# TRANSPORT OF H$^+$, NA$^+$ AND K$^+$ ACROSS THE POSTERIOR MIDGUT OF BLOOD-FED MOSQUITOES (*AEDES AEGYPTI)*

# TRANSPORT OF H$^+$, NA$^+$ AND K$^+$ ACROSS THE POSTERIOR MIDGUT OF BLOOD-FED MOSQUITOES (*AEDES AEGYPTI*)

By

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Title:     Transport of $H^+$, $Na^+$ and $K^+$ across the posterior midgut of blood-fed mosquitoes (*Aedes aegypti*)

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**Abstract**

Mosquitoes pose significant threats to human health because they act as vectors for disease causing viruses and protozoans. Indeed, *Aedes aegypti* is known as the Yellow Fever Mosquito because of its role as a vector for viral infections that kill thousands of people each year. A more thorough understanding of mosquito physiology will aid development of novel control strategies. Previous work on ion transport across the midgut has been focused primarily on larval *A. aegypti*, while research on the midgut of the adult stage is less complete. The posterior midgut of the adult female is of particular interest because it is used for the storage and digestion of the blood meal which is required for the production of eggs. This study used an array of electrophysiological methodologies such as the Scanning Ion Electrode Technique (SIET) in order to elucidate the patterns and mechanisms of $Na^+$, $H^+$ and $K^+$ transport across the posterior midgut at intervals during postprandial diuresis and digestion of the blood meal. Measurements of transepithelial potential indicated that the lumen was at its most negative (-13.2 mV) three hours after the blood meal and then gradually became less negative during the time course of digestion. $Na^+$ was absorbed (from lumen to bath) at all intervals after the blood meal (6 min, 30 min, 2h, 24 h); calculations of the electrochemical potential indicated that absorption required active transport. $H^+$ absorption at all times (6 min – 48 h) after the blood meal was also active (*i.e.* against the electrochemical gradient for $H^+$) and was greatly reduced by inhibition of carbonic anhydrase. $K^+$ transport across the midgut exhibited two distinct phases. During diuresis, luminal concentrations of $K^+$ were in the range 24 – 28 mM and secretion into the midgut was opposed by the electrochemical

gradient, indicating active transport. After diuresis, during blood meal digestion, concentrations of $K^+$ in the midgut contents were high (95 – 134 mM) and absorption of $K^+$ was favoured by the electrochemical gradient. $K^+$ absorption was sensitive to the channel blocker $Ba^{2+}$ during this period.

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make that long drive down from Ottawa to help me when I could no longer physically do

this on my own made me forever realize how lucky I am to have them.

**THESIS ORGANIZATION AND FORMAT**

This thesis is presented as a "sandwich thesis" with a general introduction and objectives of the research presented in chapter 1, followed by two research papers presented as chapter 2 and chapter 3, which are in the format of manuscripts to be submitted for publication in peer-reviewed journals. The last chapter is a general discussion of the findings of the project.

**Chapter 1:** General introduction and project objectives

**Chapter 2:** Postprandial changes in transport of $Na^+$, $H^+$ and $K^+$ across the posterior midgut of blood-fed mosquitoes (*Aedes aegypti*).

**Chapter 3:** Effects of ion transport inhibitors on transport of $Na^+$, $H^+$ and $K^+$ across the posterior midgut of blood-fed mosquitoes (*Aedes aegypti*).

**Chapter 4:** General discussion and future studies

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## Chapter 1:

**General Introduction**

 Mosquitoes are a serious health threat to the world's population causing over a million deaths annually (Isoe & Scaraffia, 2013). The majority of these deaths occur from parasites or viruses that are introduced into the host's blood stream when the mosquito is acquiring a blood meal. *Aedes aegypti* is a particularly dangerous species as it is the vector for a number of diseases such as yellow fever, dengue fever and chikungunya.

*Locating hosts and feeding*

 Only female mosquitoes spread disease because only they require blood in order to reproduce. Indeed, female mosquitoes have evolved many adaptations in order to become extremely efficient at locating hosts and feeding on blood. They detect hosts mainly by the use of chemical and visual cues. Their antennae are specifically designed to recognize $CO_2$ and 1-octen-3-ol being exhaled by the host and are also able to detect other odours in sweat. They also have heat sensors around their mouthparts that allow them to detect warm-blooded organisms while their compound eyes allow them to visualize potential hosts. Once a mosquito has found a host she will either prod around the skin looking for suitable blood vessels or bite the organism immediately. She bites by first inserting her long needle-like proboscis into the skin and then releasing saliva into the punctured blood vessel. The saliva is a mixture of secreted proteins that cause inflammation and help to prevent vasoconstriction, coagulation, platelet aggregation,

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angiogenesis as well as immune responses. It is during this deposition of saliva that the

majority of mosquito-borne parasites and viruses are introduced into the host.

*Blood components*

After the mosquito has prepared the blood vessel for feeding it will begin to ingest

a blood meal from the host into its posterior midgut for storage and digestion (Figure 1).

The blood meal is composed primarily of two different components; the plasma portion

which is high in water and $Na^+$ and the erythrocyte portion which is rich in intracellular

$K^+$ as well as the proteins which are the desired component of the meal. Studies of $^{14}$C-

protein metabolic labeling have shown that only 3% of the amino acids derived from

digestion of the blood meal proteins are allocated for oogenesis while the remainder is

converted into lipids, carbohydrates or are oxidized in order to provide energy (Zhou et

al., 2004). More than 60% of the amino acids are oxidized to carbon dioxide to provide

the energy needed for egg production (Briegel, 1985; Zhoe et al., 2004). The mosquito

doubles its body weight in less than two minutes during feeding, resulting in an array of

different physiological stresses on the mosquito (Beyenbach, 2012).

*Heat stress*

The first stress the mosquito experiences after ingestion of the blood meal is heat

stress. Mosquitoes are ectothermic animals while the majority of their suitable hosts are

endothermic. This means that when the blood meal is first ingested it retains a significant

amount of heat from the host which could be deleterious to the mosquito. Indeed, it has

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been shown that when *A. aegypti* drinks fresh blood from a human (37°C) it will begin to transcribe and translate heat shock protein 70 in response to this stress (Benoit et al., 2011). Heat shock protein 70 plays a role in supporting the normal digestion of the blood meal because when its expression is suppressed using RNA interference the rate of digestion is impaired and egg production decreases by 25% (Benoit et al., 2011).

*Diuresis*

Ingesting a large blood meal renders the mosquito barely maneuverable, easy to spot and thus at high risk of predation. The mosquito counteracts this problem by excreting 40% of the meal's water content within two hours (Williams et al., 1983). The $Na^+/K^+$-ATPase located on the basolateral membrane of the posterior midgut may contribute to diuresis (Sanders et al., 2003; Patrick et al., 2006). The sodium pump has been suggested to play a role in creation of osmotic gradients driving transcellular water transport through aquaporins 1, 2, 4 and 5 which are expressed in the midgut. Aquaporins 1, 2 and 4 are down regulated by 3 h after the blood meal, corresponding to the time by which excretion of the bulk of the blood meal-derived water has been excreted (Drake et al., 2010). Furthermore, the mosquito releases diuretic peptides from the brain such as aedeskinin and mosquito naturietic peptide into the hemolymph immediately after feeding; these then stimulate the fluid-secreting Malpighian tubules of the mosquito (Beyenbach, 2012). Through the activation of G-protein coupled receptors and the release of intracellular $Ca^{2+}$ stores, the paracellular resistance of the Malpighian tubules decreases tenfold leading to a "leaky epithelium" that allows a dramatic increase in the

transepithelial secretion of NaCl, KCl and water which are then eliminated by the

mosquito (Beyebach, 2012). The peak phase of diuresis occurs around six minutes after

the acquisition of a blood meal and steadily declines until two hours after feeding

(Williams et al., 1983). It is interesting to note that the mosquito does not completely

excrete the entire plasma/water portion of the meal, but begins to reduce the number of

water-transporting aquaporins in the posterior midgut after diuresis (Sanders et al., 2003;

Drake et al., 2010). It is believed that the mosquito retains this fluid that surrounds the

erythrocytes in the posterior midgut in order to serve as a template for the formation of

the peritrophic matrix (Sanders et al., 2003).

*The peritrophic matrix*

Soon after the end of diuresis the peritrophic matrix begins to form in the posterior

midgut. Its purposes are to protect the posterior midgut cells from mechanical and

chemical damage caused by the blood meal itself or the products of its digestion, as well

as to serve as a barrier against pathogenic infection (Pascoa et al., 2002; Villalon et al.,

2003). The peritrophic matrix surrounds the blood bolus and separates it from the midgut

epithelium in order to accomplish these beneficial tasks. However, this extra barrier

makes it difficult for digestive enzymes secreted by epithelial cells to reach the blood

meal and for digestion products to be absorbed by these same cells. When Villalon et al.

in 2003 disrupted the formation of the chitin-containing peritrophic matrix by the use of

chitinase and antibodies they found that the rate of blood meal digestion increased

indicating that the matrix was slowing down the digestion process. They suggested that

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the protection that the peritrophic matrix provided to the mosquito offsets the slower rate

of blood meal digestion associated with its presence (Villalon et al., 2003).

*Luminal pH*

As diuresis begins to level off and the peritrophic matrix starts to form, the pH

within the posterior midgut increases from 7.4 to 7.62 three hours after feeding (Billker et

al., 2000). It has been suggested that carbonic anhydrase plays a role in this pH increase

and when the posterior midgut was treated with the carbonic anhydrase inhibitors

methazolamide and acetazolamide it was found that luminal pH decreased (del Pilar

Corena et al., 2005). After three hours, however, pH levels within the lumen begin to

decrease for the remainder of the blood meal digestion, coincident with the upregulation

of the V-ATPase found on the apical membrane of the posterior midgut (Billker et al.,

2000; Sanders et al., 2003; Patrick et al., 2006). It is unknown why these pH changes

occur, but they may play a role in the activation or efficiency of the primary proteases

that cleave erythrocytes.

*Trypsin*

The two main proteases that are known to have a role in the blood meal digestion

of *A. aegypti* are early trypsin and late trypsin which releases approximately 2/3rds of

available amino acids from erythrocytes (Noriega & Wells, 1999; del Pilar Corena et al.,

2005). Early trypsin mRNA is not found in larvae or pupae, but begins to reach detectable

levels in the female 24 hours after eclosion while reaching maximal levels in two to three

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day-old adults (Noriega & Wells, 1999). The mRNA of late trypsin on the other hand is

not found in adults prior to blood feeding, but becomes transcribed only through a

complex regulatory system (Noriega & Wells, 1999). Although this system is not

completely understood, it appears that early trypsin is acting as a "sensor" that detects

whether or not the ingested meal is suitable for egg production and worth the energy-

consuming process of transcribing large amounts of late trypsin (Noriega & Wells, 1999;

Caroci & Noriega, 2003). Early trypsin translation was not triggered in mosquitoes

engorged with meals of saline, latex beads or sugar solutions and the meal was rapidly

excreted, indicating that mechanical distension of the posterior midgut is not the primary

activator of blood meal digestion (Noriega & Wells, 1999; Caroci & Noriega, 2003).

Instead it seems that free amino acids within the lumen are the primary activators of early

trypsin translation which is the beginning of the digestion process (Caroci & Noriega,

2003). These free amino acids are most likely cleaved by early trypsin and then "activate"

the transcription of late trypsin mRNA. When mosquitoes were fed a blood meal that

contained soybean trypsin inhibitor there was no transcription of late trypsin indicating

that the free amino acids cleaved by early trypsin were not present (Barillas-Mury et al.,

1995). However, when mosquitoes were fed a protein meal that had been partially

digested by bovine trypsin (which mimics the activity of early trypsin) transcription of

late trypsin occurred at control levels (Barillas-Mury et al., 1995). Interestingly, the

mosquito also uses these free amino acids as building blocks for the synthesis of late

trypsin as was shown by Schneider et al. in 1986 when they observed radioactive amino

acids being incorporated into the protease indicating that they may serve as a rate-limiting

step in the production of the enzyme. This unique regulatory system allows mosquitoes to control whether or not they commit the energy to digesting a blood meal and lets them remove low-quality meals quickly and efficiently so that they are able to acquire a more nutritious meal in the near future.

*$NH_4^+$*

After the requirements of the early digestion process have been achieved the mosquito begins to synthesize large amounts of late trypsin 8 to 36 hours post-blood feeding (Noriega & Wells, 1999). This is the time of greatest amino acid accumulation in the mosquito and represents a period of stress due to high rates of nitrogenous waste production. Amino acid catabolism releases the toxic by-product $NH_4^+$ (Scaraffia et al., 2005; Isoe & Scaraffia, 2013). $NH_4^+$ toxicity is minimized through a three-phase strategy of fixation, assimilation and excretion (Isoe & Scaraffia, 2013). In the posterior midgut $NH_4^+$ is fixed and assimilated into glutamine and alanine by glutamine synthase and alanine aminotransferase while in the fat body $NH_4^+$ is fixed and assimilated into glutamine and proline by glutamine synthase and pyrroline-5-carboxylate synthase (Isoe & Scaraffia, 2013). Proline and glutamine are known to be the most abundant amino acids in the mosquito hemolymph and during this time of high abundance proline can be utilized as an energy substrate for flight (Scaraffia & Wells, 2003; Scaraffia et al., 2005).

