cells of the middle midgut after addition of ouabain to the bathing saline (Fig. 4C), consistent with the presence of a basally located NKA. A basal NKA in this region may drive the absorption of Na^+ from cell to bath that has been reported previously (Naikkhwah and O'Donnell, 2012b).

4.3. Posterior midgut

Both immunostaining (Fig. 1A, F) and ATPase activity assays (Fig. 2B) indicated the presence of both VA and NKA in the neutral zone of the posterior midgut. The presence of VA was confirmed using SIET, which revealed a switch from net absorption of H^+ to secretion in the presence of bafilomycin (Fig. 4A). We propose that VA is present on the basolateral membrane in this region, as hypothesized by (Shanbhag and Tripathi, 2009). Since the neutral zone of the posterior midgut is adjacent to, and downstream of, the acidic midgut (Shanbhag and Tripathi, 2009), the VA may help to maintain neutrality in this region by recovery of H^+ from the lumen, thereby driving luminal pH from acidic to a neutral. Achieving neutrality in this region may also be assisted by secretion of bicarbonate generated by the hydration of CO_2 by carbonic anhydrase. The activity of carbonic anhydrase is consistent with SIET measurements, which showed that treatment with acetazolamide reduced H^+ absorption along the neutral zone of the posterior midgut (Fig. 4A).

Further downstream, at the alkaline zone of the posterior midgut, the addition of bafilomycin and acetazolamide led to a reversal from H^+ absorption to secretion, as measured by SIET (Fig. 4A). This result is in agreement with data of a previous study which reported alkalinization of the bath adjacent to the posterior midgut when bafilomycin and acetazolamide were applied to the basal side (Shanbhag and Tripathi, 2009). SIET data show direct evidence of bafilomycin-sensitive H^+ absorption in this region, and VA immunostaining was apparent in the anterior third of the posterior midgut (Fig. 1A, G). No detectable VA ATPase activity (Fig. 2B) was apparent in the alkaline zone, which may be a consequence of harvesting more posterior regions for the ATPase assay to avoid inclusion of tissue from the neutral region of the posterior midgut. Importantly, these results suggest the potential for SIET to resolve the effects of low levels of VA activity in this region of the gut or in other tissues.

In contrast to the anterior midgut and the large flat cell zone of the middle midgut, no NKA was detected by immunostaining

(Fig. 1A) or ATPase activity assays in the alkaline zone of the posterior midgut (Fig. 2B). Moreover, ouabain had no effect on the K^+ fluxes in this region (Fig. 4C), indicating a negligible role for NKA in K^+ transport.

5. Conclusion

The role of NKA in energizing transport by vertebrate epithelia has been well established, whereas the discovery of VA in the *Manduca* midgut (Wieczorek et al., 1991a, 1989) lead to numerous studies emphasizing the importance the VA in driving transepithelial ion secretion in the Malpighian tubules of insects (Dow et al., 1994; Maddrell and O'Donnell, 1992; O'Donnell et al., 1996). In subsequent studies, evidence for a role of NKA in driving ion transport by *Drosophila* Malpighian tubules suggested that the importance of NKA in transepithelial ion transport by insect epithelia might have been underestimated (Ianowski and O'Donnell, 2004; Linton and O'Donnell, 1999; Torrie et al., 2004). In this study we have confirmed the presence of NKA and VA along the *Drosophila* gut epithelium. We have also established that transport of K^+ is dependent on VA activity. We therefore propose that both ATPases contribute to the creation of favourable electrochemical gradients for transport of ions and possibly nutrients across the gut epithelial membrane.

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Chapter 3

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

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ABSTRACT

Larvae of *Aedes aegypti*, the yellow fever vector, inhabit a variety of aquatic habitats ranging from 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

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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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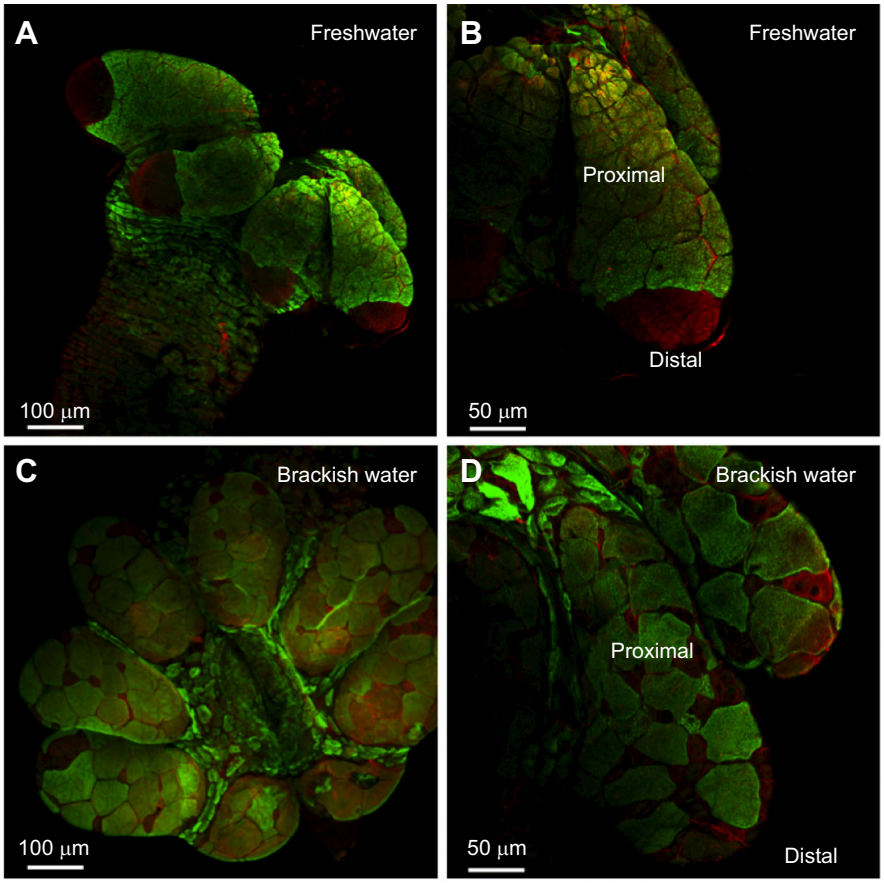

Fig. 1. Immunolocalization of ATPases in the gastric caeca in whole mounts of fourth instar *Aedes aegypti* larvae. In larvae reared in freshwater (A,B), the distal third expressed V-type H^+-ATPase (VA; red) and the proximal two-thirds expressed Na^+/K^+-ATPase (NKA; green). In larvae reared in brackish 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.

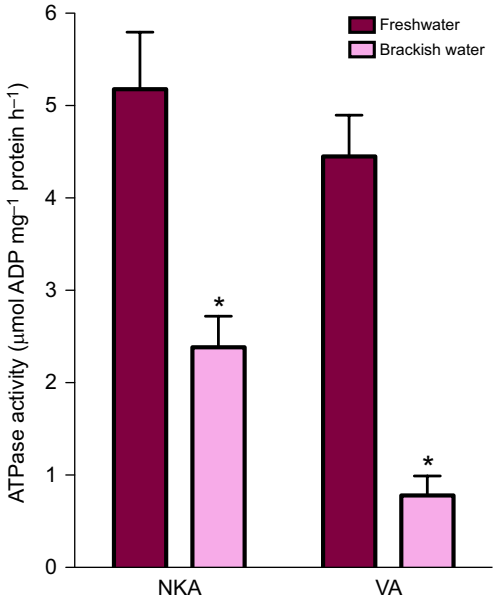

Fig. 2. NKA and VA ATPase activity for caeca of fourth instar *A. aegypti* larvae reared in freshwater or brackish water. Data are expressed as means±s.e.m. (*N*=8). *Significant differences for either NKA or VA in freshwater versus brackish water. Significance (P<0.05) was determined by two-way ANOVA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

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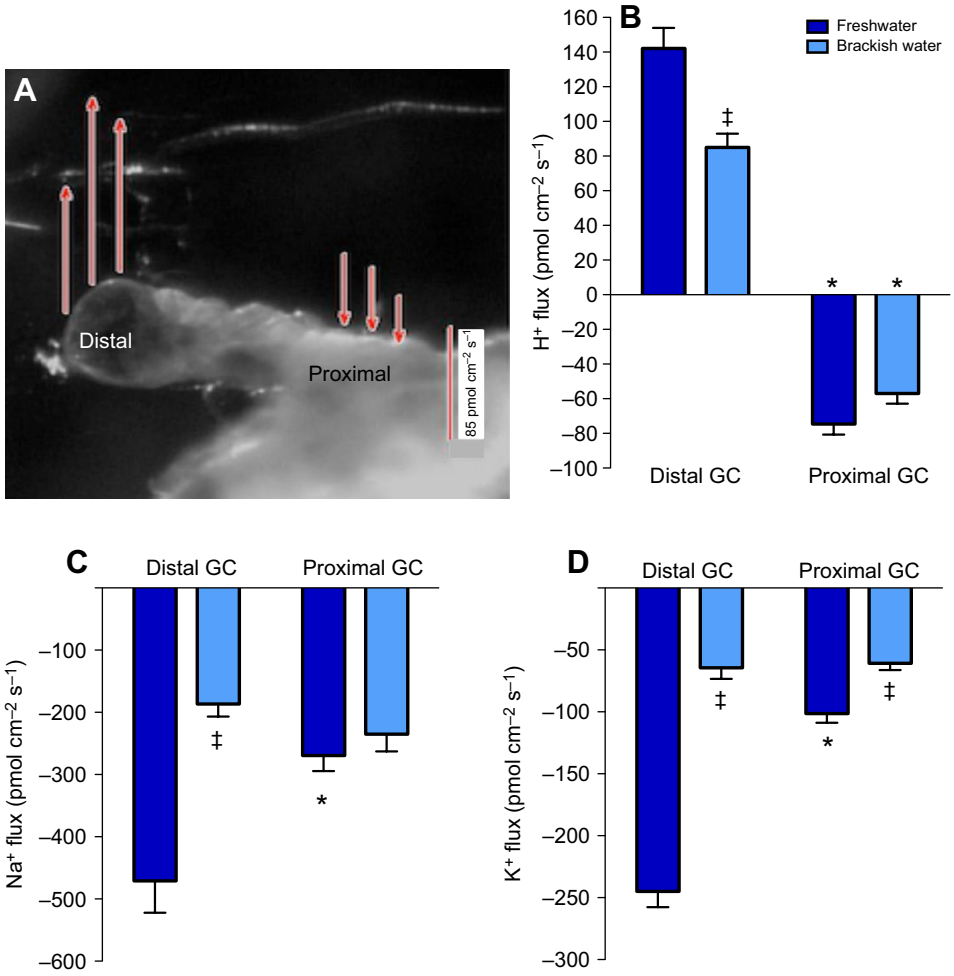

Fig. 3. Scanning ion-selective microelectrode technique (SIET) measurements of fourth instar *A. aegypti* larvae reared in freshwater or brackish water. (A) Representative example of *in vivo* H^+ transport along the surface of the gastric caecum of larvae reared in freshwater. (B) H^+, (C) Na^+ and (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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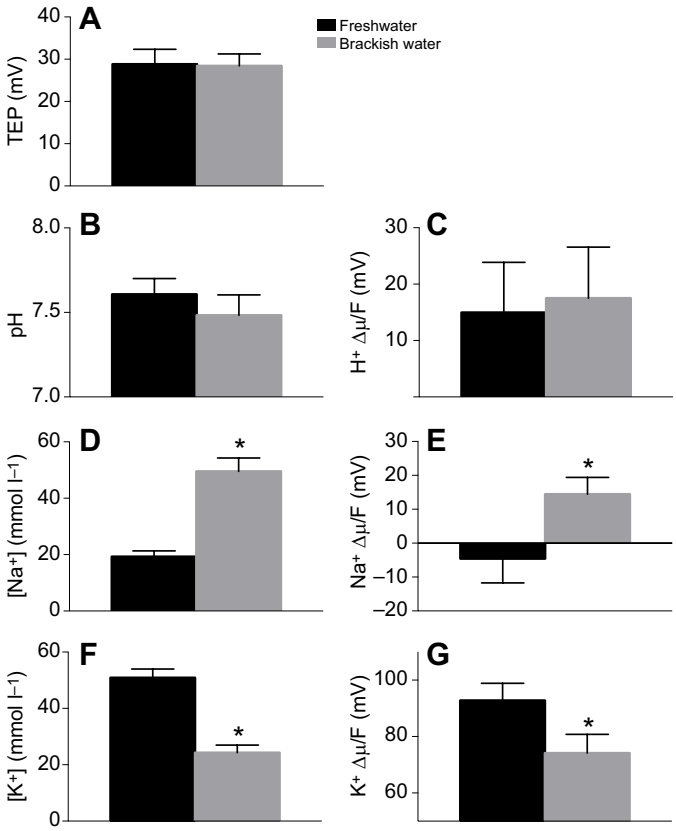

Fig. 4. TEP and transepithelial electrochemical potential for H^+, Na^+ and K^+ of fourth instar *A. aegypti* larvae reared in freshwater or brackish water. (A) Transepithelial potential difference (TEP) measurements. Luminal pH (B), and luminal (D) Na^+ and (F) K^+ concentrations, and the electrochemical 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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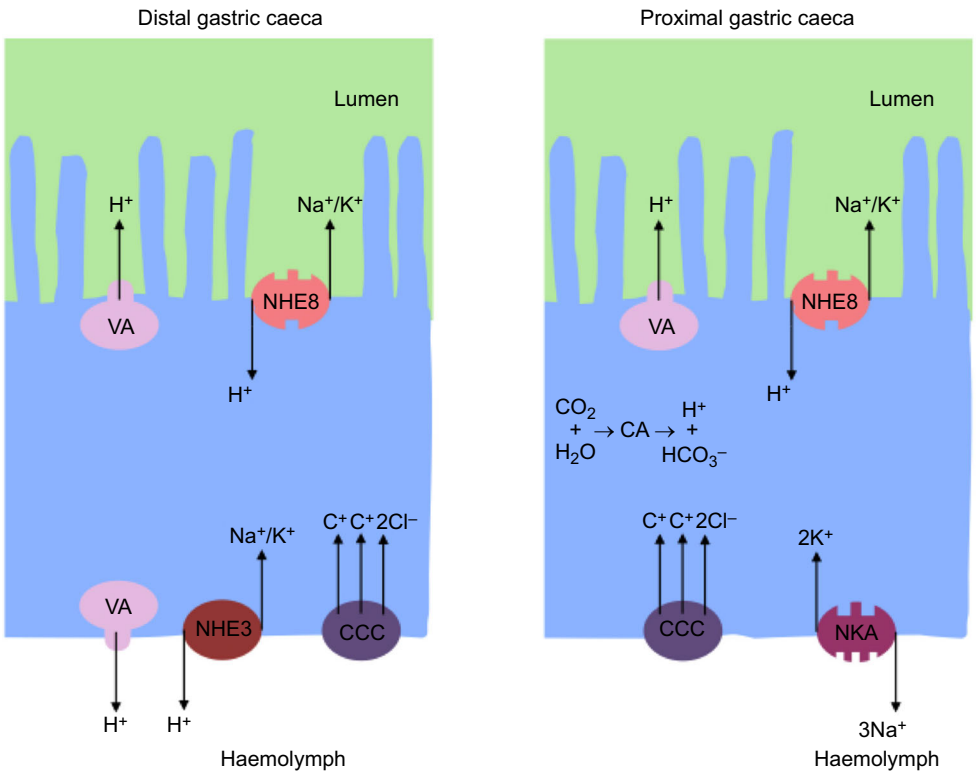

Fig. 5. Schematic diagram illustrating the role of membrane transport proteins in H^+, Na^+ and K^+ transport at the distal and proximal gastric caecum. VA, V-type H^+-ATPase; NKA, Na^+/K^+-ATPase; NHE3, *A. aegypti* Na^+/H^+ exchanger (AeNHE3); NHE8, *A. aegypti* Na^+/H^+ exchanger (AeNHE8); C^+, cation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

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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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Chapter 4

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

Natalie M. D'Silva* and Michael J. O'Donnell*

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 min 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.

KEY WORDS: Serotonin, 5-HT, Mosquito, Gastric caeca, Ion transport, cAMP

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 (Volkmann and Peters, 1989a). These gastric caeca are hypothesized to be important for digestion, nutrient and fluid reabsorption, and ion homeostasis (Wigglesworth, 1933, 1942; Ramsay, 1950; Jones and Zeve, 1968; Volkmann and Peters, 1989b).

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 (5–30% seawater) and that the larvae survive to form adults (Ramasamy et al., 2011; Jude et al., 2012; Surendran et al., 2012). *Aedes 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 (Patrick et al., 2006; D'Silva et al., 2017). In larvae reared in brackish water this regionalization 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 has been proposed 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 (D'Silva et al., 2017). As brackish water (30% seawater) 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 (Volkmann 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 (Volkmann and Peters, 1989b; Patrick et al., 2006).