*Heme and iron*

After 24 hours the peak phase of digestion is over and the mosquito now has to deal with the toxic heme groups released from lysed erythrocytes. The free iron from these cells and metabolism of the heme group can produce reactive oxygen species which cause a multitude of deleterious effects. Indeed, this is when the chemical protection provided by the peritrophic matrix is particularly useful. At this time the peritrophic matrix reaches its maximal thickness and begins to turn a brownish colour due to its binding of free heme in the lumen (Pascoa et al., 2002). Moreover, it is also during this time frame that the iron-storing protein ferritin is upregulated in the posterior midgut which then acts as a further means of binding the potentially reactive free iron (Sanders et al., 2003). Control of heme toxicity in the gut is accomplished by a combination of the formation of heme aggregates, heme degradation, antioxidant enzymes and low molecular weight free radical scavengers. In addition, low molecular weight antioxidants and heme-binding proteins in the hemolymph such as ferritin act as a second line of defence, preventing generation of free radicals and thereby resulting in a low level of oxidative stress in the other tissues (Graca-Souza et al., 2006).

*Waste products*

At 72 hours post-blood feeding the mosquito has completed its digestive process and removes the remainder of the former blood meal in the form of water, dark brown specks of the indigestible blood residue hematin and colourless crystals of ammonium urate (Van Handel, 1975).

*Larval mosquitoes*

Larval *A. aegypti* do not acquire blood meals so the physiology of their digestive system differs from that of the adult female. The main diet of mosquito larvae is plant detritus which requires its own complex system for proper digestion (Boudko et al., 2001a; Boudko et al., 2001b; del Pilar Corena et al., 2002; Jagadeshwaran et al., 2010). When the larvae ingests its meal it first enters the foregut which has a pH ~7 then it enters the anterior midgut (pH ~11) and then finally moves into the posterior midgut (pH ~7.5) where the nutrients of the meal are absorbed (Boudko et al., 2001a; Boudko et al., 2001b; del Pilar Corena et al., 2002; Jagadeshwaran et al., 2010). The highly alkaline pH of the anterior midgut is one of the most alkaline environments known in any biological system and it is believed that this environment exists in order to sterilize food from pathogens and toxins, allow digestive enzymes to work at their optimal pH, enhance the assimilation of proteins and dissociate tannin-protein complexes (Boudko et al., 2001a; Boudko et al., 2001b; del Pilar Corena et al., 2002; Jagadeshwaran et al., 2010). This highly alkaline pH has been demonstrated to be mostly the result of carbonic anhydrase and a basolateral V-ATPase as ingestion of the carbonic anhydrase inhibitor acetazolamide and application of the V-ATPase inhibitor bafilomycin decreased lumen alkalization significantly (Boudko et al., 2001a; Boudko et al., 2001b; Patrick et al., 2006). In regards to the relatively more acidic posterior midgut it was found that serotonin could stimulate both ion transport as well as the transepithelial potential bringing it back to its normal lumen-positive levels of (+75 mV) after rundown (Clark et al., 1999). In contrast to the anterior midgut, the posterior midgut has a V-ATPase located on the apical membrane that could play a role in

the acidification of that region despite the fact that application of bafilomycin on the hemolymph side had no effect on $H^+$ gradients (Boudko et al., 2001a; Patrick et al., 2006).

*Electrophysiological methods*

Although much work has already been done uncovering the patterns and mechanisms of ion transport in the digestive systems of the larval mosquito, the patterns and mechanisms of ion transport in the adult female posterior midgut are unclear. Therefore, in order to elucidate these unknowns a number of electrophysiological techniques were used in this thesis.

*Transepithelial potential measurements*

Transepithelial potential can be measured by impaling the lumen of the isolated and intact posterior midgut bathed in physiological saline with a microelectrode backfilled with 3M KCl and measuring the voltage with respect to a reference electrode placed in the bath.

*Luminal ion concentrations*

Luminal ions concentrations were measured by placing the isolated midgut under paraffin oil and then rupturing it with a pair of forceps. An ion-selective and reference electrode placed into the luminal contents can then be used to provide a measurement of ion concentration.

*Calculation of electrochemical potential*

The electrochemical potential ($\Delta\mu$/F, in mV) of an ion indicates whether the

activity of an ion is at, above or below the activity consistent with passive equilibrium. It

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 (mM), $[\text{ion}]_{\text{bath}}$ is the concentration

of the ion in the bath (mM), $z$ is the valency and TEP is the transepithelial potential (mV).

A positive value of $\Delta\mu$/F indicates that the ion concentration in the midgut lumen is in

excess of equilibrium, i.e. net passive movement from the gut lumen to bath is favoured,

whereas a negative value indicates that the luminal ion concentration is below equilibrium

and that net passive movement from the bath to the gut lumen was favoured.

*Scanning Ion-Selective Electrode Technique*

The Scanning Ion-Selective Electrode Technique (SIET) is described in detail in

the methods of chapter 2.

SIET provides a particularly effective alternative for measuring transepithelial ion

fluxes in ion-transporting epithelia that are too small to be easily studied using Ussing

chambers. SIET measures ion gradients in the unstirred boundary layer that are caused by

ion-transporting epithelia. The ion-selective electrode is placed near the transporting

epithelium (~5 μm) to record a voltage which is then compared to another voltage

measured after the microelectrode is moved 50 μm further away. A concentration gradient

($\Delta C$) of the desired ion can calculated from this voltage gradient using the equation: $\Delta C =$

$C_B \times 10^{(\Delta V/S)} - C_B$, where $C_B$ represents the background ion concentration (the average of

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the concentrations at the inner and outer limits of microelectrode excursion) in $\mu$mol cm$^{-3}$;

$\Delta V$ represents the voltage gradient measured at the tissue surface less the voltage gradient

at the reference site in $\mu$V; S is the slope of the electrode in $\mu$V. The concentration

difference can then be converted to the corresponding flux (mol cm$^{-2}$ s$^{-1}$) using Fick's

Law: $J = D\Delta C/\Delta X$, where D is the diffusion coefficient of the ion of interest and $\Delta X$ is

the excursion distance. Ion fluxes are reported as picomoles per square centimeter per

second (pmol cm$^{-2}$ s$^{-1}$) in this thesis. When an ion flux was positive it indicates that the

epithelium is absorbing the ion from the lumen to the bath whereas a negative flux

indicates that the epithelium is secreting the ion from the bath to the lumen. A schematic

diagram of the SIET process and setup is shown in figure 2.

*Objectives*

The goals of this study were to elucidate the patterns and mechanisms of ion

transport in the posterior midgut during diuresis and blood meal digestion. The three

questions to be answered were:

1: What are the patterns of Na$^+$, K$^+$ and H$^+$ transport across the posterior midgut during

diuresis and the digestion of the blood meal?

2: What are the electrochemical gradients of these three ions across the posterior midgut

during the time course of diuresis and blood meal digestion? Calculation of the

electrochemical gradients is important in determining whether measured fluxes represent

passive or active transport.

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3: What are the mechanisms of transport for these three ions across the posterior midgut during diuresis and blood meal digestion?

*Hypotheses*

Considering that much of the $Na^+$-rich plasma of the blood meal is excreted quickly during diuresis it is believed that there will be higher rates of $Na^+$ absorption during the peak phase of diuresis than during post-peak and late phases of diuresis (Williams et al., 1983). It is known that there is a $Na^+/K^+$-ATPase on the basolateral side of the posterior midgut which could play a role in this $Na^+$ transport and could be sensitive to the pharmacological inhibitor ouabain (Patrick et al., 2006).

Since the lumen of the posterior midgut becomes more basic during the first three hours after the acquisition of a blood meal it is expected that rates of $H^+$ absorption or secretion should be low during this time-frame (Billker et al., 2000). Once the lumen begins to turn more acidic following the first three hours post-feeding $H^+$ secretion should then increase as $H^+$ is being pumped into the lumen by the V-ATPase located on the apical membrane (Patrick et al., 2006). Treatment with the V-ATPase inhibitor bafilomycin could decrease the rate of this $H^+$ secretion. As digestion enters its peak phase from 8-36 hours post-feeding $H^+$ transport could become absorptive as metabolites are oxidized to $CO_2$ which could then be converted to free $H^+$ by carbonic anhydrase (Isoe & Scaraffia, 2013). Absorption of $H^+$ could be mediated by the $Na^+/H^+$ antiporter NHE3 known to exist on the basolateral membrane which has been shown to be insensitive to the pharmacological inhibitor amiloride (Pullikuth et al., 2006).

I hypothesize that $K^+$ will be secreted into the lumen during diuresis as the $Na^+/K^+$-ATPase is transporting $Na^+$ out into the hemolymph while transporting $K^+$ inwards (Patrick et al., 2006). Once blood meal digestion begins its peak phase with the introduction of late trypsin, $K^+$ concentrations within the lumen should increase as intracellular $K^+$ is being released by lysis of the erythrocytes. The luminal concentration of $K^+$ should then decrease as $K^+$ transport becomes absorptive into the hemolymph which would then allow the excess ion to be excreted.

**Figure 1.** A typical blood-fed *A. aegypti* posterior midgut. Anterior is to the left and the Malpighian tubules can be seen joining the gut at the midgut-hindgut junction.
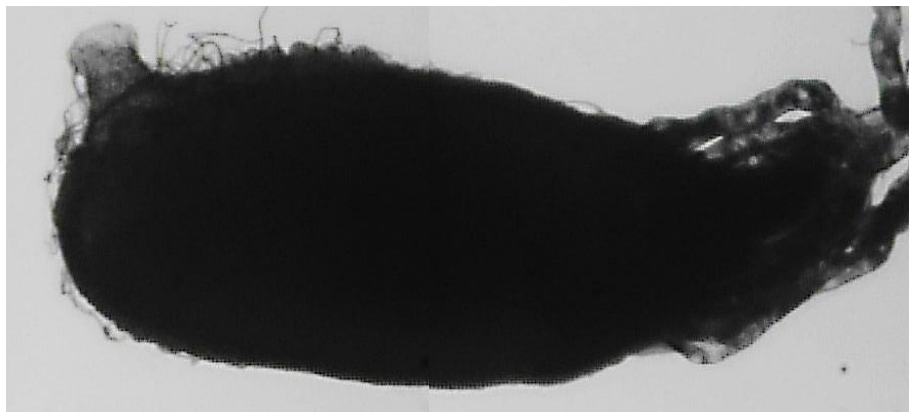
**Figure 2.** Measurement of $K^+$ fluxes using the Scanning Ion-Selective Electrode Technique (SIET). ISM, ion-selective electrode. See text for further details.
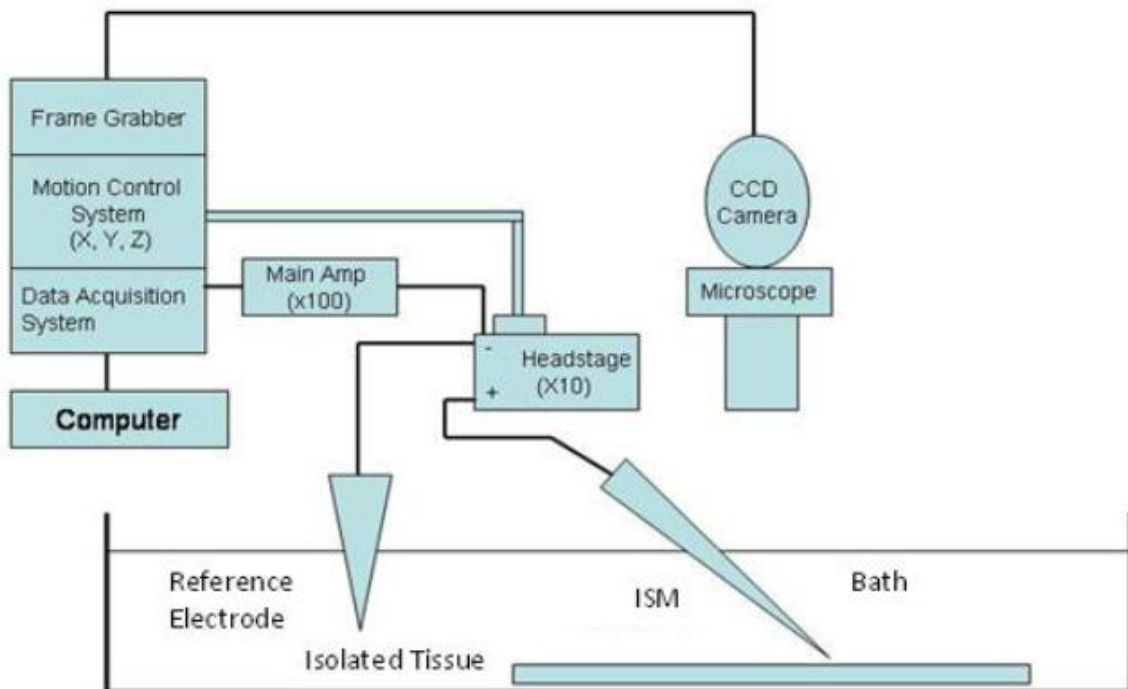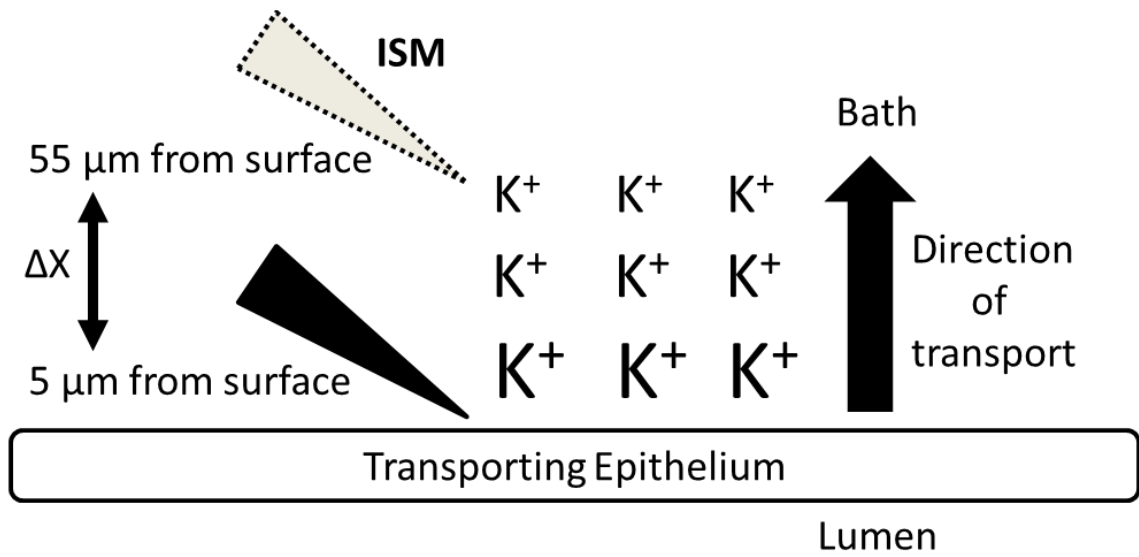
# Chapter 2

## Postprandial changes in transport of $Na^+$, $K^+$ and $H^+$ across the posterior midgut of blood-fed mosquitoes (*Aedes aegypti*)

Evan Kendal Pacey and Michael J. O'Donnell

**Abstract**

Following ingestion of a blood meal, the adult female mosquito undergoes a massive diuresis during which $Na^+$, $Cl^-$ and water are secreted at high rates by the Malpighian tubules. In the hours following completion of diuresis, digestion of the $K^+$-rich blood cells provides a source of amino acids for proteins in the developing eggs. Although the transport of inorganic ions by the Malpighian tubules of blood-fed mosquitoes has been extensively characterized, relatively little is known of the epithelial transport mechanisms responsible for movement of $Na^+$, $H^+$, and $K^+$ across the posterior midgut. In this paper we have used the Scanning Ion Electrode Technique (SIET) to measure transport of $K^+$, $Na^+$ and $H^+$ across the posterior midgut at intervals during postprandial diuresis and subsequent digestion of the blood meal. We have also measured luminal concentrations of $Na^+$ and $K^+$ and the transepithelial electrical potential at the same time points and have calculated the electrochemical potentials for $Na^+$, $K^+$ and $H^+$ across the midgut. SIET measurements reveal absorption (lumen to bath) of $Na^+$ and $H^+$ and secretion of $K^+$ for the first two hours after blood-feeding. By 24 hours after the meal, absorption of $Na^+$ and $H^+$ remains active while there is an electrochemical gradient favouring absorption of $K^+$.

**Introduction**

Adult female *Aedes aegypti* undergo rapid diuresis following ingestion of a blood meal. Diuresis removes excess $Na^+$ and water, thereby reducing the flying weight of the mosquito so that it is less susceptible to predation (Williams et al., 1983). Diuresis in *A. aegypti* peaks a few minutes after the blood meal and urine flow rates remain elevated for ~30 minutes (Stobbart, 1977; Williams et al., 1983). Following diuresis, erythrocytes within the posterior midgut are digested by two proteases known as early and late phase trypsin (Noriega & Wells, 1999). The amino acids in the blood meal proteins are converted into the amino acids incorporated into the mosquito eggs over the next 36 hours; 80% of protein digestion is completed within 24 h of feeding (Briegel & Lea, 1975). Small amounts of what is termed early trypsin are synthesized $4 - 6$ h after the blood meal, whereas large amounts of late trypsin are synthesized between 8 and 36 h after the blood meal (Noriega & Wells, 1999). Lysis of the cell membranes during digestion will also tend to release intracellular $K^+$ into the midgut lumen.

Although the secretion of fluid and ions during diuresis have been studied extensively (reviewed by Beyenbach, 2003), relatively little is known of the patterns and mechanisms of inorganic ion transport across the posterior midgut during and after diuresis. Immunohistochemical studies have revealed the locations of the $Na^+/K^+$-ATPase, the V-type $H^+$-ATPase in the adult midgut (Patrick et al., 2006), but the roles of these transporters in movement of ions across the midgut after the blood meal have not been determined. Measurements with pH microelectrodes have shown that the pH of the blood in the posterior midgut increases from 7.4 before ingestion to 7.63 by 3 hours after

ingestion, then declines to pH 7.4 by 48 hours after the blood meal (Billker et al., 2000).

It has been suggested that carbonic anhydrase activity contributes to the increase in pH in

the gut during this digestion (del Pilar Corena et al., 2005).

The purpose of this study is to examine transport of $H^+$, $Na^+$ and $K^+$ across the

midgut during specific time intervals after blood feeding (6 and 30 minutes, 1, 2, 3, 6, 12,

24, 48 and 72 hours). The intervals were selected to encompass both diuresis and the

periods during and after digestion of the blood meal. Rates of $H^+$, $Na^+$ and $K^+$ across the

posterior midgut have been measured using the scanning ion-selective electrode technique

(SIET). We have also measured $Na^+$ and $K^+$ concentrations in the blood meal and

transepithelial electrical potential at the same intervals, and have used these data to

calculate the net electrochemical potential for $Na^+$, $K^+$ and $H^+$ across the posterior midgut.

In conjunction with measurement of the direction and rate of transport for the each of

three ions, these calculations allow us to determine whether the transport of each is

consistent with thermodynamically active or passive mechanisms.

**Materials and Methods**

*Insect rearing*

*A. aegypti* larva were raised in an incubator (28°C; photoperiod 12D:12L) and fed

1 gram of brewer's yeast and 1 gram of liver extract dissolved in 50 mL of deionized

water every second day. Individual pupae were transferred to mosquito cages (24°C,

photoperiod 12D:12L; relative humidity 70-90%) and allowed to mature into adults that

were fed a solution of 10% sucrose.

Adult female mosquitoes were fed heated (37°C) sheep's blood in Alsever's solution (1:1) at pH 7.4 (Cedarlane Laboratories, Burlington, ON). Alsever's solution contains (in mM): 7.2 NaCl, 27.2 trisodium citrate dihydrate, 2.6 citric acid monohydrate and 11.4 glucose. Blood was placed in a small beaker covered with Parafilm™ through which the mosquitoes inserted their mouthparts.

*Physiological salines and dissection*

Mosquitoes were dissected using spring scissors with a 5 mm cutting edge and Dumont #5 forceps (Fine Science Tools, North Vancouver, BC) under *Aedes* saline containing (in mM): 150 NaCl, 3.4 KCl, 1.7 $CaCl_2$-$2H_2O$, 1.8 $NaHCO_3$, 1 $MgSO_4$-$7H_2O$, 25 HEPES, 5 Glucose. Saline pH was adjusted to 7.1. For measurement of $Na^+$ transport by SIET, $Na^+$ concentration was reduced to 20 mM by equimolar substitution with N-methyl-D-glucamine. Posterior midguts were removed from blood-fed adults by removal of the head and abdominal cuticle and cutting at the junction of the thorax and abdomen. Posterior midguts were then transferred to a 35 mm Petri dish filled with saline. Petri dishes were pre-coated with 70 µl droplets of 62.5 µg $ml^{-1}$ poly-L-lysine then air dried to promote adhesion of the gut to the bottom of the dish.

*Measurement of transepithelial potential and luminal $Na^+$ and $K^+$ concentrations*

Isolated midguts were ruptured using Dumont #5 forceps (Fine Science Tools, Vancouver, Canada) under paraffin oil and concentrations of $Na^+$ and $K^+$ were measured using ion-selective microelectrodes. Micropipettes used for measurements under paraffin

oil were pulled from unfilamented borosilicate glass capillaries (1.5 mm o.d, 0.84 mm i.d; WPI, Sarasota, Fl) using a vertical puller (Narishige, Tokyo, Japan) and silanized with 0.2 μl dichlorodimethylsilane placed in a 10 cm glass petri dish and inverted over batches of 14 micropipettes on a hot plate at 200 ℃. This low level of silanization rendered the glass sufficiently hydrophobic to retain the hydrophobic ionophore cocktail and prevent its displacement by capillary rise of aqueous solutions, but not so hydrophobic as to allow capillary rise of paraffin oil into the tip when the microelectrode was used under paraffin oil. $Na^+$-selective microelectrodes were calibrated in solutions of 15 mM NaCl/135 mM KCl and 150 mM NaCl. $K^+$-selective microelectrodes were calibrated in solutions of 3 mM KCl/147 mM NaCl and 30 mM KCl/120 mM NaCl. Slopes (mV) for a 10-fold change in ion concentration were (mean ± S.E.M.) 57.5±0.2 ($N$=10) for $K^+$-selective microelectrodes and 62.8±0.1 ($N$=10) for $Na^+$-selective microelectrodes.

Transepithelial potential was measured by impaling the lumen of the intact isolated gut in physiological saline with a microelectrode pulled from filamented 1.5 mm o.d., 0.84 mm i.d borosilicate capillary glass (WPI, Sarasota, Fl ) mounted on a hydraulic micromanipulator (Narishige, Tokyo, Japan). Tip resistances of the microelectrodes backfilled with 3M KCl were > 20 MΩ.

*Calculation of Electrochemical Potentials*

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

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

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Where [ion]$_{lumen}$ is the concentration of the ion in the lumen (mM), [ion]$_{bath}$ is the concentration of the ion in the bath (mM), $z$ is the valency and TEP is the transepithelial potential (mV). A positive value indicates a luminal ion concentration in excess of equilibrium, i.e. net passive movement from gut lumen to bath is favoured. A negative value indicates a luminal ion concentration below equilibrium, i.e. net passive movement from bath to gut lumen is favoured.

*Scanning Ion Electrode Technique (SIET)*

Ion-selective microelectrodes used for SIET were prepared as described previously (Donini & O'Donnell, 2005). Unfilamented borosilicate glass capillaries (1.5 mm o.d, 0.84 mm i.d; WPI, Sarasota, Fl) were pulled to tip diameters of 3 – 5 μm on a P-97 Flaming-Brown micropiptette puller (Sutter Instrument, Novato, CA), silanized with 75 μl of N, N-dimethyltrimethylsilylamine placed in a 10 cm glass petri dish and inverted over batches of 20 micropipettes at 200 °C for 20 min and stored in a desiccator. Na$^+$-selective microelectrodes were made by first backfilling the micropipettes with 150 mM NaCl and then tip filling them with a column (~200 μm) of Na$^+$ ionophore cocktail, which consisted of 10% Na$^+$ ionophore X (Fluka), 89.75% nitrophenyl octyl ether and 0.25% sodium tetraphenylborate (Messerli et al., 2008). The Na$^+$ ionophore cocktail is more selective for Na$^+$ relative to K$^+$ by a factor of $10^{2.6}$ (Messerli et al., 2008). Na$^+$-selective microelectrodes were calibrated in solutions of 15 mM NaCl/135 mM KCl and 150 mM NaCl. K$^+$-selective microelectrodes were made by first backfilling the micropipettes with 150 mM KCl and then tip filling them with a column (~200 μm) of

$K^+$ ionophore I, cocktail B (Sigma-Aldrich, St. Louis, MO). The $K^+$ ionophore cocktail is more selective for $K^+$ relative to $Na^+$ by a factor of $10^{3.9}$ (Amman et al., 1987). $K^+$-selective microelectrodes were calibrated in solutions of 3 mM KCl/147 mM NaCl and 30 mM KCl/120 mM NaCl. Slopes (mV) for a 10-fold change in ion concentration were (mean $\pm$ S.E.M.) 57.17$\pm$0.2 ($N$=10) for $K^+$-selective microelectrodes and 60.53$\pm$0.2 ($N$=10) for $Na^+$-selective microelectrodes. $H^+$-selective microelectrodes were backfilled with a solution of 100 mmol $l^{-1}$ NaCl and 100 mmol $l^{-1}$ sodium citrate at pH 6, then tip-filled with a 300–500 μm column of $H^+$ ionophore I, cocktail B (Fluka). $H^+$-selective microelectrodes calibrated in saline buffered with 25 mmol $l^{-1}$ Hepes gave slopes of 58$\pm$0.3 ($N$=10) mV per pH unit in the range pH 6.5 – pH 7.5. Reference electrodes were constructed from 10 cm borosilicate glass capillaries that were bent 1 cm from the end at a 45 degree angle to facilitate placement in the sample dish. Capillaries were filled with boiling *Aedes* saline solution containing 3-5% agar and were stored at 4°C in *Aedes* saline.