Ion transport and transepithelial potentials (TEP) 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 TEP 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 that 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 TEP, luminal pH, Na^+ concentration and K^+ concentration. Our results indicate that the effects of 5-HT 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.

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MATERIALS AND METHODS

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 per litre of 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 on a 12 h:12 h light:dark cycle. Fourth instar larvae were used for all experiments.

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 [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 (70–150 kDa, Sigma-Aldrich Canada) to aid tissue adherence to the bottom of the dish, as described by Naikkhwah and O'Donnell (2011). All experiments were performed at room temperature (23°C).

Measurement of TEP and basolateral membrane potentials

TEP and basolateral membrane potentials were measured in the gastric caecum of *A. aegypti* larvae using double-barrelled theta-glass microelectrodes [TST150, World Precision Instruments (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 three time points: within 3 min of dissection (labelled 'Initial' in the figures); 10 min after the initial measurement, before the addition of pharmacological agents (labelled 'Before' in the figures); and 15 min after addition of the agent (labelled 'After' in the figures). The agents added to the bathing saline were: 5-HT (1 µmol l^{-1}); ketanserin (5-HT antagonist, specific to 5-HT type 2 receptor (Van Nueten et al., 1983) (10 µmol l^{-1}); 8-bromo-cAMP (8-Br-cAMP), a cell permeable analogue of cAMP (Sandberg et al., 1991) (100 µmol l^{-1}); phorbol 12,13-diacetate (PE), a protein kinase C (PKC) activator (Castagna et al., 1982) (22.3 µmol l^{-1}); 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.

Measurement of TEP and luminal H^+, Na^+ and K^+ concentrations

Luminal H^+, Na^+ or K^+ concentration and TEP 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 min of dissection, after a further 10 min in saline, and finally 15 min after addition of 5-HT.

Micropipettes were pulled from theta-glass borosilicate capillaries (TST150, WPI). 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 (mmol l^{-1}): 130 NaCl, 5 KCl, 0.5 NaH_2PO_4, 0.1 Na_2HPO_4, 1.8 $CaCl_2$, 1.0 $MgCl_2$ and 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 (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 (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, 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 viewing the tip region under a stereo microscope and gently brushing the tip with tissue paper under saline (Tripathi et al., 1985; Ianowski and O'Donnell, 2006).

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 (RC) filter at 2 Hz to minimize noise resulting from the high-input impedance (>10^9 Ω) of the ion-selective barrel. V_{ref} and the difference ($V_i - V_{ref}$) were recorded using an AD converter and data acquisition system (PowerLab and LabChart software; AD Instruments, CO, USA). Ion concentrations in the gastric lumen were calculated from the measured voltages, as described previously (Ianowski et al., 2002).

Calculation of electrochemical potentials

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

$$\Delta\mu/F = RT/F \log([ion]_{lumen}/[ion]_{bath}) + zTEP, \quad (1)$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}), T is the temperature in degrees Kelvin, F is the Faraday constant (9.648×10^4 C mol^{-1}), $[ion]_{lumen}$ is the concentration of the ion in the lumen (mmol l^{-1}), $[ion]_{bath}$ is the concentration of the ion in the bath (mmol l^{-1}), z is the valency and TEP is the transepithelial potential (mV), of the caecal lumen relative to the 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.

Ion-selective microelectrodes and the scanning ion-selective electrode technique

H^+, Na^+ and K^+ scans were carried out in *A. aegypti* larval saline. As protons may diffuse freely or in association with buffers in the saline, proton transport rates were corrected for buffering using

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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 the scanning ion-selective electrode technique (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 the gastric caecum.

All initial measurements were carried out within 3 min of dissection as rates of ion secretion and absorption decayed steadily after the first 3–6 min. 5-HT (1 µmol l^{-1}) 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.

Statistical analysis

Data were plotted using GraphPad InStat (GraphPad Software, La Jolla, CA, USA). TEP, basolateral membrane potential, luminal H^+, Na^+ and K^+ concentrations and electrochemical potentials are expressed as means±s.e.m. (*N*). The parameters were plotted with respect to treatment (saline, 5-HT, ketanserin, DMSO, 8-Br-cAMP or PE) and analysed by one-way ANOVA followed by Tukey's *post hoc* test. A two-tailed paired *t*-test was used to analyse TEP for treatments (before and after 5-HT) within each concentration group. The rates of H^+, Na^+ and K^+ transport are expressed as means± s.e.m. for a number of caeca (*N*). The value for each caecum was based on the mean of measurements at three sites along the distal or proximal region of the caecum, and at each site a mean was calculated for three replicate measurements. H^+, Na^+ and K^+ transport rates were plotted against treatment (saline or 5-HT) and time and analysed by two-way ANOVA followed by Bonferroni's *post hoc* test. In all statistical tests, differences were considered significant at $P<0.05$.

RESULTS

Transepithelial potential

The lumen of the gastric caecum was at a positive potential with respect to the bathing saline (Fig. 1), consistent with previous findings (D'Silva et al., 2017). TEP declined significantly over time when left unstimulated in physiological saline, in both freshwater- (Fig. 1) 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 min indicated a one-phase exponential decay (Fig. 1B).

In larvae reared in freshwater, TEP recovered to its initial voltage within 12–15 min following addition of 1 µmol l^{-1} 5-HT (Fig. 2A), a concentration used previously on the larval midgut (Clark et al., 1999, 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 min to values not significantly different from the 'Before 5-HT' values (Fig. 2A). Addition of 0.1 and 10 µmol l^{-1} 5-HT also caused significant increases in TEP within

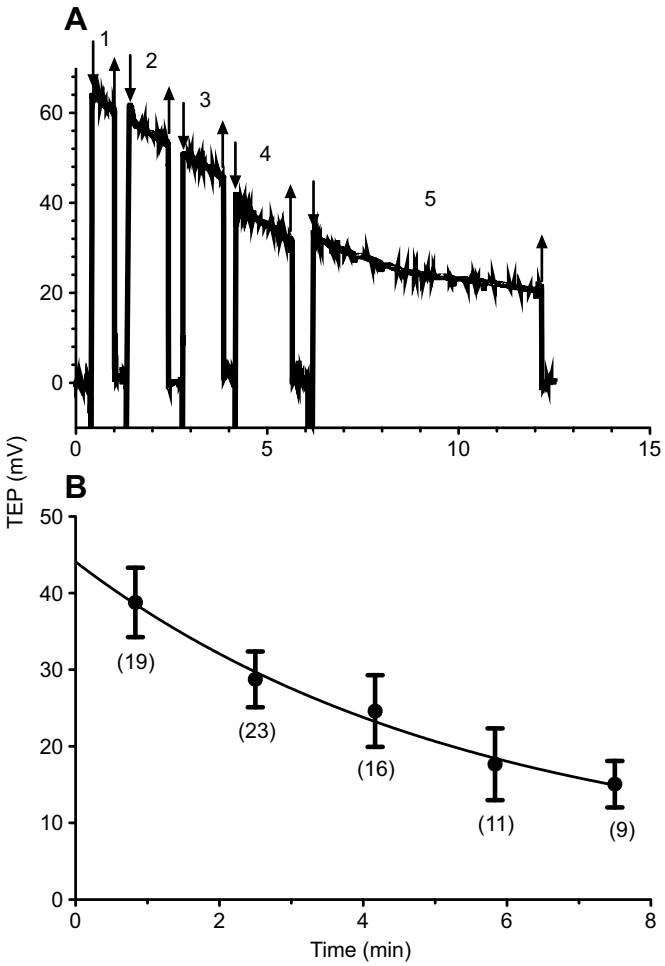

Fig. 1. Decline of TEP of the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. (A) TEP was measured for five gastric caeca within a single animal (numbered 1–5 in order of impalement). Downward arrows indicate impalement of the gastric caecum, and upward arrows indicate withdrawal 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 means±s.e.m. The data were fitted to a one-phase decay equation (r^2=0.99): TEP=(Y_0–Plateau)×exp(–*K*×time)+Plateau, where Y_0=44.09, *K*=0.1841 and Plateau=5.155. The number of gastric caeca is indicated in parentheses below each data point.

15 min (Fig. 2B). In larvae reared in brackish water, TEP recovered to its initial voltage within 15–20 min of addition of 1 µmol l^{-1} 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).

Intracellular signalling molecules

Having established the effects of 5-HT on TEP recovery, we wanted to determine the intracellular signalling molecules acting downstream of 5-HT signalling. To this end, we tested the effects of 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).

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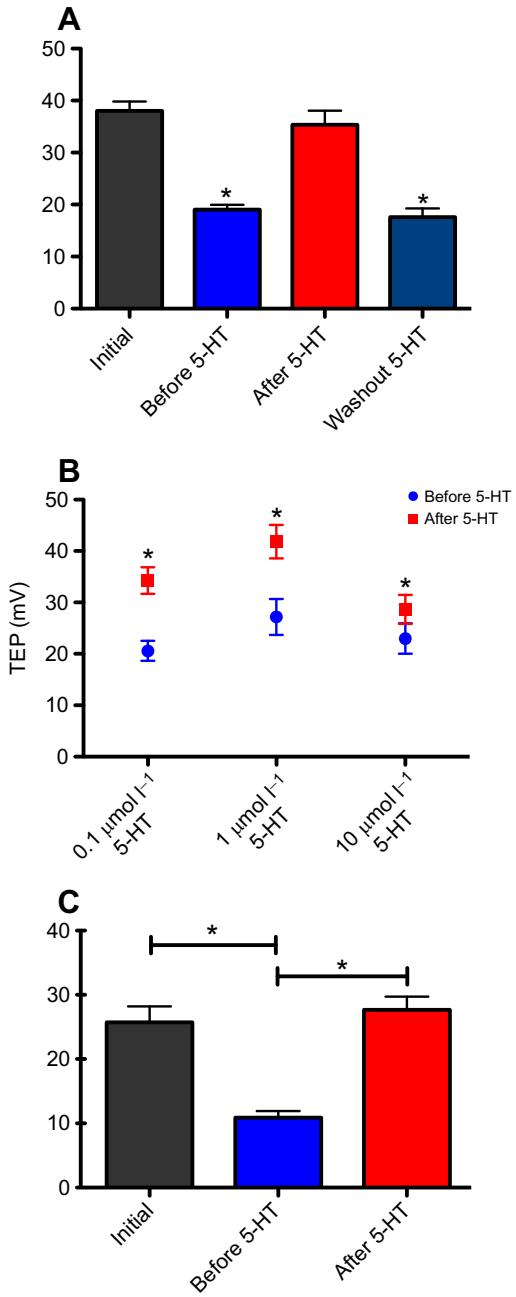

Fig. 2. Effects of 5-HT on TEP of the gastric caecum of fourth instar *Aedes aegypti* larvae. (A) TEP was measured in the absence and presence of 5-HT in larvae reared in freshwater. (B) Effects of different doses of 5-HT on the TEP of larvae reared in freshwater. (C) TEP was measured in the absence and presence of 5-HT in larvae reared in brackish water. Data are expressed as means±s.e.m. (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).

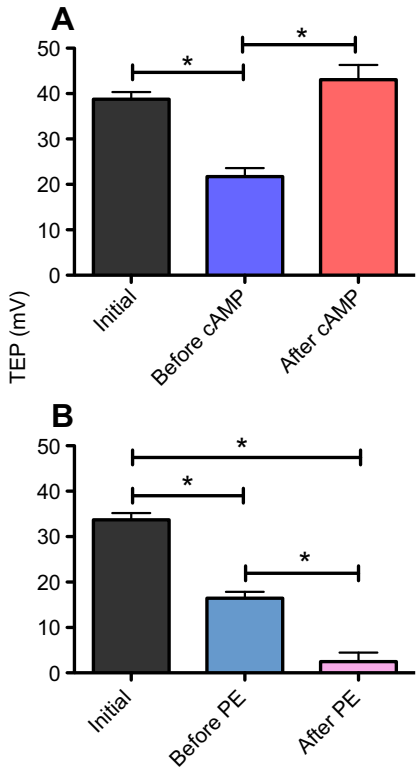

Fig. 3. TEP of the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. TEP was measured in (A) the absence and presence of cAMP, and (B) the absence and presence of PE. Data are expressed as means±s.e.m. (N=5–6). Asterisks denote significant differences between treatments; significance (P<0.05) was determined by one-way ANOVA followed by Tukey's *post hoc* test.

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 min when preparations isolated from freshwater-reared larvae were bathed in saline alone (Fig. 4). Basolateral membrane potential recovered to its initial voltage after addition of 1 µmol l^{-1} 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 from measurements of TEP and basal membrane voltage (V_b), as TEP=V_b+V_a. If V_b depolarized after the initial measurement, and V_a had remained constant, TEP would hyperpolarize. However, as 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 negatively 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.

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

As 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 microelectrodes. 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.

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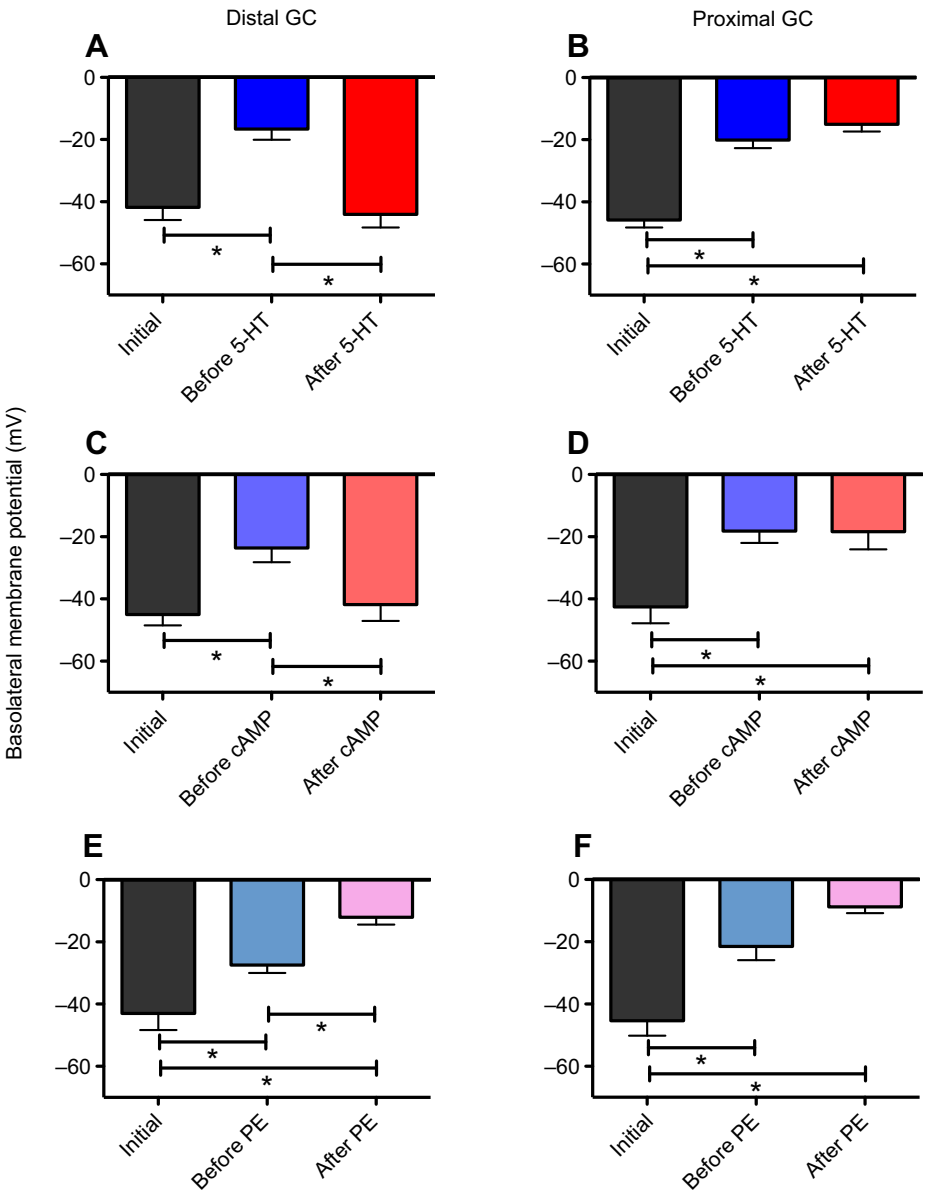

Fig. 4. Basolateral membrane potential of the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. Basolateral membrane potential was measured at the distal (A,C,E) and proximal (B,D,F) gastric caecum (GC) in the absence and presence of 5-HT (A,B), absence and presence of cAMP (C,D) and absence and presence of PE (E,F). Data are expressed as means±s.e.m. (*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.