Gradients in ion concentration created in the unstirred layer by ion transport across the posterior midgut were measured by SIET. Concentration gradients were then converted into fluxes using the Fick equation. SIET is particularly useful for spatial and temporal analysis of ion transport across epithelial preparations which show regional differentiation (Rheault & O'Donnell, 2001) or are too small for the use of Ussing chambers. The microelectrode was positioned by an orthogonal array (X, Y, Z) of computer-controlled stepper motors and moved between two points at each measurement site. The shape of dissected midgut can be approximated as a prolate spheroid and the Z-

position was adjusted during SIET scans so that measurements were taken along the

equator of the major axis. Measurements of voltage gradients were made by moving the

microelectrode tip perpendicularly from the midgut surface between two points separated

by 50 μm. Initially, the microelectrode tip was positioned within 5 μm of the tissue

surface, representing the inner limit of the microelectrode's 50 μm excursion. Positioning

was followed by a 4.0 second wait period during which no measurements were made to

allow the re-establishment of ion gradients at the tissue surface following the localized

disturbance of the microelectrode movement. A wait time of at least 3 seconds is

sufficient to allow the gradients to fully re-establish (Naikkhwah & O'Donnell, 2012).

The voltage at the microelectrode tip was recorded for 0.5 seconds following the wait

period. The microelectrode was then moved at 200 μm s$^{-1}$ to a position at the outer limit

of the 50 μm range where another wait and sample period was completed. The move,

wait, and sample cycle at both extremes of the microelectrode excursion was completed

in 9.5 seconds and was repeated three times, requiring a total of 28.5 seconds to measure

the voltage gradient at each tissue site. Depending on the size of the blood-distended

midgut, 14 – 32 sites were scanned at intervals of 60 μm. Preliminary measurements

indicated that there were no significant differences in $Na^+$, $K^+$ or $H^+$ in anterior versus

posterior regions of the posterior midgut. The microelectrode tip was moved > 2500 μm

perpendicularly away from the tissue surface to a reference site following completion of

the scans of each midgut. At this site, which is sufficiently distant from the tissue that the

influence of epithelial ion flux is negligible, the voltage gradient was recorded across the

microelectrode's 50 μm excursion in the same manner as when making measurements at

the tissue surface. Voltages at the reference site were subtracted from the voltage

gradients at the measurement sites to correct for any voltage drift during scanning. The

corrected voltage difference between the two limits of the microelectrode excursion ($\Delta V$)

was then used to calculate a corresponding concentration difference ($\Delta C$) in $\mu mol\ cm^{-3}$.

Concentration gradients for $Na^+$ and $K^+$ in the unstirred layer were calculated using the

equation:

$$\Delta C = C_B \text{ x } 10^{(\Delta V\ /S)} - C_B$$

where $C_B$ represents the background ion concentration (the average of the concentrations

at the inner and outer limits of microelectrode excursion) in $\mu mol\ cm^{-3}$; $\Delta V$ represents the

voltage gradient measured at the tissue surface less the voltage gradient at the reference

site in $\mu V$; S is the slope of the electrode in $\mu V$. The concentration difference is then

converted to flux ($mol\ cm^{-2}\ s^{-1}$) using Fick's Law: $J = D\Delta C/\Delta X$, where D is the diffusion

coefficient of the ion of interest (Donini & O'Donnell, 2005) and $\Delta X$ is the excursion

distance. In this paper, ion fluxes are reported as picomoles per square centimeter per

second ($pmol\ cm^{-2}\ s^{-1}$). For each posterior midgut, a single flux (based on 3 replicate

measurements at each of 14 – 32 sites) was calculated at each interval after feeding. Scans

were completed within ~20 minutes. Preliminary measurements showed that fluxes were

stable for > 45 minutes after dissection for all three of the ions measured ($N = 3$

preparations each; data not shown).

Proton fluxes were corrected because protons may diffuse either in a free state or

bound to the buffers (HEPES and bicarbonate) present in the saline. The actual $H^+$ flux

from a source is therefore the sum of the measured free $H^+$ flux and the unmeasured $H^+$

flux moving as $H^+$ bound to buffer. Corrections for the effects of buffers on $H^+$ flux were made using the equations provided by Messerli et al., (2006).

*Statistical Analyses*

Data are expressed as mean ± S.E.M. for the indicated number of preparations. For SIET measurements, each mean value was based on measurements at 14 – 32 sites at 60 μm along the midgut, and there were three replicate measurements at each site. Significance of differences between means of fluxes measured at different times after the blood meal was tested by means of an ANOVA with a Tukey's multiple comparisons test using $P < 0.05$ as the level of significance. Graphing of data and statistical testing was done using GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA).

**Results**

*Transepithelial potential (TEP)*

The lumen of the posterior midgut was at a negative potential with respect to the bathing saline at all intervals after the blood meal (Fig. 1). TEP increased from -4.6 mV at 6 minutes after the blood meal to -13.2 mV by 3 h. The TEP then declined from -9.2 mV at 6 h to -2.4 mV by 72 h after the blood meal. We also measured the TEP in saline in which the $Na^+$ concentration was reduced to 20 mM by equimolar replacement with N-methyl D-glucamine. The values of -8.2 mV at 30 min and -6.0 ±1.3 mV ($N = 3$) at 24 h fall within the 95% confidence intervals around the corresponding mean values in saline containing 151.8 mm saline (Student's t-test, $P > 0.05$).

*Concentrations of Na$^+$ and K$^+$ in the contents of the posterior midgut*

The concentration of Na$^+$ in the posterior midgut contents was lower than in the

bathing saline (151.8 mM), and typical values for sheep blood on which the mosquitoes

were fed (140 mM; Curran-Everett et al., 1988). Na$^+$ and K$^+$ concentrations measured by

ion-selective microelectrodes in the 1:1 mixture of sheep blood and Alsever's solution

were $121.3 \pm 4.3$ mM and $15.8 \pm 1.9$ mM, respectively ($N = 5$ samples). Na$^+$

concentration of the gut contents did not change between 6 min and 2 h after the blood

meal, but dropped from 140 mM at 2 h to 94 mM at 24 h (Fig. 2A). Luminal K$^+$

concentrations (27 – 33 mM) did not change significantly in the first 3 hours following

the blood meal, but then rose to 95 mM at 6 h and 134 mM at 12 h after the blood meal

(Fig. 2B).

*Net electrochemical potentials for Na$^+$, K$^+$ and H$^+$*

The net electrochemical potentials ($\Delta\mu/F$, mV) for Na$^+$, K$^+$ and H$^+$ were calculated

using measured transepithelial potentials and concentrations of Na$^+$ and K$^+$ in the midgut

contents and the previously reported values of luminal pH (Billker et al., 2000). A

positive value of $\Delta\mu/F$ for the ion indicates that passive movement from midgut lumen to

bath is favoured. In saline containing 151.8 mM Na$^+$ and 3.4 mM K$^+$ at pH 7.1, the

electrochemical potential for K$^+$ was positive at all points after the blood meal,

particularly after the increase in midgut content K$^+$ concentration 6 h or more after the

blood meal (Fig. 3). The electrochemical potential for Na$^+$ was -10 to -13 mV for midguts

bathed in saline containing 151.8 mM Na$^+$. However, for saline containing 20 mM Na$^+$,

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as used in SIET measurements described below, the value of $\Delta\mu/F$ shifts to 39 mV at 30 min and 34 mV at 24 h after the blood meal, indicating that passive movement from midgut lumen to bath is favoured. The electrochemical potential for $H^+$, based on our measurements of transepithelial potential and the pH of the midgut lumen determined by Billker et al. (2000), was -25 to -40 mV. The latter values indicate that passive entry of $H^+$ from bath to lumen is favoured.

*Transepithelial fluxes of $Na^+$, $K^+$ and $H^+$*

Sodium was absorbed from midgut lumen to the bath for preparations bathed in saline containing 20 mM $Na^+$ (Fig. 4A). Preliminary measurements showed that it was not feasible to measure $Na^+$ fluxes in saline containing 151.8 mM $Na^+$; as a consequence of such a high background concentration of $Na^+$ the change in unstirred layer $Na^+$ concentration produced by $Na^+$ transport across the posterior midgut epithelium was too small to be reliably measured by the SIET technique. There was no significant decline in $Na^+$ transport over the first 2 h after the blood meal (ANOVA, Tukey's multiple comparisons test). At 24 h after the blood meal, the value of $Na^+$ transport was 273.3 pmol $cm^{-2}$ $s^{-1}$ and was not significantly different from $Na^+$ transport during the first 2 hours after the blood meal (ANOVA, Tukey's multiple comparisons test). By contrast, $K^+$ was secreted for the first 2 hours after the blood meal, then absorbed into the bathing saline, and there was a linear trend ($P < 0.001$) towards increasing rates of absorption, peaking at 71 pmol $cm^{-2}$ $s^{-1}$ by 72 h after the blood meal. A one-way ANOVA followed by Tukey's multiple comparison test showed that the $K^+$ flux at 72 h was significantly

different than at earlier times. We also compared the values at 6 min, 30 min, 1, 2 and 3 h

to all fluxes at 6 h or longer after the blood meal. The rationale for these planned

comparisons was two-fold. Firstly, we expected $K^+$ transport to be lower during the

period of diuresis, which is generally complete within 2-3 h after the blood meal

(Williams et al., 1983). Secondly, the $K^+$ concentration in the midgut contents (Fig. 2B)

increased significantly at 6 h, coincident with the rise in late phase trypsin synthesis

(Noriega & Wells, 1999). The results of uncorrected Fisher's Least Squares Difference

tests indicated significant differences ($P < 0.05$) between the $K^+$ fluxes at 6 min, 30 min 1

and 2 h after the blood meal from those measured at 6, 12, 24, 48 and 72 h. There was a

trend towards increasing $H^+$ absorption, from ~50 pmol $cm^{-2}$ $s^{-1}$ for the first 6 h after the

blood meal, to a peak of 193 pmol $cm^{-2}$ $s^{-1}$ by 24 h (ANOVA, Tukey's multiple

comparisons test).

**Discussion**

The results of this study provide new information on the temporal patterns of $Na^+$,

$K^+$ and $H^+$ transport across the posterior midgut of blood-fed mosquitoes. We have also

calculated electrochemical potentials from measurements of transepithelial potential and

ion concentrations in the gut contents at the same time intervals. Taken together, the data

permit assessment of the active or passive nature of transepithelial $Na^+$, $K^+$ and $H^+$

transport across the midgut during diuresis and subsequent protein digestion. Figure 5

presents a schematic diagram showing the rates of ion transport and the corresponding

electrochemical potentials at 3 times after the blood meal: 6 min, 2 h and 24 h.

$H^+$ absorption occurs against an opposing electrochemical potential throughout the period of diuresis and blood cell digestion (Figs. 3, 5). It is important to note that our calculations of the electrochemical potential for $H^+$ are estimates, based on our measurements of transepithelial potential and the gut lumen pH values reported in Billker et al., (2000). Although the latter authors used mouse blood (pH 7.4), we used a 1:1 mixture of sheep blood:Alsever's solution which was also at pH 7.4. $H^+$ absorption across the midgut is unlikely to be a direct consequence of V-ATPase activity because immunohistochemical studies have indicated an apical location for the protein, consistent with transport of $H^+$ into the gut lumen (Patrick et al., 2006). It will be of interest in subsequent studies to examine the effects of inhibitors of transporters such as the V-ATPase and sodium proton exchangers (NHEs) on midgut $H^+$ transport. In addition, given the dependence of midgut alkalization on carbonic anhydrase activity (del Pilar Corena et al., 2005), measurement of $H^+$ fluxes in the presence of carbonic anhydrase inhibitors such as acetazolamide will be of use in evaluating the role of carbonic anhydrase in providing a source of $H^+$ for transport.

There was no significant change in the rate of transport of $Na^+$ or the concentration of $Na^+$ over the first 24 h after the blood meal. The concentration of $Na^+$ in the gut contents at 24 h after the blood meal was 94 mM, below the concentrations of 124 mM – 140 mM measured during diuresis, but still above the level of < 10 mM in many mammalian cells (Lee, 1981). This suggests that not all of the extracellular fluid in the blood meal, which contains high levels of $Na^+$, is eliminated during diuresis. In addition, it is worth noting that sheep erythrocytes contain relatively high concentrations of $Na^+$

(37 – 41 mM; Tosteson and Hoffman, 1960). High rates of $Na^+$ absorption after completion of diuresis may thus represent $Na^+$ derived both from the extracellular fluid and from intracellular sources of $Na^+$ as the blood cells are digested.

It is important to point out that for preparations bathed in saline containing 20 mM $Na^+$ the estimated electrochemical gradient (34 to 39 mV, lumen positive) favours passive movement of $Na^+$ from posterior midgut lumen to the bath (Figs. 3, 5). The electrochemical potential for hemolymph containing 96 mM $Na^+$ (Williams et al., 1983) is estimated to be ~2 mV, inside positive, assuming that the transepithelial potential remains unchanged from the values we have measured in 20 mM – 151.8 mM $Na^+$. This estimate suggests that, *in vivo*, $Na^+$ is close to electrochemical equilibrium and that either active or passive transport mechanisms, or both, may contribute to net $Na^+$ absorption. In this context, subsequent studies examining the effects of the inhibitor ouabain on $Na^+$ absorption will be of interest, given that previous immunohistochemical studies have identified the presence of the $Na^+/K^+$-ATPase in the basolateral membrane of the posterior midgut (Patrick et al., 2006).

$K^+$ is secreted across the posterior midgut at low rates in the first 2 hours after the blood meal. Given that the electrochemical potential favours passive absorption of $K^+$, this finding suggests the operation of some form of active transporter, such as the $Na^+/K^+$-ATPase (Figs. 3, 5). The concentration of $K^+$ in the gut contents (24 – 33 mM) is above that in the blood meal (15.8 mM). The difference may reflect a low level of blood cell lysis in response to early phase trypsin, plus the effects of inward transport of $K^+$. The shift from $K^+$ secretion to absorption at 3 h after the blood meal coincides with the

introduction into the posterior midgut of late phase trypsin, which is known to digest the

majority of the blood meal, releasing 2/3rds of available amino acids (Noriega & Wells,

1999; del Pilar Corena et al., 2005). Cell lysis during the period of late phase trypsin

secretion will result in release of intracellular $K^+$ from the blood cells. Indeed, $K^+$

concentrations in the gut contents were at their highest (Fig. 2B) when trypsin levels were

approaching their peak. Rates of $K^+$ absorption from gut lumen to bath were also highest

during this period (> 6 h). The electrochemical potential for $K^+$ favours passive

movement from gut lumen to bath (Figs. 3, 5), raising the possibility that $K^+$ channels

may contribute to the $K^+$ absorption across the midgut epithelium. It is worth noting that

the rate of $K^+$ absorption is ~1/3 the rate of $Na^+$ absorption at 24 h after the blood meal.

This may reflect the favourable electrochemical potential for $Na^+$ absorption when the

posterior midgut is bathed in saline containing 20 mM $Na^+$. In saline containing $Na^+$ at

the concentration found in the hemolmyph (96 mM), it seems likely that the rate of $Na^+$

absorption would be several fold lower and less than that of $K^+$, consistent with the gut

concentrations of the two ions at 24 h after the blood meal.

Future electrophysiological studies can provide further insight into the mechanisms of

$Na^+$, $H^+$ and $K^+$ transport across the midgut of blood-fed mosquitoes. In addition to

analyses of the effects of putative transport blockers on ion fluxes measured by SIET, it

will be revealing to determine the effects of changes in bathing saline ion concentrations

($Na^+$, $K^+$, $Cl^-$) on transepithelial potential. Such measurements would be of use in

estimating the contribution of conductive pathways (e.g. $K^+$ channels) to transepithelial

transport. In addition, little is known of the effects of diuretic factors on ion transport

across the posterior midgut. Mosquito natriuretic factor and leucokinin both stimulate fluid secretion by the Malpighian tubules (Beyenbach, 2003), and it would be of interest to determine the effects of these peptides on $Na^+$ transport across the posterior midgut.

**Figure captions**

**Figure 1.** Transepithelial potential of the posterior midgut of *A. aegypti* at intervals following a blood meal. Letters denote significant differences between time points (ANOVA; $P<0.05$). The height of the bar and the vertical lines indicate the mean + S.E.M. $N = 5$-$7$ blood fed adult females for each time interval.

**Figure 2.** Concentrations of $Na^+$ and $K^+$ in the contents of the posterior midgut of *A. aegypti* at intervals following a blood meal. The height of the bar and the vertical lines indicate the mean + S.E.M. ($N = 3$-$7$ blood fed adult females for each time interval). Letters denote significant differences between time points (ANOVA; $P<0.05$).

**Figure 3.** Electrochemical potential ($\Delta\mu/F$, mV) for $Na^+$, $K^+$ and $H^+$ at intervals after the blood meal. Values were calculated using the transepithelial potential measurements in Fig. 1, gut content $Na^+$ and $K^+$ concentrations (Fig. 2) and published values of luminal pH (Billker et al., 2000). A positive value indicates that passive transport from gut lumen to bath is favoured. A negative value indicates that passive transport from the bath to gut lumen is favoured.

**Figure 4.** Fluxes of $Na^+$, $H^+$ and $K^+$ across the posterior midgut of *A. aegypti* at intervals after the blood meal. Positive fluxes indicate ion absorption from the lumen to the hemolymph while negative fluxes indicate ion secretion from the hemolymph to the lumen. The height of the bars and the vertical lines indicate the mean + S.E.M. ($N$ = 3-7 blood fed adult females for each time interval). Letters denote significant differences between time points (ANOVA; $P<0.05$). Additional statistical tests for $K^+$ fluxes at different times points are described in the text.

**Figure 5.** Schematic diagram summarizing electrochemical potentials and measured fluxes for $Na^+$, $H^+$ and $K^+$ across the posterior midgut at A) 6 minutes, B) 2 hours and C) 24 hours after the blood meal. The width of the open arrows is proportional to the magnitude of the electrochemical potential.

**Figure 1:**
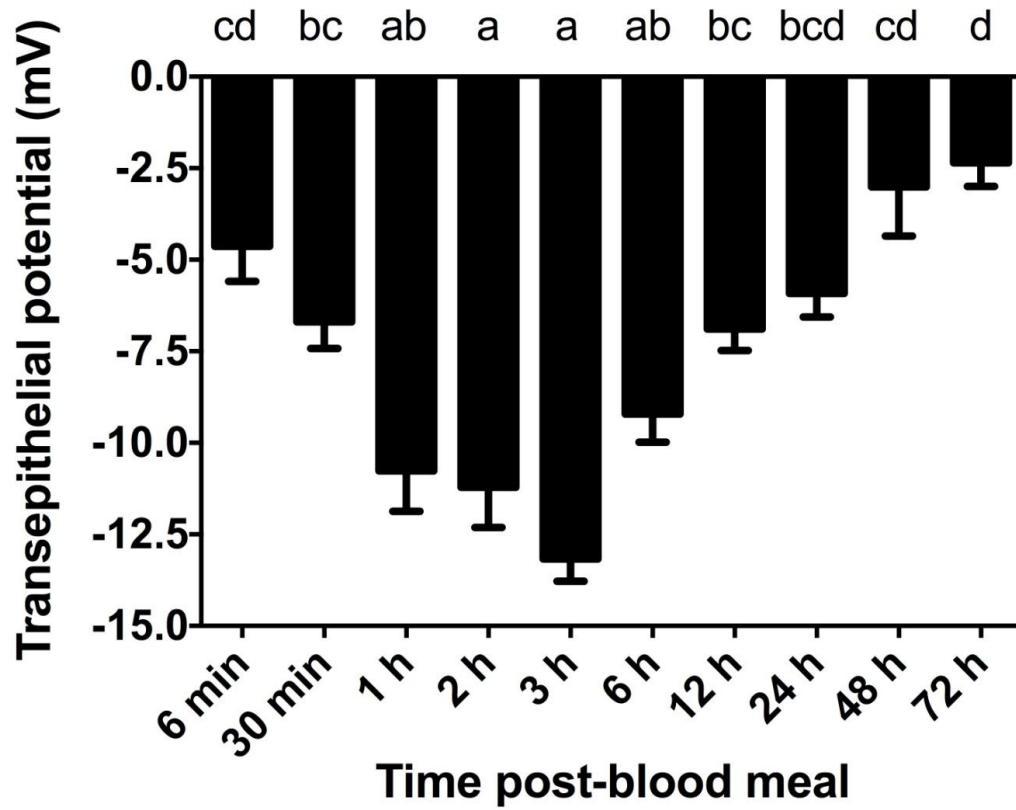
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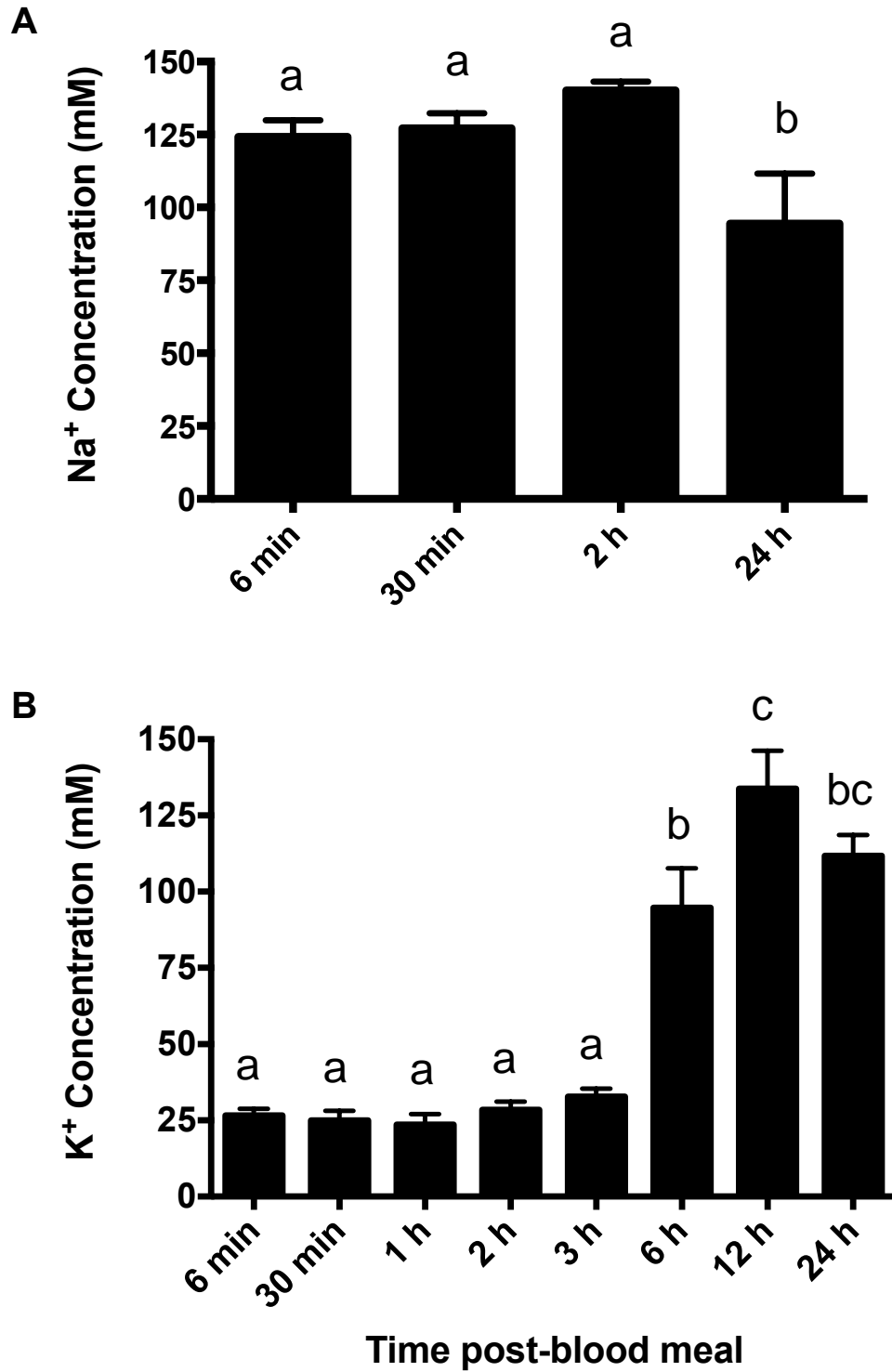
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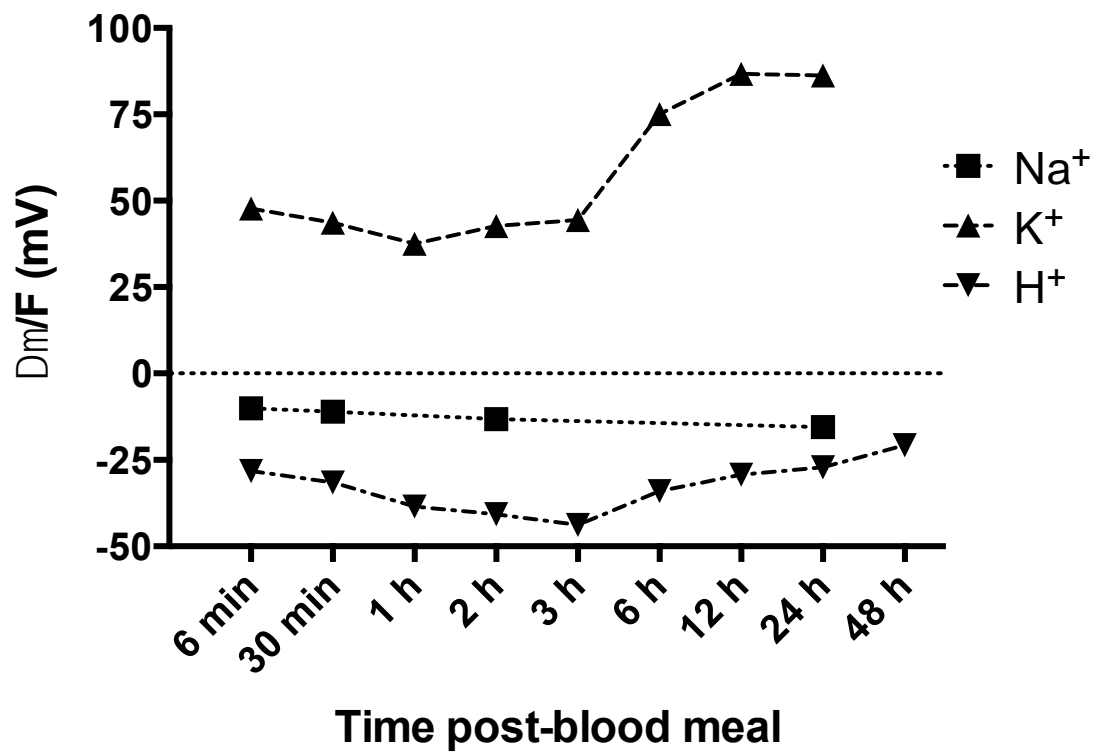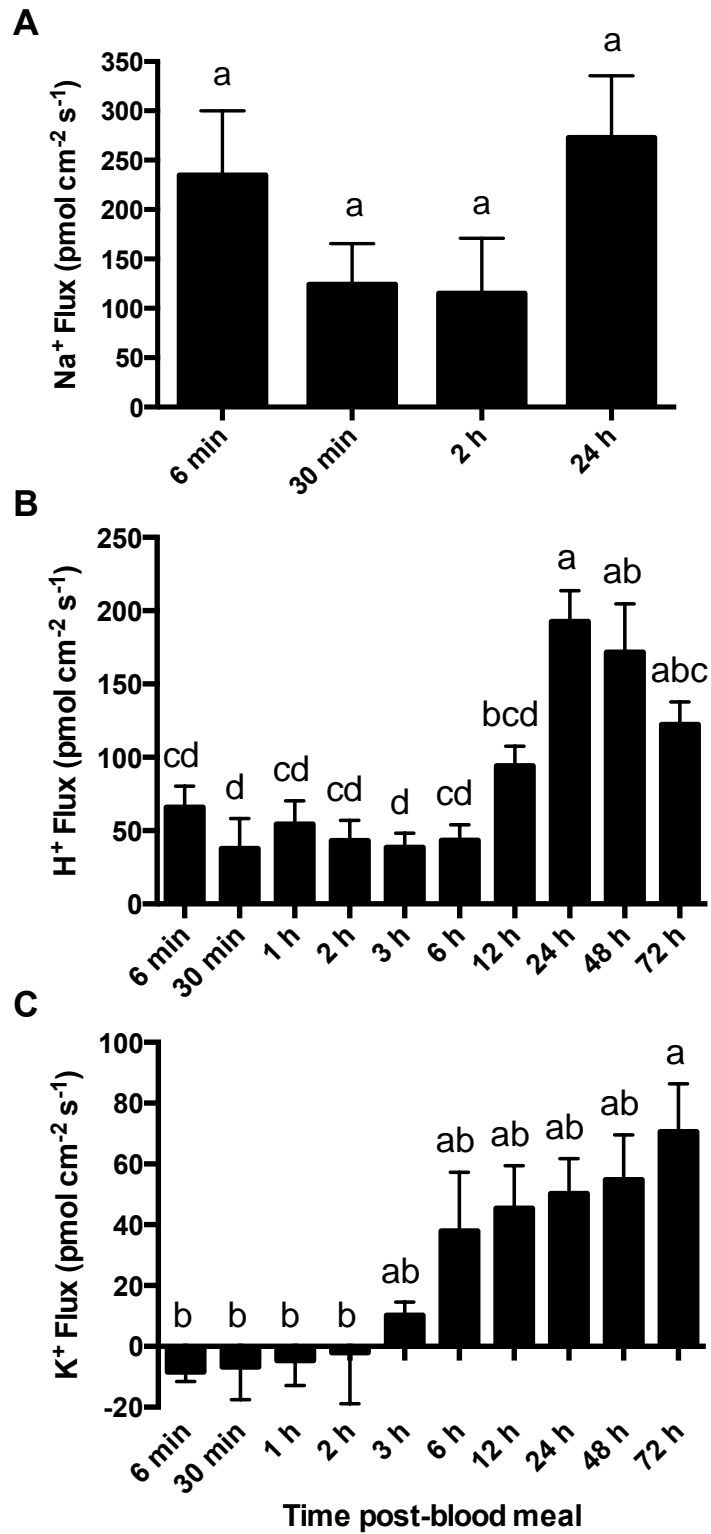
**Figure 4:**