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 µmol l^{-1} 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 µmol l^{-1} 5-HT led to a significant reduction in luminal pH in comparison with 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 µmol l^{-1} 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 µmol l^{-1} 5-HT when compared with 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 µmol l^{-1} 5-HT when compared with 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

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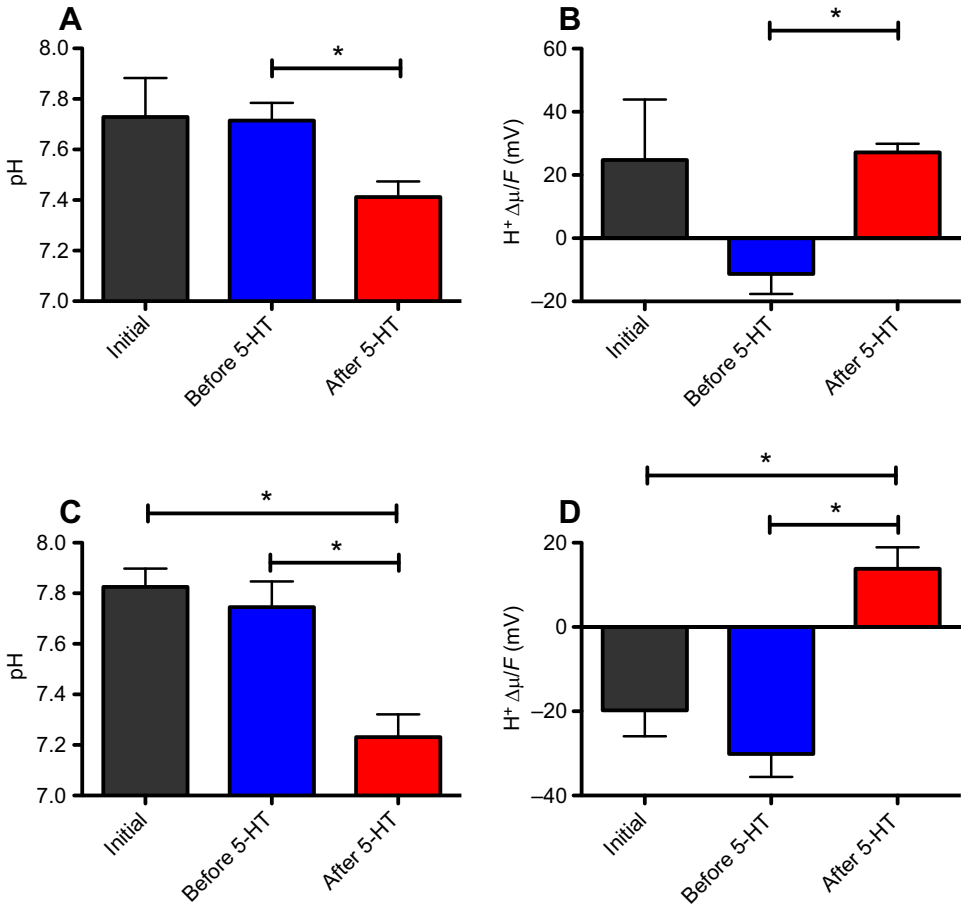

Fig. 5. Effects of 5-HT on luminal pH and electrochemical potential for H^+. Luminal pH (A,C) and electrochemical potentials (B,D) for H^+ in the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater (A,B) or brackish water (C,D). Data are expressed as means±s.e.m. (*N*=7–8). Asterisks denote significant difference between treatments; significance (*P*<0.05) was determined by one-way ANOVA followed by Tukey's *post hoc* test.

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 with the initial and 'Before 5-HT' values (Fig. 7D).

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. In addition, as 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. 8), Na^+ (Fig. 9) and K^+ (Fig. 10) 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 (Figs 9A,C, 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 min when measured in physiological saline and compared with the initial recorded value (Figs 8A, 9A and 10A). In larvae treated with 1 µmol l^{-1} 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 (Figs 8A, 9A and 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 min when measured in physiological saline (Figs 8B and 9B). The rate of secretion of K^+ did not diminish over time (Fig. 10B). The addition of 1 µmol l^{-1} 5-HT had no effect on the rates of secretion of H^+, Na^+ and K^+ in the proximal region of the caecum when compared with the rates of secretion at the corresponding time points (Figs 8B, 9B and 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 min when measured in physiological saline (Figs 8C, 9C and 10C). On addition of 1 µmol l^{-1} 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 min 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 rates of secretion of H^+, Na^+ and K^+ were significantly reduced

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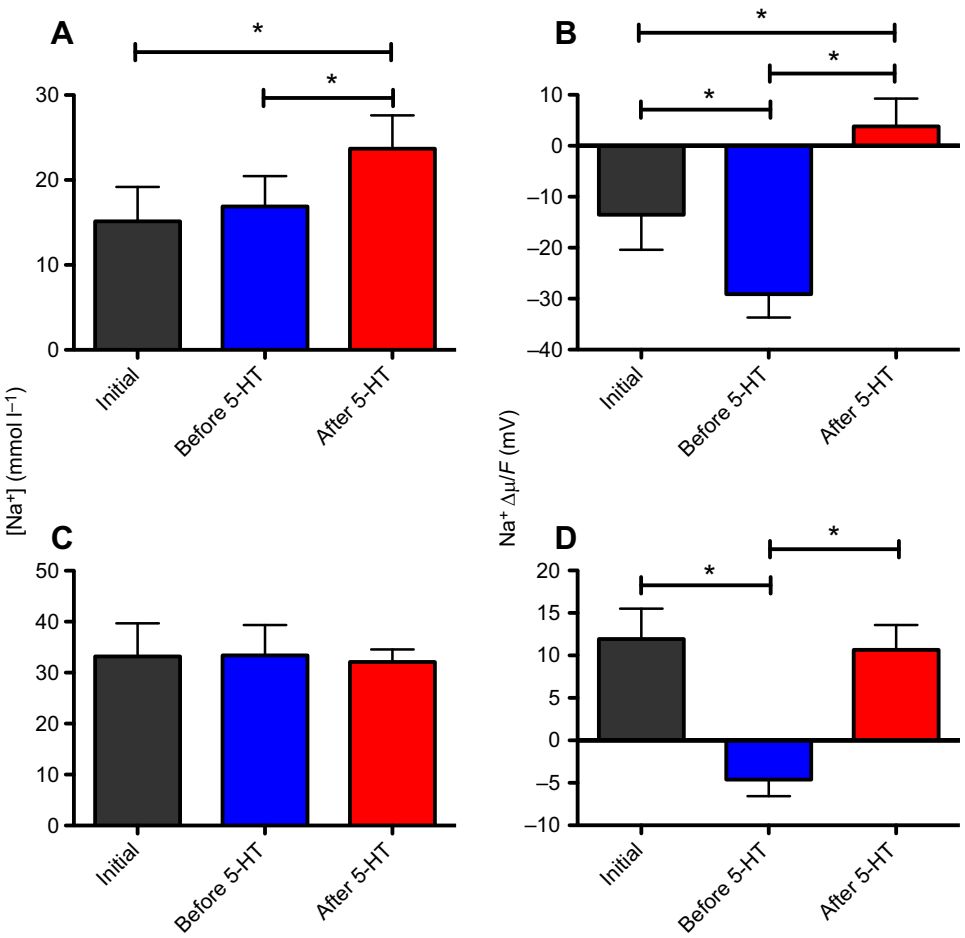

Fig. 6. Effects of 5-HT on luminal [Na^+] and electrochemical potential for Na^+. Luminal [Na^+] (A,C) and electrochemical potentials (B,D) for Na^+ in the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater (A,B) or brackish water (C,D). Data are expressed as means±s.e.m. (*N*=8–9). Asterisks denote significant difference between treatments; significance ($P<0.05$) was determined by one-way ANOVA followed by Tukey's *post hoc* test.

over time when measured in physiological saline (Figs 8D, 9D and 10D). The addition of 1 µmol l^{-1} 5-HT had no effect on the rates of secretion of H^+, Na^+ and K^+ in the proximal region when compared with the control rates of secretion at the corresponding time points (Figs 8D, 9D and 10D).

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 signalling 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 min 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 signalling is crucial for gastric caeca ion regulation *in vivo*, and that this signalling is lost when the tissue is isolated from the hemolymph and bathed with artificial saline.

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 and 10 µmol l^{-1} 5-HT (Fig. 2B). Clark et al. (1999) reported maximal stimulation of TEP in the posterior midgut of the larval *A. aegypti* at 0.1–1 µmol l^{-1}.

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 receptors regulate ion transport/fluid secretion in Malpighian tubules (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.

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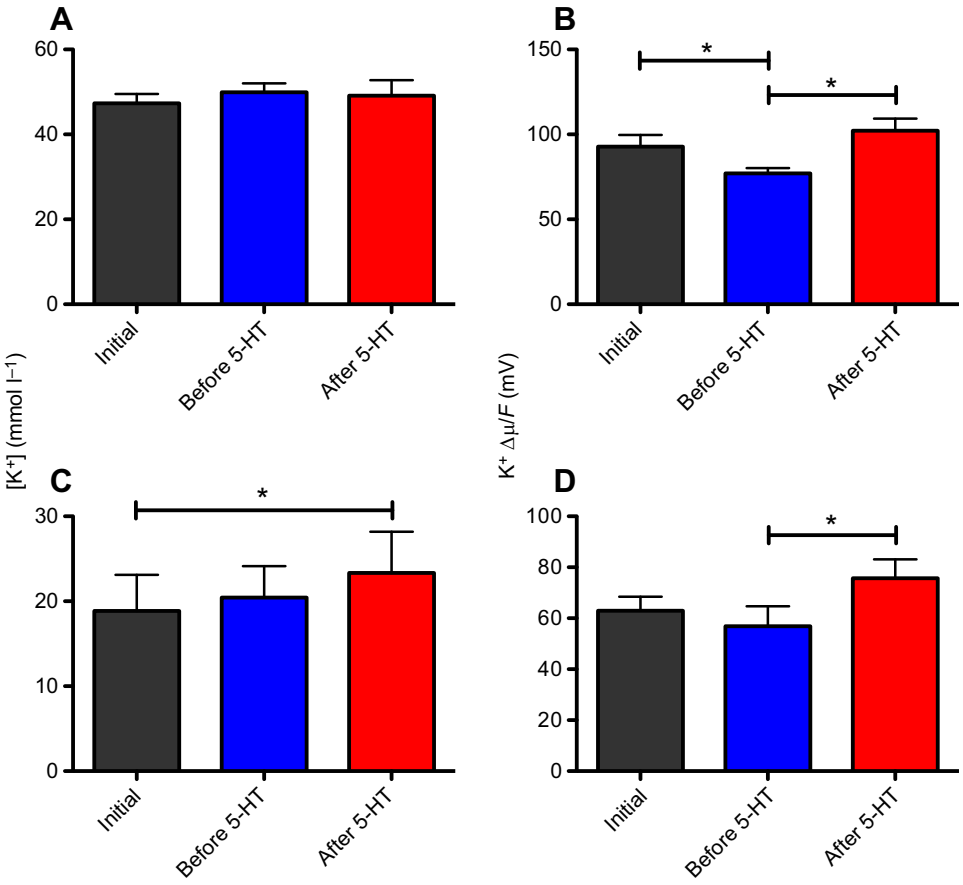

Fig. 7. Effects of 5-HT on luminal [K$^+$] and electrochemical potential for K$^+$. Luminal [K$^+$] (A,C) and electrochemical potentials (B,D) for K$^+$ in the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater (A,B) or brackish water (C,D). Data are expressed as means±s.e.m. (*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.

The mechanism of 5-HT action via intracellular signalling molecules on TEP was investigated in the gastric caeca of larvae reared in freshwater. 5-HT can act on cells and regulate their 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

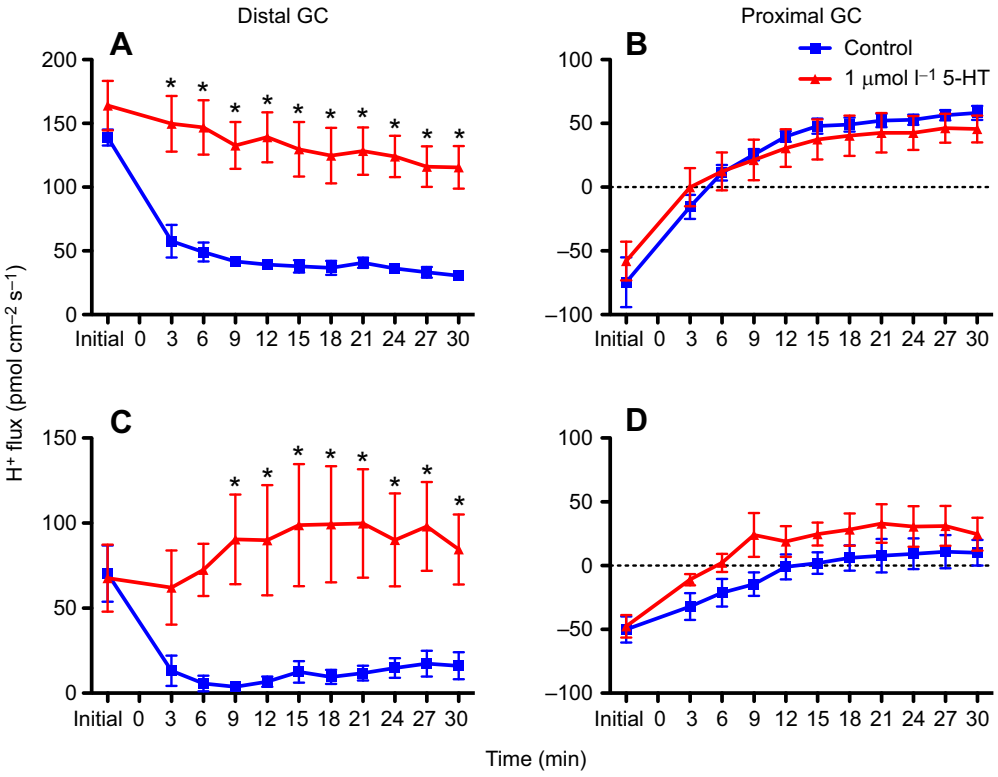

Fig. 8. Effects of 5-HT on H$^+$ transport across the gastric caecum. SIET measurements of *in vitro* H$^+$ transport rates along the surface of the distal (A,C) and proximal (B,D) gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater (A,B) or brackish water (C,D). Positive values 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*=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.

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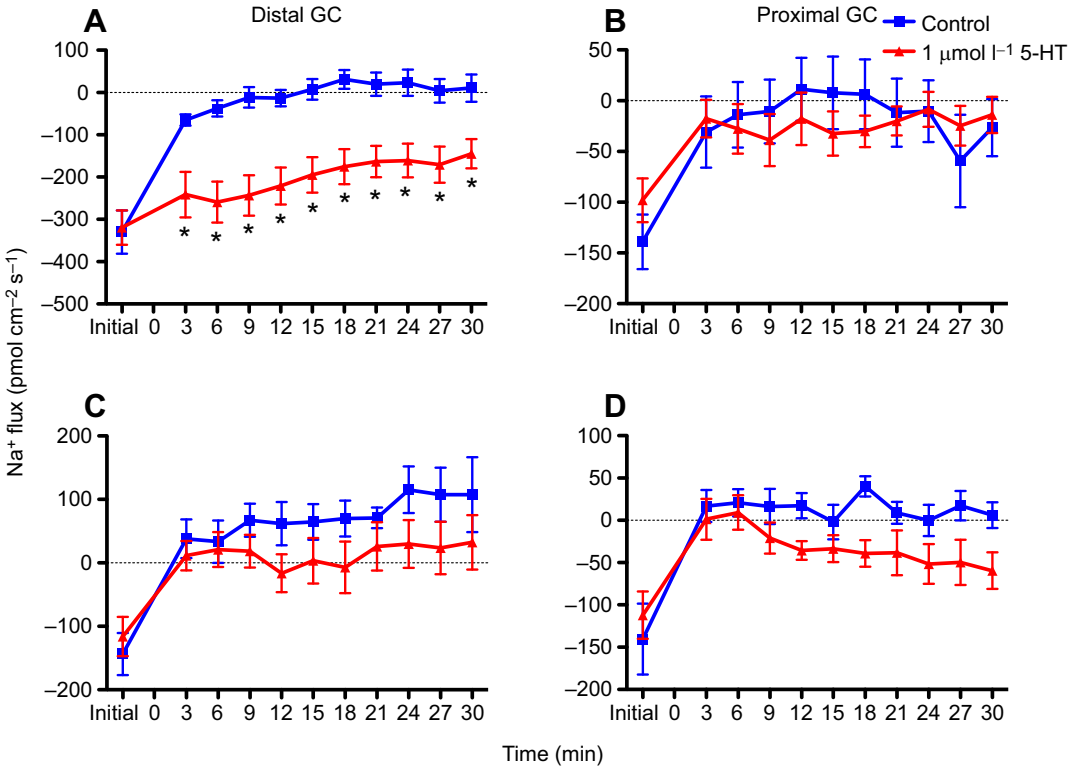

Fig. 9. Effects of 5-HT on Na^+ transport across the gastric caecum. SIET measurements of *in vitro* Na^+ transport rates along the surface of the distal (A,C) and proximal (B,D) gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater (A,B) or brackish water (C,D). Positive values 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=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.