**Figure 5:**

A)

**6 min after blood meal**

$\Delta\mu/F = 59\log([Ion]_{lumen}/[Ion]_{bath}) + zTEP$

Midgut Lumen

TEP=-4.6mV

Bath

**H⁺**

pH=7.1  $\Delta\mu H/F=-28.2mV$  pH=7.5

**K⁺**

66 pmol cm⁻² s⁻¹   Flux

26.6mM  $\Delta\mu K/F=47.7mV$  3.4mM

**Na⁺**

151.8mM  $\Delta\mu Na/F=-9.9mV$  124.3mM

235 pmol cm⁻² s⁻¹   Flux

Flux  -8.5 pmol cm⁻² s⁻¹

B)

**2 hours after blood meal**

$\Delta\mu/F = 59\log([Ion]_{lumen}/[Ion]_{bath}) + zTEP$

Midgut Lumen

TEP=-11.2mV

Bath

**H⁺**

pH=7.1  $\Delta\mu H/F=-40.7mV$  pH=7.6

**K⁺**

43 pmol cm⁻² s⁻¹   Flux

28.5mM  $\Delta\mu K/F=42.8mV$  3.4mM

**Na⁺**

151.8mM  $\Delta\mu Na/F=-13.3mV$  140.1mM

115.4 pmol cm⁻² s⁻¹   Flux

Flux  -2.2 pmol cm⁻² s⁻¹

C)

**24 hours after blood meal**

$\Delta\mu/F = 59\log([Ion]_{lumen}/[Ion]_{bath}) + zTEP$

Midgut Lumen

TEP=-5.9mV

Bath

**H⁺**

pH=7.1  ΔµH/F=-27.2mV  pH=7.5

**K⁺**

192.6 pmol cm⁻² s⁻¹  Flux

111.8mM  ΔµK/F=86.2mV  3.4mM

**Na⁺**

151.8mM  ΔµNa/F=-15.5mV  94.4mM

273.3 pmol cm⁻² s⁻¹  Flux

Flux  50.3 pmol cm⁻² s⁻¹

# Chapter 3

**Effects of ion transport inhibitors on transport of $Na^+$, $H^+$ and $K^+$ across the posterior midgut of blood-fed mosquitoes (*Aedes aegypti*).**

Evan Kendal Pacey and M. J. O'Donnell

## Abstract

The effects of epithelial ion transport inhibitors on transport of $Na^+$, $H^+$ and $K^+$ across the posterior midgut of blood-fed mosquitoes were examined. Fluxes of $Na^+$, $H^+$ and $K^+$ were measured at 6 min, 24 h and 72 h after the blood meal, respectively, corresponding to the times at which absorption of each ion (midgut lumen to bath) are maximal. Fluxes of each ion were measured before and after application of each drug to the bathing saline. $Na^+$ absorption was reduced 51% by the $Na^+/K^+$-ATPase inhibitor ouabain (1 mM) and $K^+$ transport was reversed from absorption to secretion in the presence of the $K^+$ channel blocker $Ba^{2+}$ (5 mM). $H^+$ transport was reduced 80% by the carbonic anhydrase inhibitor acetazolamide (1 mM). There was no effect of the $Na^+$:$K^+$:2$Cl^-$ cotransport inhibitor bumetanide (100 μM) on transport of $Na^+$, $K^+$ or $H^+$ or of the V-type $H^+$-ATPase inhibitor bafilomycin (5 μM) on $H^+$ transport. Amiloride (1 mM) also had no significant effects on either $H^+$ or $Na^+$ absorption. A working model of transepithelial ion transport across the posterior midgut proposes that net transport of $Na^+$ and $K^+$ from cell to hemolymph is mediated by basolateral $Na^+/K^+$-ATPase and $K^+$

channels. $H^+$ and $HCO_3^-$ produced from the actions of carbonic anhydrase on cellular $CO_2$ are transferred into the hemolymph and midgut lumen, respectively.

**Introduction**

Ingestion of a blood meal is required for egg production by the mosquito *Aedes aegypti*. During a post prandial diuresis, much of the $Na^+$, $Cl^-$ and water in the blood plasma is absorbed across the posterior midgut into the hemolymph, then eliminated through secretion of $Na^+$-rich urine by the Malpighian tubules. Following diuresis, $K^+$ released from digestion of the blood cells is transported across the posterior midgut and secreted at low rates by the tubules. Although the mechanisms and control of ion transport by the Malpighian tubules have been the subject of intense study (reviewed by Beyenbach & Piermarini, 2011), much less is known of the mechanisms of ion transport across the posterior midgut. Immunohistochemical studies have revealed expression of basolateral P-type $Na^+/K^+$-A TPase and apical V-type $H^+$-ATPase in each cell of the adult posterior midgut (Patrick et al., 2006). Carbonic anhydrase is associated with the posterior midgut and carbonic anhydrase inhibitors reduce midgut pH in blood-fed mosquitoes, suggesting that the enzyme is implicated in maintenance of the lumen pH (del Pilar Corena et al., 2005). In addition, an isoform of sodium/proton exchanger 3 (NHE3) is found on the basolateral membrane of the anterior and posterior midgut of adult female *A. aegypti* (Pullikuth et al., 2006).

Our previous study analysed transport of $Na^+$, $K^+$ and $H^+$ across the posterior midgut using the scanning ion-selective electrode technique (SIET). Electrochemical gradients for each ion calculated from measurements of transepithelial electrical potential and luminal ion concentrations indicate that $Na^+$, $H^+$, and $K^+$ absorption are thermodynamically uphill at specific times after the blood meal, consistent with active ion

transport, whereas $Na^+$ and $K^+$ may move passively in response to favourable

electrochemical gradients at other times (Chapter 2). In this study, therefore, we have

examined the effects of drugs known to block specific ion transporting ATPases or

channels which may mediate transport of $Na^+$, $K^+$ and $H^+$. Absorption of $Na^+$ across the

midgut of blood-fed *Rhodnius prolixus*, for example, is reduced 81% by the $Na^+/K^+$-

ATPase inhibitor ouabain (Farmer et al., 1981). In many insect epithelia, however, fluxes

of $Na^+$ and $K^+$ are energized by $H^+$ gradients created by a V-type $H^+$-ATPase (reviewed

by Harvey, 2009). Secretion of $Na^+$ and $K^+$ driven by the resulting $H^+$ gradients across the

apical membrane of Malpighian tubules can be inhibited by application of the

sodium:proton exchanger (NHE) inhibitor amiloride applied to the bathing saline

(Maddrell & O'Donnell, 1992). Given the involvement of carbonic anhydrase in

maintenance of midgut pH, we have also measured the effects of the carbonic anhydrase

inhibitor acetazolamide on $H^+$ fluxes. Lastly, because epithelial $K^+$ channels implicated in

secretion or absorption of $K^+$ by insect Malpighain tubules and the midgut are known to

be blocked by barium ions (Leyssens et al., 1994; Schirmanns & Zeiske, 1994; Haley &

O'Donnell, 1997), we have examined the effects of $Ba^{2+}$ on absorption of $K^+$ across the

posterior midgut. In each experiment, ion fluxes across the isolated posterior midgut have

been measured by SIET before and after application of each drug.

**Materials and Methods**

*Insect rearing*

Insects were reared as previously described in chapter 2.

*Physiological salines and dissection*

Physiological salines were prepared and dissections were performed as described previously in chapter 2.

*Pharmacological reagents*

Stock solutions of the ion transport inhibitors were prepared at 10 times the desired final concentration. Solutions of acetazolamide and ouabain were prepared in saline. Amiloride stock solution was prepared in warmed saline (~60° C) to facilitate dissolution. Salines containing $Ba^{2+}$ were prepared by addition of $BaCl_2$ to sulphate-free saline prepared by substitution of $MgCl_2$ for $MgSO_4$. Bumetanide and bafilomycin were prepared in ethanol; the final concentration of ethanol was < 1%. Control experiments indicated no significant effects of 1% ethanol on ion transport across the posterior midgut.

*Scanning Ion Electrode Technique (SIET)*

The methods for preparation of SIET were as described in chapter 2.

*Statistical Analyses*

Statistical analyses were done as described in chapter 2.

**Results**

The effects of each of the drugs used in this study were analysed at specific time intervals after the blood meal. In order to increase the scope for detection of drug effects, we selected the times at which the flux of each ion maximal. For $Na^+$, $H^+$ and $K^+$, the times selected were 6 min, 24 h and 72 h, respectively, based on previous SIET measurements of postprandial ion flux (Chap. 2). Fluxes were measured 10 – 15 minutes after addition of each drug to the bathing saline. Previous studies have shown that $Na^+$, $K^+$ and $H^+$ fluxes are stable for > 45 minutes after dissection (Chap. 2). Control experiments showed no effect of the addition of the vehicle for bumetanide and bafilomycin (1% ethanol in saline) on $H^+$, $Na^+$ or $K^+$ absorption ($N = 3$ preparations each; data not shown).

*$H^+$ fluxes*

Absorption of $H^+$ across the posterior midgut was reduced 80% by the carbonic anhydrase inhibitor acetazolamide (Fig. 1). There was no effect of either the NHE inhibitor amiloride or the V-ATPase inhibitor bafilomycin (Fig. 1).

*$Na^+$ fluxes*

Absorption of $Na^+$ across the posterior midgut was unaffected by amiloride, bafilomycin or the $Na^+$:$K^+$:$2Cl^-$ cotransporter inhibitor bumetanide (Fig. 2). However, addition of ouabain (1 mM) to the bathing saline resulted in a 51% decrease in the rate of $Na^+$ absorption.

*$K^+$ fluxes*

Absorption of $K^+$ across the posterior midgut was reversed by application of $Ba^{2+}$ to sulphate-free saline (Fig. 3). In control saline, there was no effect of either bafilomycin or bumetanide on $K^+$ absorption.

**Discussion**

The results of this study provide new insight into the mechanisms of ion transport across the posterior midgut of blood-fed mosquitoes. A role for the $Na^+/K^+$-ATPase in $Na^+$ absorption during diuresis is suggested by the inhibition of $Na^+$ transport by ouabain. Although we have not measured the effects of ouabain on $K^+$ transport, it is worth noting that $K^+$ is secreted from bath to midgut lumen during diuresis (Chap. 2). Thus, the $Na^+/K^+$-ATPase may energize both $K^+$ secretion and $Na^+$ absorption during the postprandial diuresis in blood-fed mosquitoes.

$K^+$ is absorbed from the midgut at 6 h or longer after the blood meal, coincident with an increase in late trypsin activity (Noriega & Wells, 1999) and a consequent increase in $K^+$ concentration in the midgut contents from ~25 mM to 95 mM at 6 h and 134 mM at 12 h after the blood meal (Chap. 2). The reversal of $K^+$ absorption by $Ba^{2+}$ suggests the involvement of $Ba^{2+}$-sensitive $K^+$ channels in $K^+$ absorption, in response to an electrochemical potential favouring passive movement of $K^+$ from gut lumen to the bathing saline. $Ba^{2+}$ blocks $K^+$ channels in the midgut epithelium of *Manduca sexta* (Schirmanns & Zeiske, 1994) and also blocks $K^+$ transport in both the secretory (Leyssens

et al., 1994) and reabsorptive (Haley & O'Donnell, 1997) segments of insect Malpighian tubules.

Although the V-type $H^+$-ATPase is present in the apical membrane of the posterior midgut (Patrick et al., 2006), there was no effect of the V-ATPase inhibitor bafilomycin on fluxes of $Na^+$, $H^+$ or $K^+$ at selected intervals after the blood meal. These findings suggest that, in contrast to the Malpighian tubules, the V-type $H^+$-ATPase is not the primary means by which ion transport across the apical membrane is energized. In the related species *A. stephensi*, more than 80% of midgut ATPase activity is inhibited by ouabain (MacVicker et al., 1993), suggesting that the $Na^+/K^+$-ATPase may be the primary energizer of transepithelial ion transport across the posterior midgut of *Aedes*. One caveat is that bafilomycin was applied to the bathing saline rather than the gut lumen in our experiments. However, bath application of bafilomycin effectively inhibits the V-ATPase on the apical membrane of Malpighian tubules of multiple species (Beyenbach et al., 2000). Bafilomycin is also known to inhibit transport dependent upon an apical $H^+$-ATPase in the tardigrade midgut when applied from the basolateral side (Halberg & Møbjerg, 2012). This suggests that sufficient quantities of the drug are able to diffuse through epithelial tissues to reach sites on the apical membrane, but further studies using perfused gut preparations and luminal application of bafilomycin are required to define the functional roles of the apical V-ATPase in transport across the posterior midgut.

The $Na^+$:$K^+$:$2Cl^-$ cotransporter on either the basolateral or apical membrane contributes to secretion or absorption, respectively, of the three ions in different types of epithelia (Epstein et al., 1983). Bumetanide is well-known as an inhibitor of basolateral

$Na^+$:$K^+$:$2Cl^-$ cotransport in the Malpighian tubules of dipterans (Scott et al., 2004; Ianowski & O'Donnell, 2004) and other species (e.g. crickets; Coast, 2012). In humans, it is used as a "loop diuretic", and is effective at blocking salt reabsorption in the thick ascending loop of Henle. Since both $K^+$ and $Na^+$ are transported in the same direction across the posterior midgut of blood-fed mosquitoes following completion of diuresis, we examined the effects of bumetanide. Addition of bumetanide to the bathing saline had no effect on $Na^+$ absorption at 6 min post-blood meal, or on $K^+$ absorption at 72 h. For bumetanide, as for bafilomycin described above, further experiments are required to determine the effectiveness of the drug if applied from the luminal side.

A working model of ion transport across the posterior midgut is present in Figure 4. The model incorporates the results of the present study, as well as those of a previous publication (Chap. 2). We propose that a basolateral $Na^+$/$K^+$-ATPase is responsible for $Na^+$ absorption and $K^+$ secretion during diuresis, and that $Ba^{2+}$-sensitive basolateral $K^+$ channels mediate transport from cell to hemolymph across the basolateral membrane. The ion transporters mediating transport of $Na^+$ and $K^+$ across the apical membrane are unknown. Proton gradients established by the apical $H^+$-ATPase may be used to drive amino acid uptake into the cells through an apical proton amino acid transporter (PAT) that is present in the midgut epithelial cells of *A. aegypti* (AaePAT1; Evans et al., 2009). We suggest that $HCO_3^-$ and $H^+$ resulting from acetazolamide-sensitive carbonic anhydrase activity exit the cell across the apical and basolateral membrane, respectively. An apical $Cl^-$/$HCO_3^-$ exchanger is a candidate transporter mediating $HCO_3^-$ movement into the lumen in exchange for luminal $Cl^-$. Transfer of $HCO_3^-$ into the gut lumen may contribute

to maintenance of an alkaline pH, as proposed by del Pilar Corena et al., (2005). $Cl^-$ may then exit into the hemolymph via basolateral $Cl^-$ channels (Fig. 4). Both $K^+$ and $Cl^-$ conductances have been identified in the basolateral membrane of the *Drosophila* Malpighian tubule (Ianowski & O'Donnell, 2004). The $H^+$ resulting from carbonic anhydrase may exit basolaterally, perhaps through the NHE3 exchanger which is present on the basolateral surface of the midgut and is insensitive to amiloride (Pullikuth et al., 2006).

It is worth noting that $Na^+$ absorption across the midgut of the blood-feeding hemipteran insect *Rhodnius prolixus* is inhibited 81% by 1 mM ouabain (Farmer et al., 1981). The latter authors also propose that $K^+$ moves passively from cell to hemolymph. There are thus similarities in the transport models for midgut ion transport in blood-feeders in two different insect orders (Diptera and Hemiptera). It is also worth noting that $Na^+$ transport by the Malpighian tubules of both *Rhodnius* and *Aedes* during diuresis is energized by a bafilomycin-sensitive apical V-type $H^+$-ATPase. There are thus striking differences in the means by which primary active transport is energized in epithelia of the midgut versus the Malpighian tubule.

**Figure captions**

**Figure 1.** Fluxes of $H^+$ across the posterior midgut of *A. aegypti* before and after drug treatment. Open bars indicate fluxes before drug treatment while closed bars indicate fluxes $10 - 15$ minutes after the addition of drug to the bath. Positive fluxes indicate ion absorption from the lumen to the hemolymph. The asterisks denote significant differences ($P < 0.05$) relative to non-treated posterior midguts. $N = 3$-7 blood fed adult females for each drug.

**Figure 2.** Fluxes of $Na^+$ across the posterior midgut of *A. aegypti* before and after drug treatment. Open bars indicate fluxes before drug treatment while closed bars indicate fluxes $10 - 15$ minutes after the addition of drug to the bath. Positive fluxes indicate ion absorption from the lumen to the hemolymph. The asterisks denote significant differences ($P < 0.05$) relative to non-treated posterior midguts. $N = 3$-7 blood fed adult females for each drug.

**Figure 3.** Fluxes of $K^+$ across the posterior midgut of *A. aegypti* before and after drug treatment. Open bars indicate fluxes before drug treatment while closed bars indicate fluxes $10 - 15$ minutes after the addition of drug to the bath. Positive fluxes indicate ion absorption from the lumen to the hemolymph while negative fluxes indicate ion secretion from the hemolymph to the lumen. The asterisks denote significant differences ($P < 0.05$) relative to non-treated posterior midguts. $N = 5$-7 blood fed adult females for each drug.