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, however, is similar to the Malpighian tubules, where stimulation of diuretic activity by 5-HT in larval tubules is

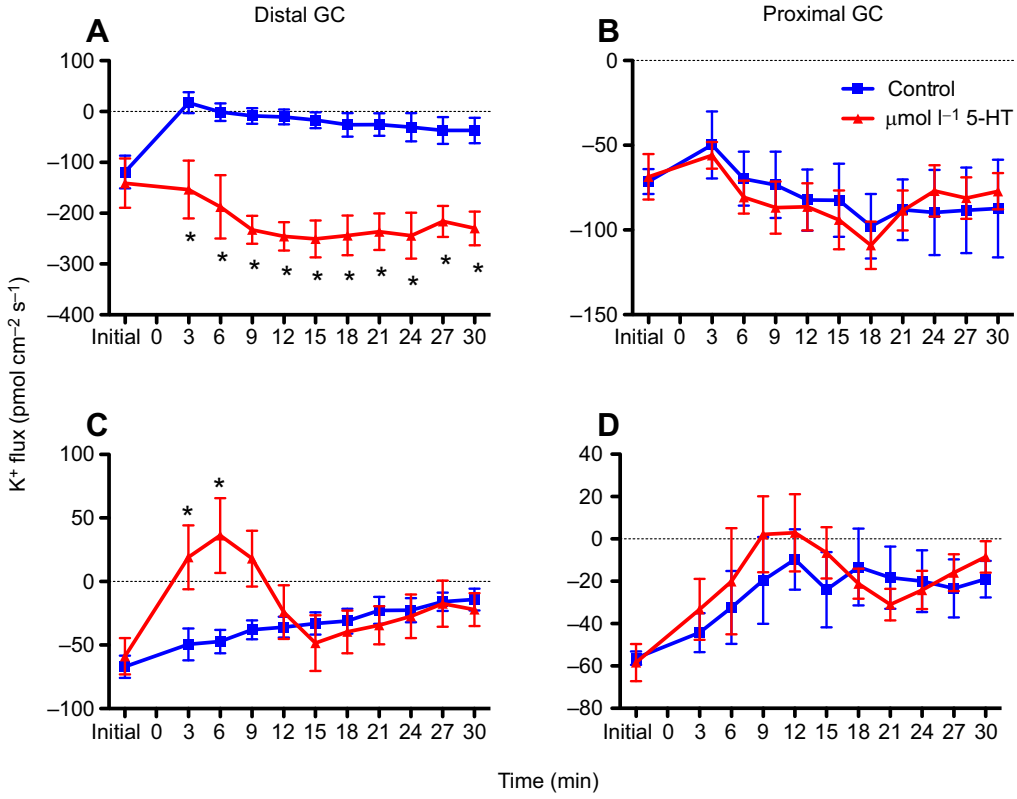

Fig. 10. Effects of 5-HT on K^+ transport across the gastric caecum. SIET measurements of *in vitro* K^+ transport rates along the surface of the distal (A,C) and proximal (B,D) gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater (A,B) or brackish water (C,D). Positive values 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=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.

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mediated through cAMP (Clark and Bradley, 1996, 1997, 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 signalling molecules. Freshwater larvae were chosen for this part of the study as 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 (Patrick et al., 2006; D'Silva et al., 2017). 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–D). Immediately after isolation, basolateral membrane potential was approximately −40 mV for both types of cells. This potential decreased within 3–6 min 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. In addition, TEP recovers fully in the presence of 5-HT or 8-Br-cAMP (Figs 2A, 3A). As 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. Fig. 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 (O'Donnell et al., 1996; Wieczorek et al., 1999; Coast et al., 2001; Dames et al., 2006; Voss et al., 2010; Baumann and Walz, 2012).

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 suggest 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

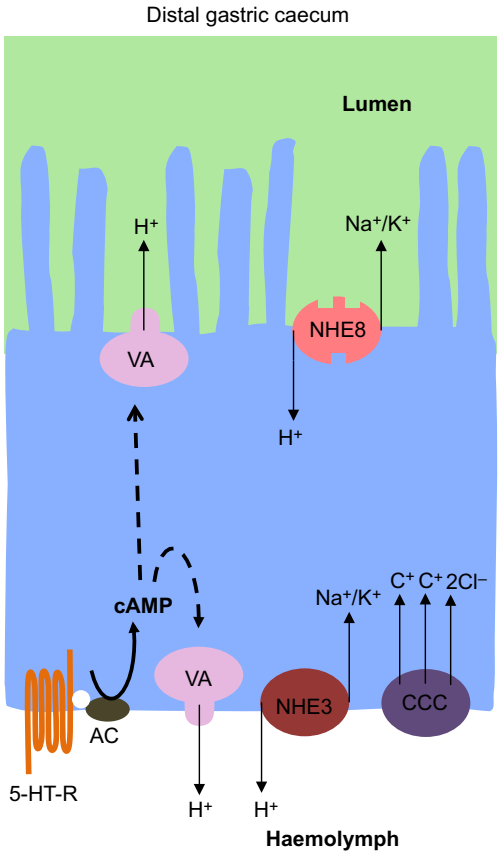

Fig. 11. Schematic diagram illustrating the role of 5-HT on activation of VA in the distal gastric caecum. AC, adenylyl cyclase; VA, V-type H^+ ATPase; NHE, Na^+/H^+ exchanger (for *A. aegypti*: AeNHE8, AeNHE3); CCC, cation chloride cotransporter; 5-HT-R, 5-HT receptor. Modified from D'Silva et al. (2017).

therefore tested the effects of 5-HT on H^+, Na^+ and K^+ transport rates along the distal and proximal regions, for both freshwater- and 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 (Volkmann 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). In addition, 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

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secretion of Na^+ or K^+ into the lumen via an apically expressed Na^+/H^+ anti-porter. 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; Fig. 11), consistent with the increase in Na^+ and K^+ fluxes at the distal gastric caecum of freshwater larvae. Again, there is similarity to the role of CCC in uptake of ions into Malpighian tubule cells (Ianowski et al., 2002, 2004). Taken together, our data 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 mmol l^{-1}; Fig. 6C) in comparison with larvae reared in freshwater (15 mmol l^{-1}; Fig. 6A). However, luminal K^+ concentration is lower in the caecum of larvae reared in brackish water (19 mmol l^{-1}; Fig. 7C) compared with freshwater (47 mmol l^{-1}; 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).

Although 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, as larvae reared in brackish water experience much smaller ionic gradients for passive movements of ions and water when compared with freshwater larvae, they do not need to hyper-regulate as 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 with brackish water larvae. Freshwater larvae ingest large amounts of dilute media, thus osmotically favouring movement of water from the midgut into the haemolymph and 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 as 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 (Volkmann 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 gastric caecum indicates that other neuromodulators or hormones may be responsible for sustaining the transport activity of the proximal gastric caeca *in vivo*.

Conclusions

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; Patrick et al., 2006; Pullikuth et al., 2006; Kang'ethe et al., 2007). 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.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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Supplementary information

Supplementary information available online at
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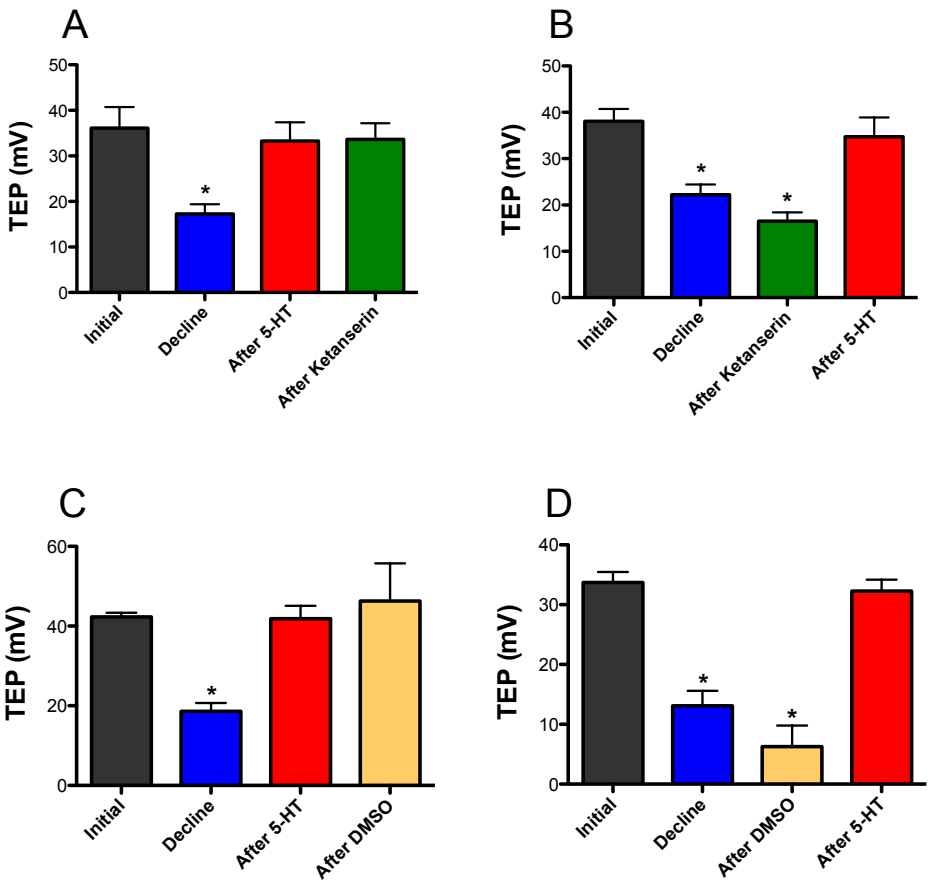

Figure S1: Transepithelial potential (TEP) of the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. An initial TEP was measured, followed after 10 mins by a measurement during the decline in TEP. TEP was then measured (A) after the addition of 5-HT followed by ketanserin, (B) 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 ± 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.

Chapter 5

Ion transport across the distal gastric caecum of larval *Aedes aegypti*

Natalie M. D'Silva and Michael J. O'Donnell

Abstract

We provide the first measurements of the effects of specific ion-transporter inhibitors on rates of transport of H^+, Na^+ and K^+, across the distal gastric caecum of larval *Aedes aegypti*, a vector of yellow fever. Measurements in this study were carried out with preparations stimulated with 5-hydroxytryptamine (5-HT, 10^{-6} M) in order to maintain stable rates of H^+, Na^+, and K^+ transport rates across the distal caecum in control preparations. The specific vacuolar-type H^+-ATPase (VA) inhibitor, concanamycin (50 μM) reduced H^+ efflux by 50 %. TEP, V_b, and H^+, Na^+ and K^+ fluxes declined after the addition of a addition of another vacuolar-type H^+-ATPase (VA) inhibitor (N-ethylmaleimide, 1 mM), consistent with a primary role for VA in energizing ion transport across the distal gastric caecum. H^+, Na^+ and K^+ fluxes declined after the addition of amiloride (cation:H^+ antiporter inhibitor), and Na^+ and K^+ secretion also declined after the addition of bumetanide (cation chloride cotransporter (CCC) inhibitor). We propose that apically expressed VA and cation:H^+ antiporter (AeNHE8) are coupled, analogous to the coupling of apical VA and cation:nH^+ antiporter in Malpighian tubules. This coupling of VA and AeNHE8 in the apical membrane leads to the removal of intracellular Na^+ or K^+, thus creating favourable ion gradients to promote the activity of two transporters in the basal membrane, cation:H^+ antiporter (AeNHE3) and cation chloride cotransporter (CCC). We have developed a working model to describe the roles of VA, AeNHE8, AeNHE3 and CCC in transport of H^+, Na^+ and K^+ across the distal gastric caecum.

1. Introduction

Larval *Aedes aegypti* possess eight globular extensions of the midgut called gastric caeca, located immediately posterior to the cardia and anterior to the midgut proper (Volkmann and Peters, 1989a). The gastric caeca are hypothesized to be important in ionoregulation and digestion (Jones and Zeve, 1968; Ramsay, 1950; Volkmann and Peters, 1989b; Wigglesworth, 1933; Wigglesworth, 1942). Gastric caeca are comprised predominately of two cell types, the ion transporting and the digestive cells. The ion transporting cells are located in the distal third of the caecum, and the digestive cells are located in the proximal two-thirds of the caecum (Volkmann and Peters, 1989a). The regionalization of these cells corresponds to the expression of vacuolar-type H^+-ATPase (VA) and Na^+/K^+-ATPase (NKA) along the gastric caeca (D'Silva et al., 2017; Patrick et al., 2006). Immunohistochemical techniques have revealed that VA is expressed along the apical and basal membrane of the distal third (VA-rich cells), and on the apical membrane of the proximal cells, whereas NKA is expressed on the basal membrane of the proximal two-thirds (NKA-rich cells) of the gastric caecum (D'Silva et al., 2017; Patrick et al., 2006). In addition to VA and NKA, other inorganic ion transporters, including the cation proton exchangers (NHEs), AeNHE8 (Kang'ethe et al., 2007) and AeNHE3 (Pullikuth et al., 2006), and cation chloride co-transporter (CCC) (Filippov et al., 2003) have been identified in the gastric caecum using immunohistochemical techniques.

In this study we elucidate the ion transport mechanisms of the distal gastric caecum by measuring changes in ion transport rates and transmembrane potentials in response to ion transport inhibitors. In the gastric caecum, there is

a precipitous decline of TEP, V_b, and ion fluxes across the caecum within 3-6 mins of dissection (D'Silva and O'Donnell, 2018). 5-hydroxytryptamine (5-HT) restores the TEP, V_b, and the H^+, Na^+, and K^+ fluxes across the distal, but not proximal, gastric caecum. Taken together, these results suggest that endogenous 5-HT may be critical for maintaining ion transport of the distal gastric caecum *in vivo*, and that this signaling is lost when the tissue is isolated from the haemolymph and bathed with artificial saline (D'Silva and O'Donnell, 2018). Since 5-HT restores ion fluxes in the distal caecum and not the proximal caecum, this study focuses on ion transport across the distal gastric caecum.

The distal gastric caecum shows similarities to the Malpighian tubules in the expression of specific inorganic ion transporters in the cells. In both tissues, VA is expressed on the apical membrane, and AeNHE3 and CCC are expressed on the basolateral membrane (Kang'ethe et al., 2007; Pullikuth et al., 2006; Scott, 2004). In Malpighian tubules, the apical VA provides the proton motive force to drive exchange of luminal H^+ for cellular Na^+ or K^+ (Beyenbach, 2001; Beyenbach, 2003; Beyenbach et al., 2000; Kang'ethe et al., 2007). AeNHE8 is located on the apical membrane of the gastric caecum (Kang'ethe et al., 2007), and has been proposed to provide a means for exchange of luminal H^+ for cellular Na^+ or K^+ (D'Silva and O'Donnell, 2018), analogous to the role of sodium/proton antiporters (NHAs) in Malpighian tubules of larval mosquitoes (e.g. AgNHA1) (Rheault et al., 2007; Xiang et al., 2012).

In this study we have used inhibitors of specific ion transporters to assess the roles of VA, CCC, cation/proton antiporters, and anion transporters, in transport of H^+, Na^+ and K^+ across the distal gastric caecum. Fluxes of H^+,

Na^+, and K^+, along the distal gastric caecum have been measured using the Scanning Ion-Selective Electrode Technique. We have also used microelectrodes to measure transepithelial potential and basolateral membrane potential of the distal cells of the gastric caecum.

2. Materials and methods

2.1 Raising larvae

Aedes aegypti (Linnaeus) eggs were hatched and held in freshwater (dechlorinated Hamilton tap water). The eggs were hatched in rectangular tubs, and fed a 1:1 ground liver powder:dry 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 was dissected out in *A. aegypti* larval saline (in mM: 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 and basolateral membrane potentials

Transepithelial potential (TEP) was measured after impalement of the gastric caecum, and basolateral membrane potentials (V_b) were measured after impaling the distal cells of the gastric caecum of *A. aegypti* larvae using double-barrelled theta-glass microelectrodes (TST150, WPI, New Haven, CT, USA). After pulling, both barrels were filled with 150 mmol l^{-1} KCl and one barrel was connected through a chlorided Ag wire to the headstage of a high impedance electrometer (HiZ 223, Warner Instruments, Hamden CT). The natural bevel resulting from the prominent spear-like projection of the central septum of theta glass facilitates impalement, allowing multiple impalements using a single electrode.

Measurements were carried out within 3 minutes of dissection, in *A. aegypti* larval saline containing 1μM 5-HT. After the initial measurement, N-ethylmaleimide (NEM; VA inhibitor; 1 mM) was added to the bathing saline, and the consequent effects on TEP and V_b were measured at 3 min intervals up to 9 min.

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

H^+, Na^+ and K^+ scans were carried out in *A. aegypti* larval saline containing 5-HT (1 μM). 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 H^+-selective, Na^+-selective and K^+-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 of the gastric caecum.

All initial measurements were carried out within 3 minutes of dissection, in *A. aegypti* larval saline containing 1μM 5-HT. After the initial measurement, a pharmacological agent or DMSO (carrier for the pharmacological agents; final concentration v/v 0.01 %) was added to the bathing saline, and the consequent effect on H^+, Na^+ or K^+ were measured at 3 min intervals up to 9 min using SIET. The pharmacological agents added to the bathing saline were: concanamycin (50 μM; Onken et al., 2008), NEM (1 mM; (O'Donnell and Sharda, 1994), bumetanide (100 μM; Pacey and O'Donnell, 2013), amiloride (200 μM; Onken et al., 2008), or 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS; 1 mM; Onken et al., 2008). The pharmacological agents did not alter the slope of the H^+, Na^+, or K^+ - selective microelectrodes.

2.5 Statistical analysis

Data were plotted using Graphpad InStat (Graphpad Software Inc., La Jolla, CA). TEP, V_b, and rates of H^+, Na^+ and K^+ transport are expressed as mean ± s.e.m. (N). For transport rates measured by SIET, 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 the value was the mean of 3 replicate measurements. TEP, V_b, and H^+, Na^+ and K^+ transport rates were plotted against treatment (DMSO or pharmacological agent) and time, and analyzed by repeated measures one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1 VA inhibitor, NEM, inhibits TEP and V_b

Measurements of TEP and V_b were carried out in the presence of 1 μM 5-HT since 5-HT prevents the decline of both parameters in preparations bathed in saline (D'Silva and O'Donnell, 2018). TEP was significantly reduced in the presence of 1 mM NEM (Fig. 1A), but was unaffected by 50 μM concanamycin ($N = 7$; $p = 0.28$). After 9 mins of incubation with NEM, both TEP and V_b declined to values near zero mV (Fig. 1A, B).

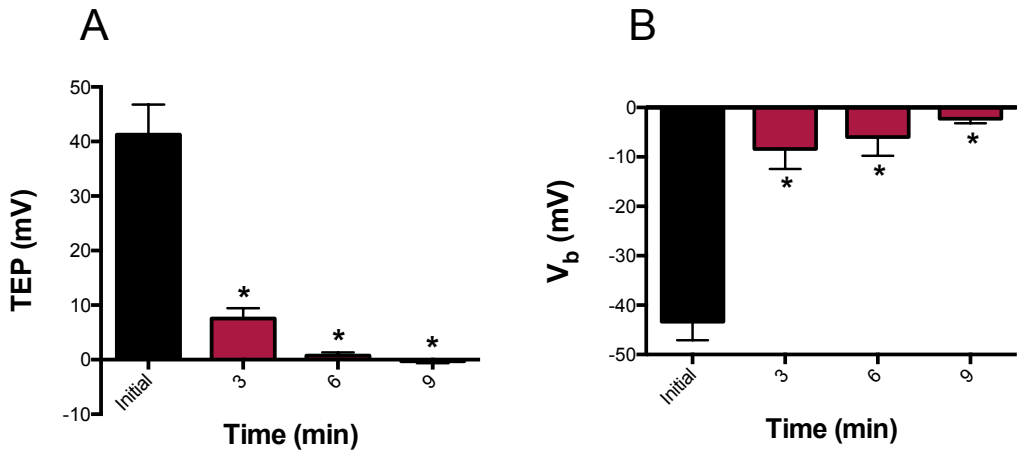

Figure 1: Effects of 1 mM NEM on TEP (A), and V_b of the distal cells (B), of the gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. Data are expressed as means±s.e.m. (N=5–6). Asterisks denote significant differences between treatments; significance ($P<0.05$) was determined by repeated measures one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test

3.2 Effects of pharmacological transport inhibitors on transport rates of H^+, Na^+ and K^+ along the distal gastric caecum

All measurements were carried out in the presence of 1 μM 5-HT to prevent the decline of transport rates of H^+, Na^+ and K^+ along the distal gastric caecum (D'Silva and O'Donnell, 2018). Transport rates for H^+ (Fig. 2A-E), Na^+ (Fig. 3A-D) and K^+ (Fig. 4A-D) were measured along the haemolymph-facing surface of the distal gastric caecum of larval *A. aegypti*. The first set of measurements were carried out in the absence of inhibitors, and are called 'initial' values; they served as the controls within each group. In the presence of 5-HT, 'initial' SIET measurements revealed that H^+ was absorbed (into the bath) (Fig. 2A-E), and Na^+ and K^+ were secreted (bath to lumen) (Fig. 3A-D; Fig. 4A-D), consistent with previous findings (D'Silva et al., 2017).

The rate of absorption of H^+ was significantly reduced within 3 minutes by concanamycin (Fig. 2B), NEM (Fig. 2C) and amiloride (Fig. 2D), while DIDS (Fig. 2E) showed no effect. The secretion rates of Na^+ and K^+ were significantly reduced within 3 minutes by NEM, amiloride and bumetanide (Fig. 3B-D; Fig. 4B-D). The addition of DMSO, the carrier for amiloride, bumetanide, DIDS, and concanamycin, had no effect on the rates of transport of H^+, Na^+ and K^+, which did not differ significantly from transport rates measured in saline alone, at the corresponding time points. Rates of ion transport measured with DMSO (final concentration v/v 0.01% in saline) or saline alone were therefore pooled together as 'control' (Fig. 2A, 3A, 4A).

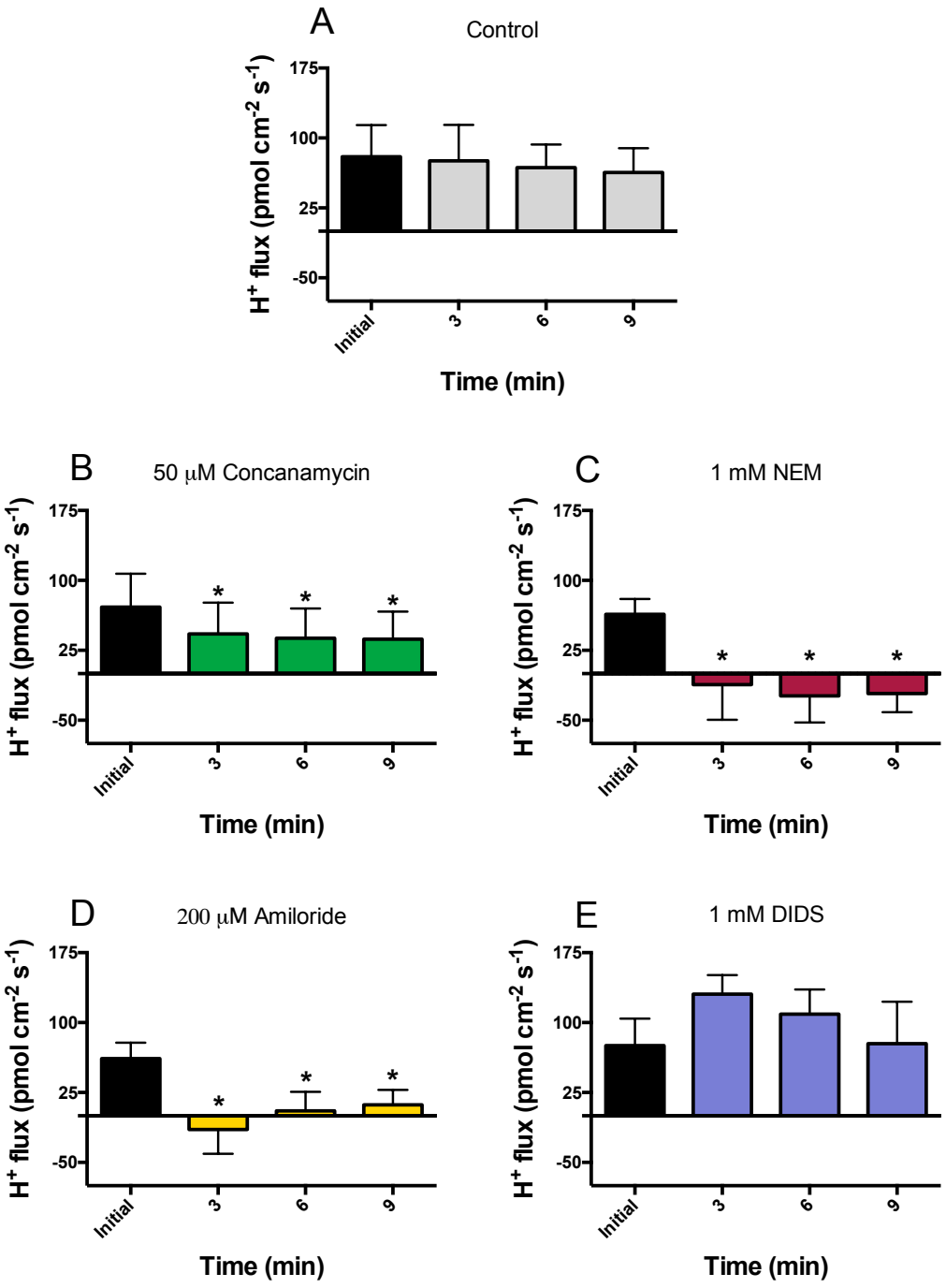

Figure 2: Scanning Ion-Selective Microelectrode Technique (SIET) measurements of *in vitro* H^+ transport rates along the surface of the distal gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. Data are expressed as means±s.e.m. (N=5–11). Asterisks denote significant differences between treatments (3', 6', 9') and the initial value; significance ($P<0.05$) was determined by repeated measures one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test

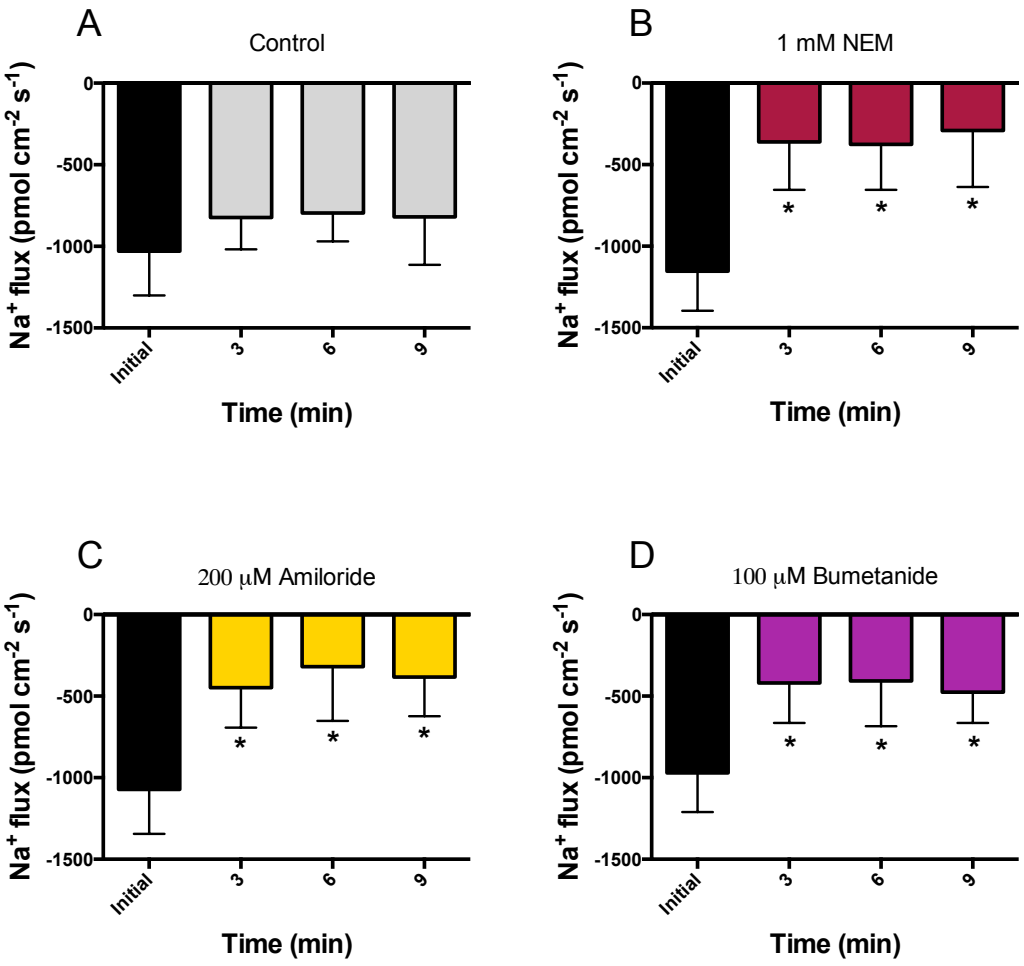

Figure 3: Scanning Ion-Selective Microelectrode Technique (SIET) measurements of *in vitro* Na^+ transport rates along the surface of the distal gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. Data are expressed as means±s.e.m. (N=5–11). Asterisks denote significant differences between treatments (3', 6', 9') and the initial value; significance ($P<0.05$) was determined by repeated measures one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test

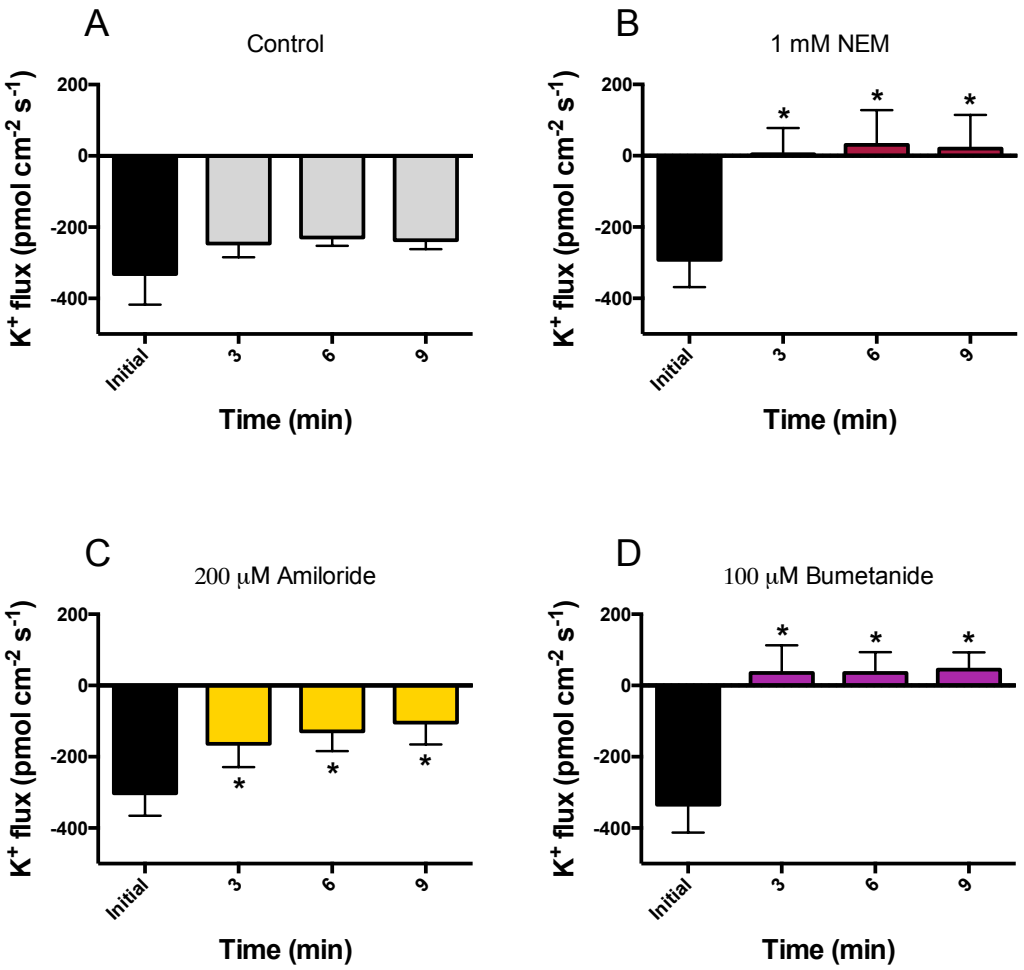

Figure 4: Scanning Ion-Selective Microelectrode Technique (SIET) measurements of *in vitro* K^+ transport rates along the surface of the distal gastric caecum of fourth instar *Aedes aegypti* larvae reared in freshwater. Data are expressed as means±s.e.m. (N=5–6). Asterisks denote significant differences between treatments (3', 6', 9') and the initial value; significance ($P<0.05$) was determined by repeated measures one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test

4. Discussion

This study provides the first comprehensive analysis of the effects of inhibitors of inorganic ion transporters in the distal gastric caecum. While many studies have hypothesized that the distal gastric caecum is ionoregulatory in

nature (Patrick et al., 2006; Pullikuth et al., 2006; Volkmann and Peters, 1989a; Volkmann and Peters, 1989b), and others have identified ion transporters in the gastric caecum using immunohistochemical methods (Filippov et al., 2003; Kang'ethe et al., 2007; Patrick et al., 2006; Pullikuth et al., 2006), this is the first study to investigate the contribution of each of these transporters to H^+, Na^+, and K^+ transport across the distal gastric caecum.

One problem that impedes the analyses of ion transport mechanisms in isolated larval *Aedes* gastric caeca is that within 3-6 mins of isolation of the entire midgut, the TEP, and V_b depolarize, and the transport rates of H^+, Na^+ and K^+ decline precipitously (D'Silva and O'Donnell, 2018; D'Silva et al., 2017). In a previous study we found that 5-HT restored TEP to original values, and in the distal gastric caecum restored V_b and the transport rates of H^+, Na^+ and K^+ of *in vitro* preparations (D'Silva and O'Donnell, 2018). Isolated midguts were therefore incubated in 5-HT prior to electrophysiological measurements, thus allowing us to analyse the ion transport mechanisms of the stimulated distal gastric caecum. 5-HT acts via an intracellular second messenger, cAMP, to stimulate VA activity (Fig. 5) (D'Silva and O'Donnell, 2018). This is similar to the action of 5-HT in other insect tissues, where 5-HT acts via cAMP (Clark and Bradley, 1996; Clark and Bradley, 1997; Clark and Bradley, 1998; Donini et al., 2006; Gioino et al., 2014; Ruiz-Sanchez et al., 2015), and increased intracellular cAMP levels lead 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; Wieczorek et al., 1999). Fig. 5 summarizes the immunohistochemical evidence for ion transporters (Filippov et al., 2003; Kang'ethe et al., 2007; Patrick et al., 2006;

Pullikuth et al., 2006) and the proposed action of 5-HT on the VA present on the apical and basal membranes of the distal gastric caecum (D'Silva and O'Donnell, 2018). Fig. 5 also summarizes the ion transport mechanisms described below.

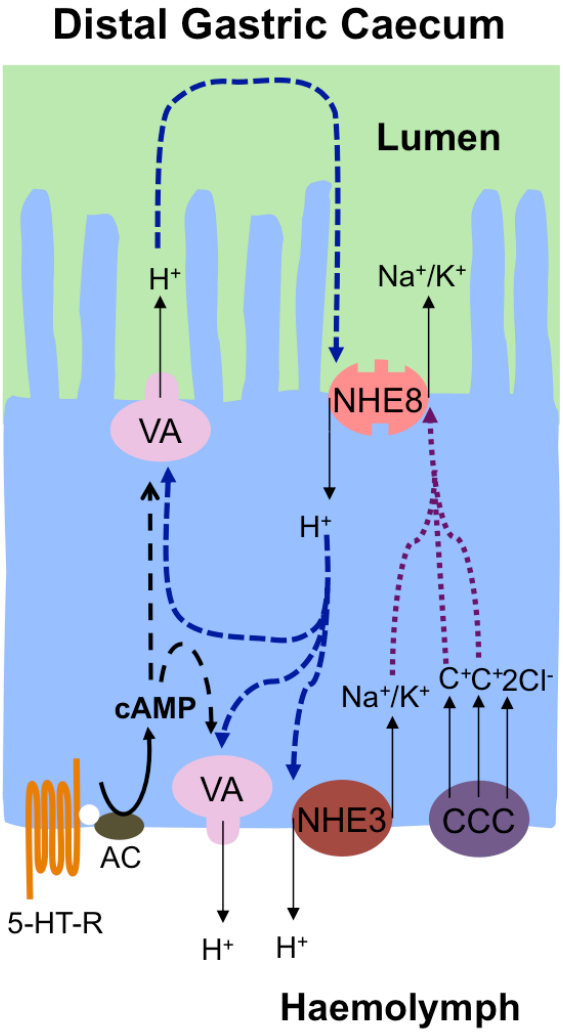

Figure 5: Schematic diagram illustrating the role of VA in energizing transepithelial transport across the distal gastric caecum. Blue arrows indicate H^+ transport and purple arrows indicate Na^+ or K^+ movement. AC, adenylyl cyclase; VA, V-type H^+ ATPase; NHE, Na^+/H^+ exchanger (for A. aegypti: AeNHE8, AeNHE3); CCC, cation chloride cotransporter; 5-HT-R, 5-HT receptor. Modified from D'Silva and O'Donnell (2018).

4.1 Ion transport mechanisms of the distal gastric caecum

In the *Aedes* distal gastric caecum (Fig. 5), VA and AeNHE8 are expressed along the apical membrane, and VA, AeNHE3, and CCC are expressed on the basal membrane (Filippov et al., 2003; Kang'ethe et al., 2007; Patrick et al., 2006; Pullikuth et al., 2006). This expression of ion transporters is similar to that of *Aedes* Malpighian tubules. In Malpighian tubules, ion and fluid transport is energized by an apically expressed VA, along with a secondary ion transport system, an apical cation/proton antiporter, that exchanges luminal H^+ for cellular Na^+ or K^+ (Beyenbach, 2001; Beyenbach, 2003; Beyenbach et al., 2000; Kang'ethe et al., 2007). In the Malpighian tubule model, the apical VA secretes H^+ into the lumen, leading to a H^+ gradient across the apical membrane which drives the transport of cation:nH^+ across the apical membrane via the apical NHA (Rheault et al., 2007; Xiang et al., 2012). Overall, this leads to cycling of protons across the apical membrane.

Concanamycin significantly reduced H^+ absorption across the distal gastric caecum (Fig. 2B); however, the concentration (50 μM) required to inhibit 50% H^+ absorption is high relative to that used to inhbit other epithelia such as Malpighian tubules (e.g. 10 μM, Dijkstra et al., 1994) . Another plecomacrolide, bafilomycin, was previously shown to have no effect on the TEP of the gastric caecum at a concentration of 10 μM (Boudko et al., 2001). Given the excessive cost of using high concentrations of concanamycin, a cheaper alternative, NEM, was used to determine the impacts of VA inhibition on TEP, V_b, H^+, Na^+ and K^+ fluxes.

NEM abolished the TEP and V_b of the distal gastric caecum (Fig. 1A, B) and significantly reduced H^+ absorption as well as Na^+ and K^+ secretion across the distal gastric caecum (Fig. 2C, 3B, 4B), suggesting that VA is responsible for maintenance of transmembrane electrical potential differences and the secondary active transport of Na^+ and K^+ across the distal gastric caecum. This finding is consistent with the action of apical VA in the *Aedes* Malpighian tubule model where direct inhibition of VA by bafilomycin or indirect inhibition by lowering ATP levels through application of dinitrophenol, an uncoupler of oxidative phosphorylation, inhibited secondary active transport of Na^+ and K^+ across the apical membrane (Beyenbach et al., 2000).

We therefore propose that the functions of the apical VA and AeNHE8 in the distal gastric caecum are coupled, analogous to the coupling of apical VA and NHA in Malpighian tubules. In the distal gastric caecum the coupling of the apical VA and AeNHE8 drives the secretion of Na^+ or K^+ out of the cytosol and into the lumen (Fig. 5), thereby creating a favourable electrochemical gradient, which facilitates the function of transporters on the basolateral membrane. Furthermore, removal of intracellular Na^+ or K^+ by the AeNHE8 would lower intracellular levels of these ions, thus driving uptake of Na^+ or K^+ by basally expressed AeNHE3 and CCC from the bathing saline (Fig. 5). Therefore, inhibition of the apical VA by NEM would lead to subsequent reduction in Na^+ or K^+ secretion, as observed in this study (Fig. 3B, 4B). These results provide evidence that apical VA and AeNHE8 operate such that there is overall absorption of H^+ and secretion of Na^+ or K^+ (Pullikuth et al., 2006), which is

also consistent with measured net rates of transport of H^+ and Na^+ or K^+ across the distal caecum (D'Silva and O'Donnell, 2018; D'Silva et al., 2017).

When either of these NHEs, AeNHE8 or AeNHE3, are expressed heterologously in cell culture, amiloride inhibits AeNHE8 (Piermarini et al., 2009), but does not inhibit Na^+ uptake by AeNHE3 (Pullikuth et al., 2006). The effects of amiloride (Fig. 2D, 3C, 4C) are thus consistent with the ion transport model, in which inhibition of AeNHE8 by amiloride results in subsequent effects on the AeNHE3 and CCC thereby inhibiting Na^+ or K^+ entry and H^+ extrusion across the basolateral membrane.

In addition to amiloride, bumetanide (CCC inhibitor) also affected the Na^+ and K^+ transport rates with K^+ secretion being fully inhibited (Fig. 3D, 4D). Some evidence of a reversal (Fig. 4D) perhaps reflects outward leak of K^+ through channels. Since NKA is absent in the distal gastric caecum (D'Silva et al., 2017; Patrick et al., 2006), our results suggest that the AeNHE8 coupled to apical VA is the main driver of Na^+ or K^+ gradient across the apical membrane, and that CCC appears to be the primary site of K^+ entry across the basal membrane.

Together with basally expressed VA, expression of AeNHE3 on the basal membrane of the distal gastric caecum may be indicative of a supportive role for cation/proton exchange in H^+ absorption across the distal gastric caecum (Pullikuth et al., 2006). While both AeNHE3 and VA are expressed on the basal membrane (D'Silva et al., 2017; Patrick et al., 2006; Pullikuth et al., 2006), we postulate that AeNHE3 activity is not coupled to basal membrane VA activity, but rather functions in parallel to basal VA to absorb H^+ from the cytosol to the haemolymph (Fig. 5). There is evidence for carbonic anhydrase in the larval distal

gastric caecum of another mosquito, *Anopheles gambiae*, (Smith et al., 2007). The absorption of H^+ at the distal caecum may therefore be enhanced due to the production of high levels of cellular H^+ by the actions of carbonic anhydrase in this region. The AeNHE3 therefore aids in regulating cytosolic pH, and may be indirectly affected by the activity of the apical VA, and consequently AeNHE8 (Fig. 5) (Pullikuth et al., 2006).

We determined the effects of DIDS on H^+ transport rates because cytosolic and luminal pH may also be influenced by anion transporters. DIDS is a relatively non-specific inhibitor of anion transport, and is known to inhibit both Cl^- channels and Cl^-/HCO_3^- exchangers (Cabantchik and Greger, 1992). DIDS however had no effect on the rates of H^+ absorption (Fig. 2E). These observations suggest that an anionic pathway may not play a major role in maintaining the pH of caecal lumen via the distal cells. This finding is in contrast to the adjacent midgut, where anion exchangers are responsible for strong alkalinization of the anterior midgut lumen by contributing to acid efflux (Boudko et al., 2001b).

4.2 Use of VA inhibitors

We used NEM, a non-specific H^+-ATPase inhibitor to elucidate the role of VA in the distal gastric caecum. One of the challenges we faced in this study was that only relatively high concentrations (50 μM) of concanamycin were required to inhibit 50% of H^+ absorption. Furthermore, another plecomacrolide, bafilomycin, had no effect on the H^+ transport across the gastric caecum at a concentration of 10 μM (Boudko et al., 2001). Since concanamycin and

bafilomycin inhibit VA by binding to the membrane associated V_o subunit c (Bowman and Bowman, 2002; Hanada et al., 1990; Huss et al., 2002; Rautiala et al., 1993), it is possible that they may not be able to easily access the intramembrane binding site, and may therefore require a very high concentration in order to exert an effect. Since these plecomacrolides are very expensive, it was not feasible to use them for all experiments. A cheaper drug, NEM, was therefore chosen for our study as it also inhibits eukaryotic VA (Feng and Forgac, 1992; Mellman et al., 1986). NEM inhibits VA by binding to the cysteinyl residue on the cytosolic V_1 A subunit (Arai et al., 1987; Bowman and Bowman, 1986; Mandala and Taiz, 1986; Moriyama and Nelson, 1987), and may therefore have easy access to its binding site in comparison to the plecomacrolides which bind to an intramembrane binding site. At concentrations between 0.1 – 1 mM, NEM may have an effect on P-type ATPases, however, it will inhibit all VA (Hunt and Sanders, 1996). Furthermore, NEM was used in other organisms at doses within the 0.1 – 1 mM range, for example, in the Malpighian tubules of ants, NEM (0.5 mM) inhibited VA thus leading to the depolarization of TEP, (Dijkstra et al., 1994). In *Trypanosoma cruzi* cells, NEM (0.5 mM) inhibited the efflux of H^+ by VA, leading to rapid depolarization of the cells; bafilomycin (2 μM) also depolarized the cells, however, it's effects on H^+ efflux was not measured (Van Der Heyden and Docampo, 2002). In mouse macrophages, acid extrusion by VA is sensitive to NEM (1 mM) (Swallow et al., 1988). It is important to bear in mind that while the effects of 1 mM NEM reported in our study include the inhibition of VA, it may also include effects on other ATPases

that may be present in the *Aedes aegypti* distal gastric caecum. However, since our results indicate that both NEM and concanamycin had similar qualitative effects on H^+ absorption, and immunolocalization of VA was observed on both the apical and basolateral membranes of the distal gastric caecum, whereas immunolocalization of NKA, a P-type ATPase, was not observed in this region (Patrick et al., 2006), we can assume that the bulk of the measured effects of NEM is primarily due to the inhibition of VA by NEM.

5. Conclusions

Together, our results suggest that VA is the primary ionomotive ATPase in the distal gastric caecum. As described in our ion transport model (Fig. 5), the apical VA and AeNHE8 function together to exchange luminal H^+ for cellular Na^+ or K^+, similar to their action of VA and cation/proton antiporters in the apical membrane of Malpighian tubules. This coupling of the apical VA and AeNHE8 leads to the removal of intracellular Na^+ or K^+, therefore promoting the secretion of Na^+ or K^+ into the cell by basally expressed AeNHE3 and CCC.

In addition to 5-HT, other endocrine modulators may also contribute to functional control of the distal gastric caecum; therefore the mechanisms of ion transport discussed here may not describe the full picture. Since the expression of ion transporters in the distal gastric caecum and Malpighian tubules are very similar, future studies would include exploring the roles of neuropeptides known to stimulate the Malpighian tubule, like diuretic hormones (Beyenbach, 2003).

6. References

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Chapter 6

General discussion

This thesis has explored the roles of two ion-motive ATPases, VA and NKA, in promoting transepithelial ion transport across the gut epithelia of larval *Drosophila melanogaster* and the gastric caecum of larval *Aedes aegypti*. The studies in this dissertation present immunohistochemical evidence for the expression of VA and NKA, and have also functionally characterized the two ATPases by means of ATPase assays and multiple electrophysiological methods. Taken together, the results support the roles of these ATPases in driving ion transport in the larval midgut of *Drosophila melanogaster*, and the gastric caecum of *Aedes aegypti*.

The pronounced regionalization of VA and NKA along the distal and proximal gastric caecum of *Aedes aegypti* larvae reared in freshwater (Patrick et al., 2006) allowed for a side-by-side comparison of the two regions. Furthermore, in view of the paucity of studies on ion transport and acid base balance of the gastric caecum, in contrast to the extensive knowledge on the midgut (Boudko et al., 2001; Clark et al., 1999; Clark et al., 2000; Corena et al., 2002; Izeirovski et al., 2009; Jagadeshwaran et al., 2010; Linser et al., 2009; Onken and Moffett, 2009; Onken et al., 2004a; Onken et al., 2006; Onken et al., 2008; Onken et al., 2009), Malpighian tubules (Beyenbach, 2003; Beyenbach et al., 2000; Beyenbach and Piermarini, 2011; Clark and Bradley, 1996; Clark and Bradley, 1997; Clark and Bradley, 1998; Hegarty et al., 1991; Piermarini et al., 2017; Piermarini et al., 2009; Ramsay, 1950; Scott, 2004; Veenstra, 1988; Weng et al., 2003), and anal papillae (Del Duca et al., 2011; Donini et al., 2007; Donini and O'Donnell, 2005; Hopkins, 1967; Koch, 1938; Stobbart, 1971), this thesis provides new insights into the functions of this understudied region of the digestive tract.

1. Roles of VA and NKA in ion transport across insect gut epithelia

In order to examine the contributions of VA and NKA to ion transport across insect gut epithelia I first examined the expression patterns of the two ATPases, after which I used the SIET technique in combination with the application of ion transport inhibitors to assess the functional roles of the two ATPases (Chapters 2 - 5).

Previous studies on the *Drosophila melanogaster* gut reported that VA is present in the acidic region of the middle midgut (Overend et al., 2016) and in the posterior midgut (Shanbhag and Tripathi, 2005), and that NKA is expressed along the middle midgut (Dubreuil et al., 2000). Prior to the study which comprises Chapter 2 of this thesis, the expression patterns of VA and NKA in other regions of the gut were unknown. I therefore examined expression patterns of VA and NKA in other regions of the *Drosophila melanogaster* gut such as the caeca, anterior midgut, middle midgut, and posterior midgut (Chapter 2). Immunohistochemistry revealed that VA is expressed throughout the gut, from the caeca to the posterior midgut, and that NKA is expressed in the caeca, anterior midgut, middle midgut (large flat cells) and posterior midgut (neutral zone). Furthermore, ATPase activities of VA and NKA are quantitatively similar within each region of the gut, suggesting that both ATPases may be important for establishing favourable electrochemical gradients for transport of ions across the gut epithelial cells.

In a previous study, H^+ was transported from the cell to the bathing saline along the posterior midgut of *Drosophila melanogaster*, and this transport was reduced by the VA inhibitor bafilomycin (Shanbhag and Tripathi, 2005). These findings were confirmed in chapter 2, which also demonstrated that bafilomycin-sensitive VA is present in other regions of the gut - the caeca and anterior midgut. Furthermore, bafilomycin, as well as

the carbonic anhydrase inhibitor acetazolamide, inhibited K^+ absorption in the caeca and anterior midgut, suggesting that K^+ transport in these regions is dependent on the activity of VA. Ouabain, an NKA inhibitor, increased K^+ absorption across the anterior midgut and large flat cells of the middle midgut, indicating a role for NKA in these regions.

In *Aedes aegypti*, a previous study revealed striking regionalization of VA and NKA along the gastric caecum of larvae reared in freshwater, with VA being expressed along the apical membrane throughout the caecum, and on the basal membrane of the distal one-third, while NKA is expressed on the basal membrane of the proximal two-thirds (Patrick et al., 2006). The basal expression of VA and NKA was confirmed in Chapter 3; additionally, VA and NKA ATPase activities were shown to be quantitatively similar in the gastric caecum, suggesting that both ATPases may be important for driving ion transport across the caecum of *Aedes aegypti* larvae. Furthermore, since there is clear regionalization of the two ionomotive ATPases, I was able to measure and compare the ion transport rates of the two regions, the distal and proximal caecum (Chapter 3, 4). I found that the ion transport rates declined precipitously within 3-6 mins after dissection of the gut from the animal and isolation in saline, but that this decline was prevented by addition of 5-HT to the bathing saline (Chapter 4). I therefore chose to investigate the action of ion transport inhibitors in this region only (Chapter 5). One of the challenges I faced was that while concanamycin, a VA inhibitor, which was reported to have an inhibitory effect on the VA expressed in the *Aedes aegypti* larval anterior midgut (Onken et al., 2004b; Onken et al., 2006; Onken et al., 2008), did have an effect on the H^+ transport rates of the gastric caecum, a very high concentration (50 μM) was needed in order to reduce the magnitude of the flux by approximately 50%. Furthermore, another

plecomacrolide, bafilomycin, which was used in the *Drosophila melanogaster* study included in this thesis (Chapter 2), was previously shown to have no effect on the TEP of the gastric caecum at a concentration of 10 μM (Boudko et al., 2001). Since concanamycin and bafilomycin inhibit VA by binding to the membrane associated V_o subunit c (Bowman and Bowman, 2002; Hanada et al., 1990; Huss et al., 2002; Rautiala et al., 1993), it is possible that they may not be able to easily access the intramembrane binding site in the gastric caecum, and therefore require a very high concentration in order to exert an effect. Additionally, the concentrations required to inhibit VA in the *Aedes aegypti* gastric caecum (Chapter 5) proved to be very expensive, which is why I used a cheaper alternative, NEM, which inhibits VA by binding to the V_1 A subunit (Arai et al., 1987; Bowman and Bowman, 1986; Mandala and Taiz, 1986; Moriyama and Nelson, 1987). At concentrations between 0.1 – 1 mM, NEM may have an effect on P-type ATPases, however, it will inhibit all VA (Hunt and Sanders, 1996). Therefore it is important to bear in mind that the results reported for NEM (Chapter 5) is a consequence of inhibition of VA, but may also include effects on other ATPases that may be present in the *Aedes aegypti* distal gastric caecum. However, given that VA is expressed on both the apical and basolateral membranes of the distal gastric caecum, and no immunolocalization of NKA, a P-type ATPase, was observed in this region (Patrick et al., 2006), and also given that both NEM and concanamycin had similar qualitative effects on H^+ absorption, we assume that the bulk of the measured effects of NEM is primarily due to the inhibition of VA by NEM (Chapter 5). Furthermore, NEM was used in other organisms at doses within the 0.1 – 1 mM range, for example, NEM (0.5 mM) was used in the characterization of VA in the Malpighian tubules of ants, where it

depolarized the TEP of the tubules (Dijkstra et al., 1994). In *Trypanosoma cruzi* cells, NEM (0.5 mM) inhibited the efflux of H^+ by VA, leading to rapid depolarization of the cells; bafilomycin (2 μM) also depolarized the cells, however, it's effects on H^+ efflux was not measured (Van Der Heyden and Docampo, 2002). In mouse macrophages, acid extrusion by VA is also sensitive to NEM (1 mM) (Swallow et al., 1988).

In *Aedes aegypti*, application of NEM resulted in declines of TEP, and V_b of the distal gastric caecum to near zero voltages, suggesting that VA is the main driver of the transmembrane electrical potentials of the distal caecum. Furthermore, NEM significantly reduced H^+ absorption, as well as Na^+ and K^+ secretion across the distal gastric caecum.

In both *Drosophila melanogaster* and *Aedes aegypti*, the dependence of Na^+ or K^+ transport on VA activity is consistent with the model developed by Wieczorek et al. (Wieczorek, 1992; Wieczorek et al., 1999; Wieczorek et al., 1991; Wieczorek et al., 1989). According to this model, apically expressed VA drives the secretion of H^+ into the lumen, thereby establishing an electrochemical gradient for H^+ across the apical membrane. An apically expressed cation:nH^+-antiporter, uses the voltage generated by VA to transport H^+ back into the cell in exchange for Na^+ or K^+.

In vertebrates, an electroneutral cation:nH^+-antiporter (n=1; NHE) is typically located on the basolateral membrane, and transports cellular H^+ out of the cell in exchange for Na^+ into the cell, thus aiding in intracellular pH regulation (Orlowski and Grinstein, 2004). This exchange is mediated by NKA, which is typically expressed on the basolateral membrane; NKA transports Na^+ out of the cell in exchange for K^+ into the cell, thus keeping intracellular Na^+ concentrations low.

NHE uses the large Na^+ gradient established by NKA across the basolateral membrane, to import Na^+ into the cell and export H^+ out of it in a 1:1 stoichiometry (Orlowski and Grinstein, 2004).

In insect cells where NKA immunolocalization was not observed, such as the distal cells of the *Aedes aegypti* gastric caecum, the basolateral NHE is likely driven by the combined actions of apically expressed VA and the cation:nH^+ antiporter, which work in tandem to reduce intracellular Na^+ or K^+ concentrations (Chapter 5). This creates a Na^+ or K^+ gradient favouring entry of Na^+ or K^+ via NHE on the basolateral membrane. Therefore the VA and cation:nH^+ antiporter work together to mimic the role of NKA in generating transmembrane Na^+ gradients. The Na^+ gradient created by the combined action of apical VA and cation:nH^+ antiporter differs from the Na^+ gradient created by basolateral NKA in mammalian cells, in that it creates the gradient by transferring Na^+ into the lumen instead of the haemolymph.

2. Ion transport mechanisms: *Aedes aegypti* gastric caecum

Given that the gastric caecum of *Aedes aegypti* larvae reared in freshwater has two types of cells, the distal cells with VA expressed on both the apical and basolateral membranes, and the proximal cells which express VA on the apical membrane and NKA on the basal membrane (Patrick et al., 2006), I decided to compare the two types of cells. As noted above, one of the challenges I faced was that the TEP and ion transport rates declined precipitously within 3-6 mins of dissection (Chapter 4). Previous studies on the anterior and posterior midgut regions also reported

declines in TEP, which was prevented by 5-HT (Clark et al., 1999). Furthermore, putative 5-HT receptor (AaSER-1) is expressed in the gastric caecum (Petrova and Moffett, 2016); however, the localization of this receptor to the distal and proximal regions was not determined. I therefore investigated the effects of 5-HT on the gastric caecum and showed that 5-HT is critical for maintaining TEP of the caecum (Chapter 4). I further investigated the effects of 5-HT on the distal and proximal cells of the gastric caecum, by measuring the basolateral membrane potentials (V_b), and transport rates of H^+, Na^+, and K^+ across the distal and proximal regions. I found that 5-HT stimulation lead to recovery of V_b in the distal cells but not the proximal cells, and that 5-HT prevented the decline of H^+, Na^+, and K^+ transport rates at the distal caecum but not the proximal caecum (Chapter 4). Subsequent to the publication of Chapter 4, serotonergic axons were shown to innervate the distal and proximal gastric caecum, linking them longitudinally and transversely (Petrova et al., 2017), suggesting that 5-HT may coordinate functions between both regions of the caecum. While there are no measurable effects of 5-HT on ion transport along the proximal gastric caecum, 5-HT may control functions like gut motility (Onken et al., 2004a), thus allowing for mixing of the luminal contents. Together, these results suggest that endogenous 5-HT is important for maintaining ion transport, as well as TEP of the gastric caecum *in vivo*. This signalling is lost when the gastric caecum is isolated from the animal and bathed in artificial saline. In addition to 5-HT, I have shown that cAMP, a second messenger for 5-HT, has similar effects on the TEP and V_b as 5-HT. I therefore propose that the actions of 5-HT are mediated through cAMP, similar to the action of 5-HT in Malpighian tubules, where it acts via intracellular cAMP (Clark and Bradley, 1996; Clark

and Bradley, 1997; Clark and Bradley, 1998; Donini et al., 2006). I also propose that intracellular cAMP leads to the activation of VA and an increase in H^+ transport, similar to the action of cAMP on VA in other species (Baumann and Walz, 2012; Coast et al., 2001; Dames et al., 2006; O'Donnell et al., 1996; Voss et al., 2010; Wieczorek et al., 1999). cAMP first activates PKA, which then mediates phosphorylation of the VA subunit C that undergoes a conformational change, which gives it an enhanced affinity for the V_1 complex. Once the phosphorylated C subunit stably binds to the V_1 complex, this complete V_1 complex then associates with the membrane bound V_0 complex to form an active VA (Baumann and Walz, 2012; Tiburcy et al., 2012; Voss et al., 2010). In order to test this model in the *Aedes aegypti* gastric caecum, a PKA inhibitor like H-89 (Tiburcy et al., 2012) could be added to the bathing saline after stimulating the caecum with either 5-HT or cAMP. Inhibition of the actions of 5-HT or cAMP by H-89 would support a role for PKA in activation of VA in the caecum in response to stimulation with 5-HT. Overall, these results suggest that the function of the gastric caecum may be controlled in part by the effects of 5-HT, and that VA may be the main driver of electrochemical potentials and ion transport across the distal gastric caecum. Other neuromodulators or hormones that contribute to functional control of the caecum, particularly the proximal gastric caecum, have yet to be identified.

Figure 1 is a summary figure for the gastric caecum that summarizes the immunohistochemical evidence for inorganic ion transporters (Filippov et al., 2003; Kang'ethe et al., 2007; Patrick et al., 2006; Pullikuth et al., 2006), and the ion transport mechanisms described in the studies presented in this thesis (Chapters 3, 4, 5). Briefly, VA is expressed along the apical and basal membrane of the distal third (VA-

rich cells; Fig. 1), and on the apical membrane of the proximal cells, whereas NKA is expressed on the basal membrane of the proximal two-thirds (NKA-rich cells; Fig. 1) of the gastric caecum (D'Silva et al., 2017; Patrick et al., 2006). AeNHE8 is expressed along the apical membrane throughout the gastric caecum (Kang'ethe et al., 2007), AeNHE3 is expressed on the basolateral membrane of the distal caecum (Pullikuth et al., 2006), and CCC is expressed along the basolateral membrane throughout the gastric caecum (Filippov et al., 2003) (Fig. 1).

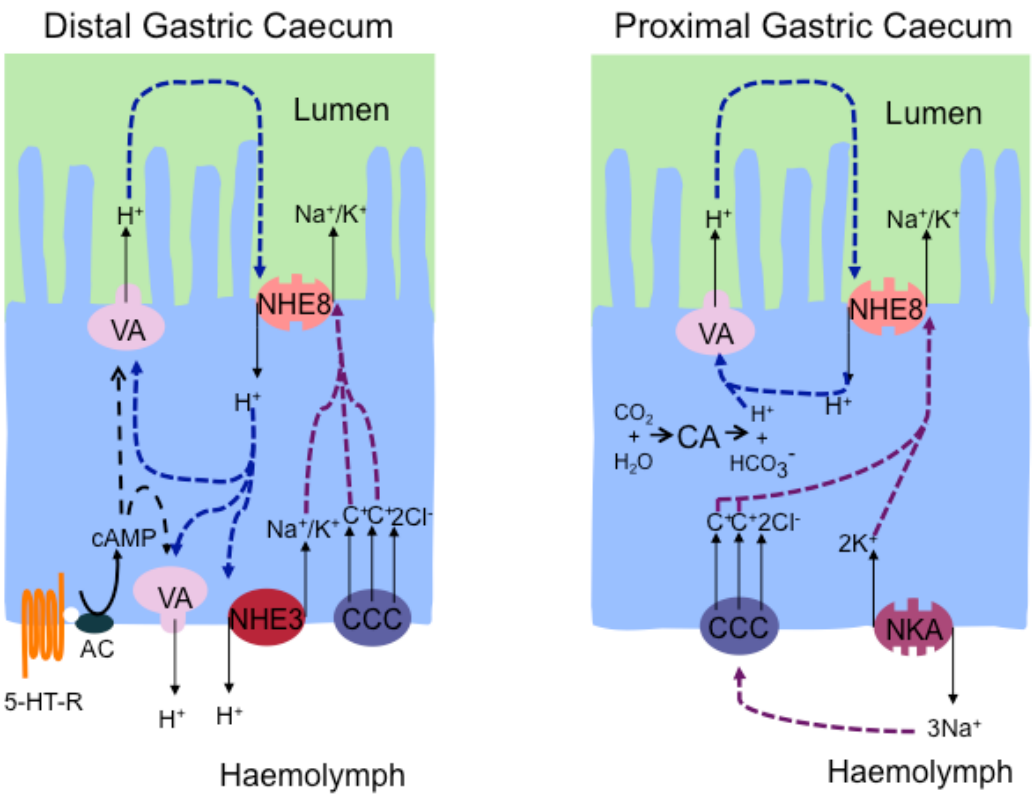

Figure 1: Schematic diagram illustrating the roles of VA and NKA in energizing transepithelial transport across the distal and proximal gastric caecum. Blue arrows indicate H^+ transport and purple arrows indicate Na^+ or K^+ movement. VA, vacuolar-type H^+-ATPase; NHE, Na^+/H^+ exchanger (for *Aedes aegypti*: AeNHE8, AeNHE3); CCC, cation chloride cotransporter; 5-HT-R, 5-HT receptor; AC, adenylyl cyclase;

cAMP, cyclic adenosine monophosphate; CA, carbonic anhydrase.

Since the transporters that have been identified are cation transporters, transporting H^+, Na^+, or K^+, I used SIET to measure the rates of transport of these ions across the distal and proximal gastric caecum (Chapter 3). I found that H^+ is absorbed (from lumen to bath) across the distal gastric caecum, and secreted (from bath to lumen) across the proximal gastric caecum. Na^+ and K^+ are secreted along both regions, with significantly higher secretion rates at the distal caecum compared to the proximal gastric caecum. The directions of ion transport are consistent with those expected based on the expression patterns of ion transporters reported by immunohistochemical studies (Filippov et al., 2003; Kang'ethe et al., 2007; Patrick et al., 2006; Pullikuth et al., 2006).

I used inhibitors of specific ion transporters in order to assess the roles of VA, AeNHE8, and CCC, in the transport of H^+, Na^+, and K^+ across the distal gastric caecum (Chapter 5). These experiments were done in the presence of 5-HT, which maintains stable rates of ion transport across the distal caecum in control preparations. However, it is important to note that other neuromodulators or hormones may also contribute to the functional control of the distal gastric caecum; therefore, the mechanisms of ion transport discussed here may not paint the complete picture.

At the distal gastric caecum (Fig. 1) cycling of H^+ will occur at the apical membrane due to the combined action of apical VA and AeNHE8, with overall absorption of H^+ in this region mediated by the combined actions of apical AeNHE8, and basolateral AeNHE3 and VA. As described earlier using the model developed by Wieczorek et al. (section 1), apically expressed VA and AeNHE8 work in concert to

regulate intracellular Na^+ or K^+ by secreting these ions into the lumen in exchange for H^+ absorbed into the cell. A low intracellular Na^+ or K^+ concentration, combined with the negative V_b, will facilitate the action of AeNHE3 and CCC on the basolateral membrane to transport Na^+ and/or K^+ into the cell (Fig. 1). Overall this will result in secretion of Na^+ and K^+ across the distal gastric caecum.

The effects of amiloride on the transport rates of Na^+ and K^+ are consistent with dependence of Na^+ and K^+ secretion on the action of apical AeNHE8. Amiloride inhibits AeNHE8 (Piermarini et al., 2009) but not AeNHE3 (Pullikuth et al., 2006), and, in the distal gastric caecum, amiloride leads to reduction in the rate of H^+ absorption, and reductions in Na^+ and K^+ secretion rates. The effects of amiloride thus support the ion transport model (Fig. 1), in which inhibition of AeNHE8 would lead to an increase of intracellular Na^+ and/or K^+, thereby affecting the electrochemical gradients required to facilitate the action of AeNHE3 and CCC on the basolateral membrane and thus reducing overall secretion Na^+ and/or K^+. Bumetanide also inhibited Na^+ and K^+ transport rates, with K^+ secretion being reversed from secretion to absorption, suggesting that CCC may be the primary site of K^+ entry into the cell, with possible leaks out of the cell via K^+ channels across the basolateral membrane of the distal gastric caecum. Furthermore, there is a basolateral VA in the distal gastric caecum, which probably acts in tandem with the AeNHE3 to regulate cytosolic pH. I propose that AeNHE3 and basally expressed VA function in parallel to regulate cytosolic pH by transporting H^+ out of the cells and into the haemolymph (Fig. 1). Additionally, there is evidence for carbonic anhydrase in the larval distal gastric caecum of another mosquito, *Anopheles gambiae*,

(Smith et al., 2007); therefore, absorption of H^+ at the distal caecum may be enhanced due to the production of high levels of cytosolic H^+ by the actions of carbonic anhydrase in this region.

At the proximal gastric caecum (Fig. 1), cycling of H^+ will occur at the apical membrane, with secretion of Na^+ and/or K^+ into the lumen, due to the combined action of apical VA and AeNHE8, as described by the Wieczorek model. However, in the proximal caecum there is overall secretion of H^+, which is probably enhanced due to the production of intracellular H^+ by the actions of cytoplasmic carbonic anhydrase in this region (Fig. 1) (Seron et al., 2004). Furthermore, there is no evidence for a basolateral VA or AeNHE3 in this region that would aid in maintaining the intracellular pH of these cells. Similar to the distal gastric caecum, apical VA and AeNHE8 probably aid in maintaining low intracellular Na^+ concentration, and these actions are supplemented by basolateral NKA which transports Na^+ out of the cell in exchange for K^+ into the cell. This Na^+ gradient probably drives the action of the basolateral CCC in the proximal caecum. In order to test this model using pharmacological tools, we first need to identify factors or conditions which prevent the decline of ion transport across the proximal caecum *in vitro*, thus permitting the effects of inhibition of specific transporters to be assessed by comparison with stable transport rates in control preparations not exposed to the inhibitors.

3. *Aedes aegypti* gastric caecum: effects of rearing salinity

As mentioned earlier, there is a striking regionalization of VA and NKA along the gastric caecum, with VA expressed along the basal membrane of the distal third (VA-rich

cells), and NKA expressed along the basal membrane of the proximal two-thirds (NKA-rich cells) (Chapter 3; Patrick et al., 2006). The regionalization of VA and NKA expression corresponds to two morphologically distinct cell types, ion transporting and digestive cells, which were described in an ultrastructural study (Volkmann and Peters, 1989a). The digestive cells were originally called resorbing/secreting (Volkmann and Peters, 1989a), but are referred to as digestive cells throughout this thesis, in order to avoid confusion with cells that absorb and secrete inorganic ions (Chapter 3).

Interestingly, the expression patterns of VA and NKA of the gastric caecum are dependent on the salinity of the rearing medium (Chapter 3). VA and NKA are not regionalized in larvae reared in brackish water, but instead, small VA-rich cells are interspersed between the NKA-rich cells leading to a mosaic-like expression pattern. The apparent decrease in size of the distal VA-rich region, as well as the size of the VA-rich cells (Chapter 3), is consistent with a previous ultrastructural study which showed a decrease in size of the distal ion transporting cells in high rearing salinities (Volkmann and Peters, 1989b). Furthermore, the mosaic pattern is similar to that of a salt-tolerant anopheline, *Anopheles stephensi*, where the ion transporting cells are scattered among the digestive cells of the gastric caecum (Volkmann and Peters, 1989a). Therefore, rearing salinity alters the structure and organization of cells in the gastric caecum, as well as the expression of ATPases along the gastric caecum. The variation in ATPase expression in response to rearing salinity is also observed in the rectum of anopheline mosquito larvae, which undergo a dramatic shift in rectal NKA localization when reared in freshwater versus brackish water (Smith et al., 2008). Additionally, the regionalization of ATPases is also altered when larvae are transferred from freshwater to brackish water. This ability to

alter the expression the ATPases in response to ambient salinity is important to maintaining ionoregulation by the rectum, and aids the larvae in adapting to different environments (Smith et al., 2008). Therefore, it is plausible that the rearrangement of ATPases of the gastric caecum in response to rearing salinity is important for maintaining ionic homeostasis in the cells and/or lumen of the caecum.

In order to test if the altered regionalization of ATPases in the gastric caecum is correlated with changes in ATPase activity, I measured the enzyme activities for VA and NKA for the two rearing conditions. I found that the ATPase activities for VA and NKA are significantly higher in the freshwater group (Chapter 3). The differences in organization of cell type, as well as ATPase activities, may be due to the differences in osmotic gradients experienced by the freshwater and brackish water larvae. While freshwater is hypoosmotic in comparison to larval haemolymph, brackish water (30% seawater) is roughly isosmotic to the larval haemolymph (Clark et al., 2004). Freshwater larvae therefore face an osmotic challenge due to ingestion of hypoosmotic media, which leads to an osmotic gradient favouring movement of water from the midgut into the haemolymph, as well as loss of ions from body fluids into the surrounding water. On the other hand, brackish water larvae experience much smaller osmotic gradients compared to freshwater larvae (Clark et al., 2004). The luminal fluid of the caecum is hyperosmotic compared to the haemolymph (Ramsay, 1950), which indicates that despite ingesting fluid that is hypotonic compared to the haemolymph, the gastric caecum is able to maintain a hypertonic environment in its lumen. In order for the caecum to maintain a hypertonic lumen, it secretes H^+, Na^+, and K^+ into the caecal lumen of larvae reared in either freshwater or brackish water, with ion transport rates being significantly higher in

freshwater larvae (Chapter 3, 4). Therefore, I propose that freshwater larvae require a higher ATPase activity in order to maintain secretion of ions into the caecal lumen to counteract dilution by the ingested hypotonic media, whereas in brackish water larvae there is a reduced need for ionomotive ATPases due to the elevated cation concentrations in the ingested media.

The luminal Na^+ or K^+ concentrations vary with rearing salinity, whereas luminal pH is maintained at ~7.6 in either freshwater or brackish water larvae (Chapter 3). In freshwater larvae, the luminal Na^+/K^+ ratio is ~0.4, with K^+ being actively secreted into the lumen while Na^+ is at electrochemical equilibrium. In contrast, the luminal Na^+/K^+ ratio is ~2.0 in brackish water larvae, and the caecum actively secretes Na^+ into the lumen while reducing active secretion of K^+. Evidence of this drastic change in the luminal Na^+/K^+ ratio is also seen in the *Aedes aegypti* larval Malpighian tubules and rectum (Ramsay, 1953). The Na^+/K^+ ratio is ~0.3 for Malpighian tubules, and ~0.2 for the rectal lumen, of larvae reared in distilled water or high-K^+ environments. The luminal K^+ concentrations are much higher than that of the haemolymph, while the luminal Na^+ concentration is lower than that of the haemolymph (Ramsay, 1953), indicating that the larvae tend to conserve haemolymph Na^+ when reared in low-Na^+ environments. However, when the larvae are reared in a high-Na^+ environment, the Na^+/K^+ ratio of the Malpighian tubule fluid increases to ~0.8, and the Na^+/K^+ ratio of the rectal fluid increases to ~6 (Ramsay, 1953). This indicates that the larvae are able to adapt to the environment they are in, and tend to conserve haemolymph Na^+ in dilute or low-Na^+ environments, but are able to utilize Na^+ when it is readily available.

Overall, these results, in conjunction with the higher osmotic pressure observed in the caecum (Ramsay, 1950), indicate that the caecum is capable of ionoregulation in different rearing salinities. Ionoregulation in the caecum probably serves to maintain its own luminal milieu in order to provide an environment conducive for digestion and nutrient absorption. For example, a L-amino acid transporter is expressed on the apical membrane of the gastric caecum, which transports large neutral and basic amino acids, with neutral amino acid uptake being partially Na^+ dependent, while basic amino acid uptake is completely Na^+ independent (Jin et al., 2003). While neutral amino acid uptake can occur in the absence of Na^+, it is significantly higher in the presence of the ion. The capacity for both Na^+ dependent and Na^+ independent amino acid transport may indicate that amino acid uptake can be maintained in both fresh water and brackish water environments.

4. Function and regulation of the *Aedes aegypti* gastric caecum: future directions

The *Aedes aegypti* gastric caecum is primarily made up of two types of cells, the ion transporting cells forming the distal third, and the digestive cells along the proximal two-thirds (Volkmann and Peters, 1989a). The ion transporting cells are characterised by long slender microvilli on the apical surface, which are densely populated by mitochondria, resembling the cells of the Malpighian tubules (Volkmann and Peters, 1989a). Furthermore, the distribution of inorganic ion transporters in the ion transporting cells (Fig. 1) resembles that of the Malpighian tubule, where VA is expressed on the apical membrane, and AeNHE3 and CCC are expressed on the basolateral membrane (Kang'ethe et al., 2007; Pullikuth et al., 2006;

Scott, 2004). As described earlier (section 3) these cells become smaller when larvae are reared in an environment that is isosmotic (brackish water) with its haemolymph, suggesting that these cells may be involved in water transport across the caecum. While overall, there is evidence to suggest that the gastric caecum absorbs water (Ramsay, 1950; Wigglesworth, 1933a), given the similarities of the ion transporting cells to the Malpighian tubule cells, it is possible that these cells may also secrete water into the caecal lumen. As observed in larvae reared in a hypoosmotic environment (freshwater), the ion transport rates are much higher in the ion transporting cells as opposed to the digestive cells (Chapters 3, 4). Since the osmotic gradient favours movement of water from the lumen into the haemolymph, the high secretion rates of Na^+ and K^+ across these cells may be correlated with fluid secretion into the lumen, which may be important in supporting digestion by luminal fluid circulation. I have demonstrated that the ion transporting cells of the distal caecum are controlled in part by 5-HT (section 2), however, other endocrine factors may also contribute to functional control of these cells. Since the ion transporting cells of the *Aedes aegypti* caecum show similarities to the cells of the Malpighian tubule, I suggest exploring the roles of endocrine factors known to regulate insect Malpighian tubules (Beyenbach, 2003; Coast, 2007; Coast, 1996; Coast et al., 2002; Dow and Davies, 2003; O'Donnell and Spring, 2000; Park, 2012; Schooley et al., 2012).

In addition to exploring the effects of endocrine factors on the ion transporting cells, we have yet to discover what regulates the digestive cells of the proximal caecum. The digestive cells differ from the ion transporting cells in that the mitochondria of these cells do not extend into the microvilli on the apical membrane, and the cytoplasm is more

electron dense (Volkmann and Peters, 1989a). Furthermore, in contrast to the ion transporting cells which have only smooth endoplasmic reticulum, the digestive cells have both smooth and rough endoplasmic reticulum, as well as Golgi fields (Volkmann and Peters, 1989a). This suggests that the digestive cells may secrete proteins (Volkmann and Peters, 1989a). Previous studies on the gastric caeca of insects proposed that the caecum is involved in secretion of digestive enzymes, and digestion and absorption of nutrients (Biagio et al., 2009; Caldeira et al., 2007; Day et al., 2008; Weidlich et al., 2015; Weidlich et al., 2013; Wigglesworth, 1933b; Wigglesworth, 1942; Wolfersberger, 1984; Woodring et al., 2009; Woodring et al., 2007; Woodring and Weidlich, 2016). The release of digestive enzymes along the insect gut may be controlled by neuropeptides. For example, allatostatin-A stimulates the release of amylase and trypsin in crickets (Woodring et al., 2009) and carbohydrate digesting enzymes in cockroaches (Aguilar et al., 2003; Fusé et al., 1999), while leucokinin-II inhibits amylase and protease release in lepidopterans, whereas leucokinin-VIII stimulates protease release (Harshini et al., 2002). In *Aedes aegypti*, the effects of neuropeptides on TEP were explored in the anterior midgut (Onken et al., 2004a). *Aedes aegypti* allatostatins (allatostatin A 1-5), neuropeptide F, and proctolin, all reduced the TEP of the anterior midgut, whereas, *Aedes aegypti* allatotropin, head peptides I and III, and short neuropeptide F had no effects on the TEP (Onken et al., 2004a). Since the luminal environments of the caecum and the anterior midgut are very different, the action of neuropeptides may also differ in the two regions. I showed that despite the proximity of the caecum to the anterior midgut, the caecum maintains its lumen at a pH of 7.6 and at a lumen-positive electrical potential

(Chapter 3), which is in contrast to the adjacent anterior midgut that has a highly alkaline (pH 10.5 – 11) lumen that is at a negative potential with respect to the bathing saline (Clark et al., 1999; Dadd, 1975; Zhuang et al., 1999). The luminal environments likely differ drastically because the midgut and caecum may digest and absorb different nutrients (Wigglesworth, 1942). I therefore suggest exploring the roles of these neuropeptides, as well as other endocrine factors known to regulate digestion in insects (Lehane et al., 1995; Spit et al., 2012). A simple way to identify potential endocrine factors that may exert an action on the caecum is to use RT-PCR (reverse transcription polymerase chain reaction) or *in situ* hybridization to determine expression of their receptors, thus giving us a clear indication of whether there is a potential site of action, before testing the effects on the caecum. Alternatively, one could also use immunohistochemistry to identify expression of neuropeptide receptors along the gastric caecum. Antibodies for *Aedes aegypti* neuropeptide receptors for leucokinin (Lu et al., 2011; Pietrantonio et al., 2005), calcitonin-like diuretic hormone (Kwon et al., 2012; Kwon and Pietrantonio, 2013), and corticotropic releasing factor-like diuretic hormone (Jagge and Pietrantonio, 2008) have been synthesized. Localizing the expression of these receptors using immunohistochemistry would allow us to explore, first, if the receptor for the peptide is present, and second, whether there is regionalization of peptide action along the caecum.

5. Significance

Collectively, these studies provide novel information for caeca and midgut-specific actions of VA and NKA in *Drosophila melanogaster* and *Aedes aegypti*. First, I

have shown that both VA and NKA contribute to ion transport across the *Drosophila melanogaster* gut. Since transport of K^+ is altered by inhibition of VA or NKA, I propose that both ATPases contribute to the creation of favourable electrochemical gradients for the transport of ions across the *Drosophila melanogaster* gut. I also propose that the dependence of cation transport on VA activity is consistent with the Wieczorek model, in both *Drosophila melanogaster* and *Aedes aegypti*. Furthermore, in cells where NKA immunolocalization was not observed, like the distal cells of the *Aedes aegypti* gastric caecum, apical VA can work in tandem with an apically expressed cation:nH^+ antiporter to maintain low intracellular Na^+ concentrations. These findings may contribute to a new avenue of research, exploring the role of NKA in the insect gut. Since the resolution of the 'ouabain paradox' (Torrie et al., 2004), only a handful of studies have investigated the role of NKA in influencing ion transport in the osmoregulatory tissues (Hine et al., 2014; Jonusaite et al., 2013; Kolosov et al., 2018), however, apart from the studies presented in this thesis, the role of NKA in energizing ion transport in the insect gut epithelium remains to be investigated.

I have also demonstrated that the expression patterns and enzyme activities of VA and NKA differ when *Aedes aegypti* larvae are reared in freshwater or brackish water. It would be interesting to investigate if transferring larvae reared in freshwater in to brackish water, or vice versa, will have any effect on the expression patterns or enzyme activities of VA and NKA over the spans of hours or days. This would provide insights into how the larvae adapt to changing salinities in the wild; for example, larvae in tidal pools may face dilution of their surrounding media by rainfall, or an increase in salinity due to evaporation or inflow of seawater. 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 salinities can provide the foundation for development of novel larvicides.

Furthermore, this thesis provides some insight into the ion transport mechanisms of the gastric caecum, an organ that has been understudied in insects. More work is needed to fully understand the regulation of the gastric caecum of *Aedes aegypti*. I have demonstrated that 5-HT regulates the distal gastric caecum, however, the roles of other endocrine factors in stimulating ion transport also need to be investigated for both the distal and proximal regions, as outlined earlier. I hope that this work will open a broader avenue of research into the caecal ion transport mechanisms of other insect species as well.

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