**Figure 4.** Possible mechanisms of ion transport in the posterior midgut of *A. aegypti* during diuresis and digestion of the blood meal. Ion and water channels are indicated by cylinders, exchangers by ovals, cotransporters by rectangles and ATPases by circles.
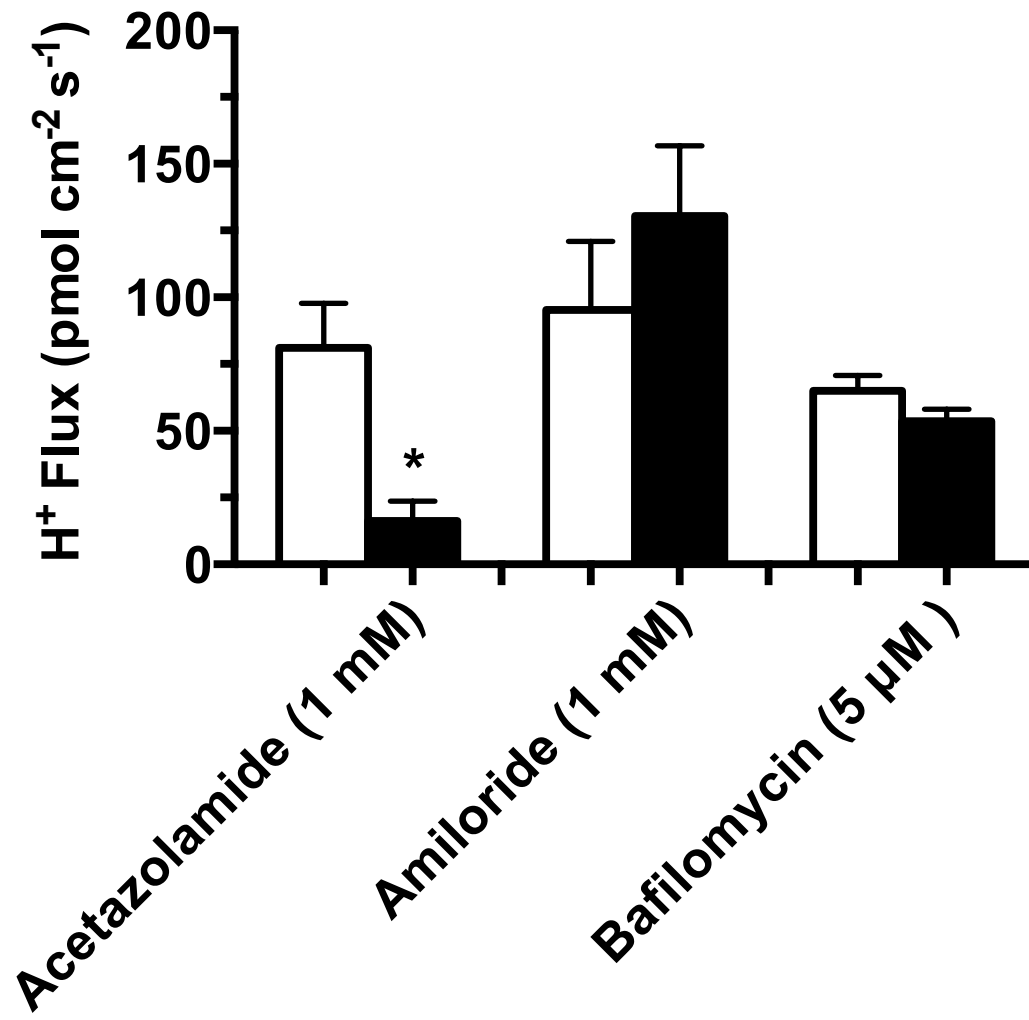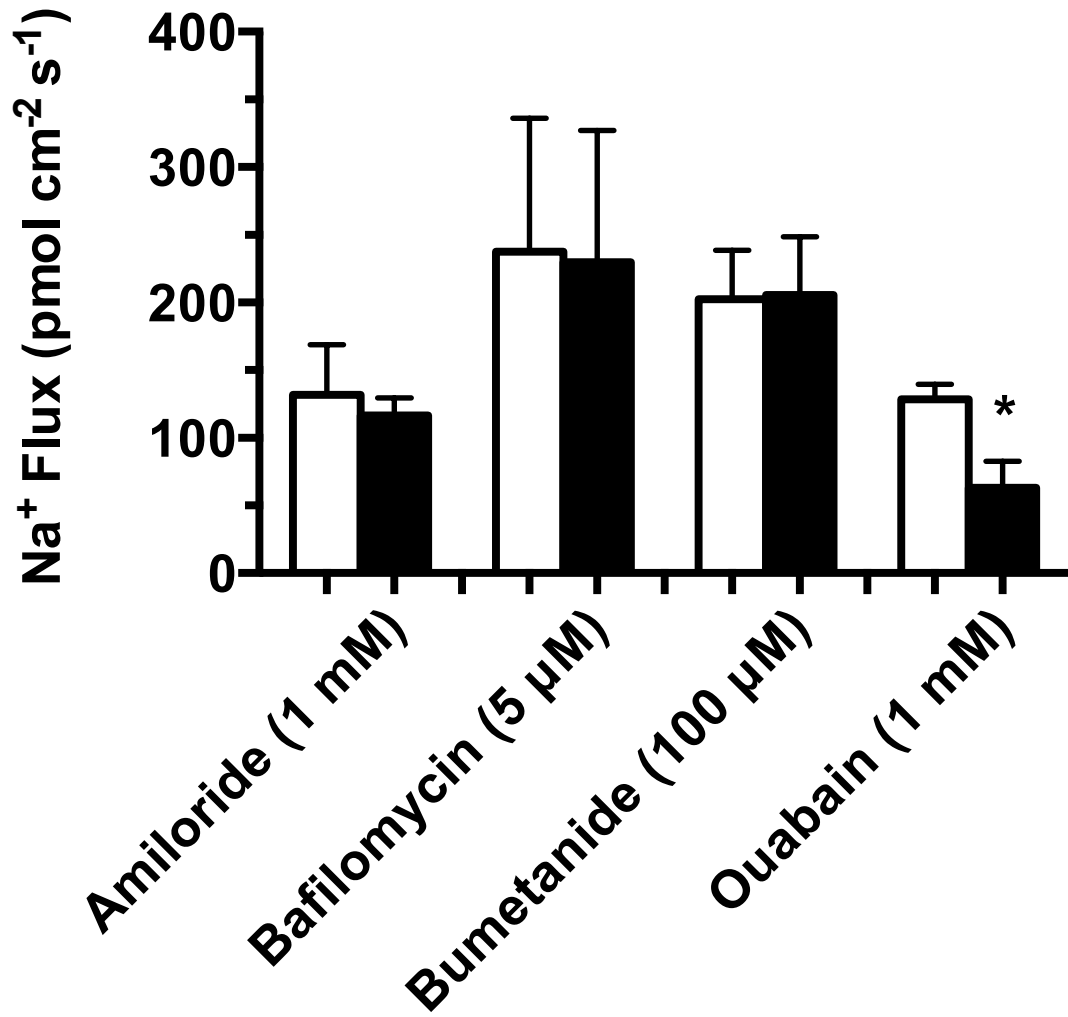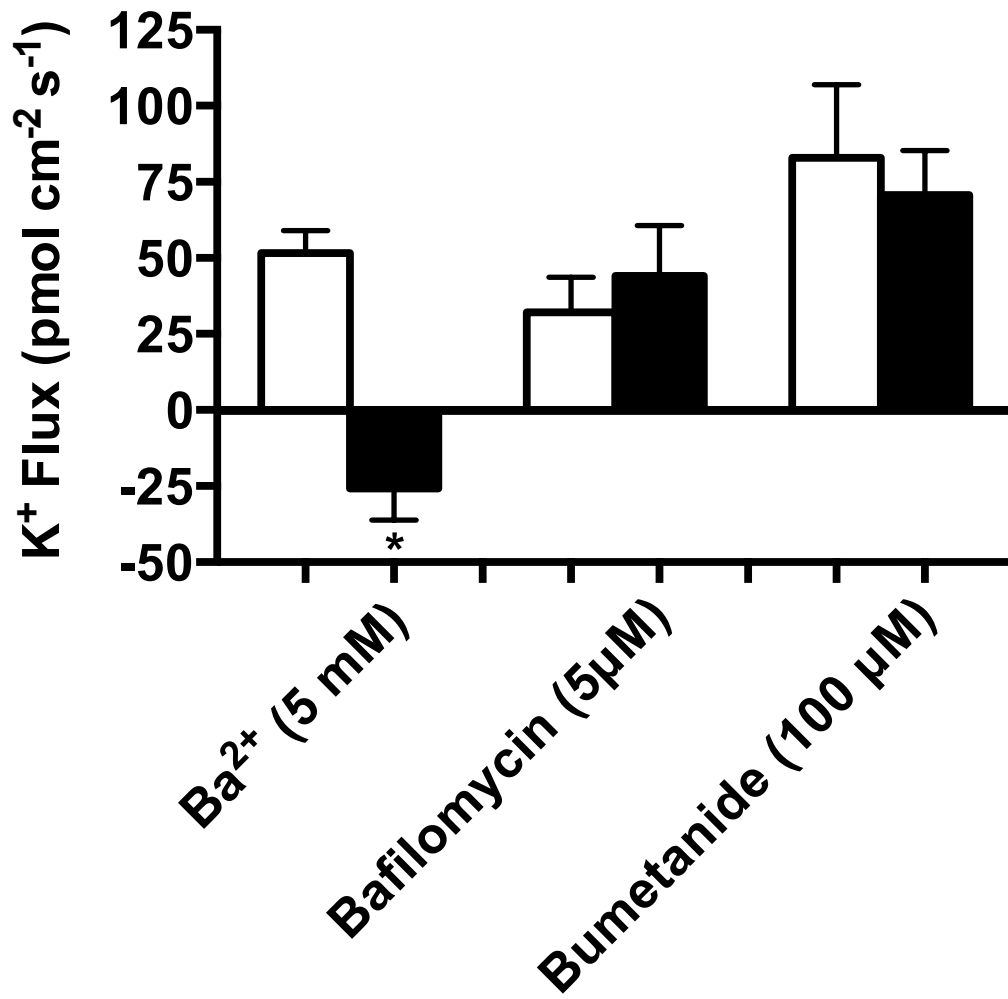
**Figure 1:**

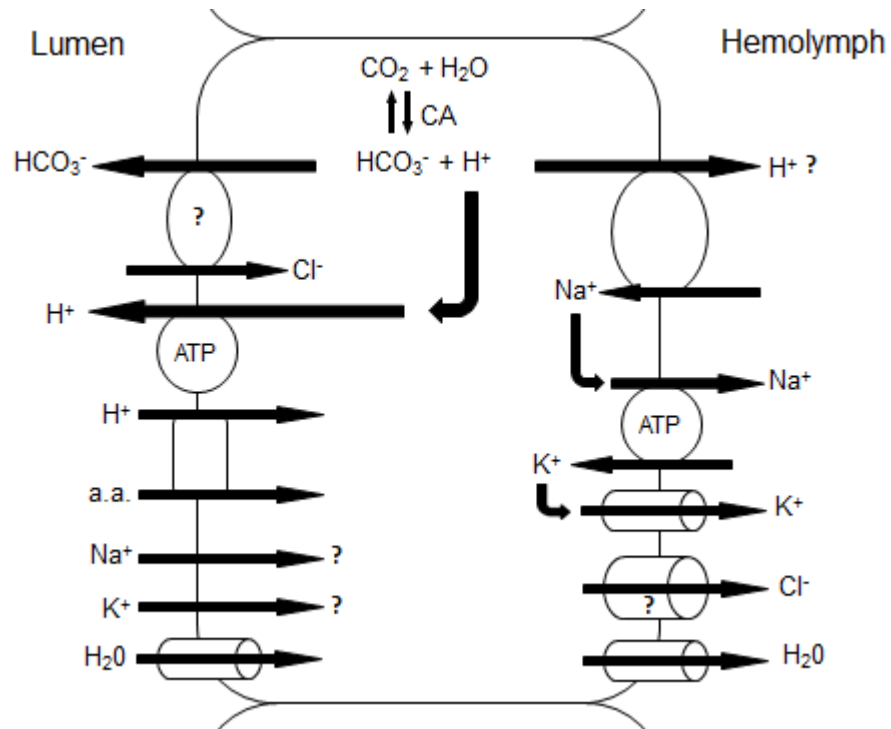**Figure 2:**

**Figure 3:**

**Figure 4:**

# Chapter 4

**General Discussion**

This thesis has examined the patterns and mechanisms of $Na^+$, $H^+$ and $K^+$ transport across the posterior midgut of *A. aegypti* during postprandial diuresis and blood meal digestion. This general discussion integrates the results from chapters 2 and 3 and extends the discussion of the working model that has been presented in Chapter 3 (Fig. 4). In addition, suggestions for subsequent research that could test specific proposals in the model are presented.

*A working model of ion transport across the posterior midgut of blood-fed mosquitoes: transport across the basolateral membrane.*

The ouabain-sensitive $Na^+/K^+$-ATPase plays a significant role in the transport of $Na^+$ and appears to be the primary energizer of secondary active transport systems in the apical or basolateral membranes. As noted in chapter 3, transport of $Na^+$ and water across the midgut of blood-fed *Rhodnius prolixus* is also ouabain-sensitive (Farmer et al., 1981). Thus, it appears that although the bafilomycin-sensitive V-type ATPase plays a cardinal role in transport of energizing secretion of water and ions by the Malpighian tubules in both mosquitoes and *R. prolixus* (reviewed in O'Donnell, 2008; Beyenbach et al., 2010), the ouabain-sensitive $Na^+/K^+$-ATPase is the primary driver of water and ion transport across the midgut, particularly during the period of diuresis. It would be of interest in future studies to examine the effects of ouabain on $Na^+$ absorption at later time intervals, after the completion of diuresis.

Since the electrochemical potential for $K^+$ favours absorption of $K^+$ from lumen to bath at all times after the blood meal, some form of ATP-dependent pump is required for secretion of $K^+$ during the first 2 hours after the blood meal. The stoichiometry of the basolateral $Na^+/K^+$-ATPase is appropriate for secretion of $K^+$. Although the $Na^+/K^+$-ATPase may continue to energize $Na^+$ absorption at later intervals, the dramatic increase in luminal $K^+$ concentration by 6 hours after the blood meal and the corresponding increase in the electrochemical potential favouring $K^+$ absorption may mask the $K^+$ transport by the $Na^+/K^+$-ATPase. At these times, $K^+$ is absorbed through a $Ba^{2+}$-sensitive pathway (i.e. $K^+$ channels). It is worth noting that secretion of $K^+$ was restored in the presence of $Ba^{2+}$, consistent with continued operation of the $Na^+/K^+$-ATPase after inhibition of $Ba^{2+}$-sensitive $K^+$ channels. In future work, the effects of changes in bathing saline $K^+$ concentration on transepithelial potential could be used to determine if $K^+$ channels are present in the basolateral membrane. Similarly, the effects of changes in bathing saline $Cl^-$ concentration could be used to assess whether a conductive paracellular or transcellular pathway for $Cl^-$ is present. Paracellular $Cl^-$ absorption could be driven by the lumen-negative transepithelial potential. Alternatively, the effects of $Cl^-$ channel blockers such as diphenylamine-2-carboxylate and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) on changes in transepithelial potential produced by varying bathing saline $Cl^-$ concentration could be used to assess transcellular $Cl^-$ transport through basolateral $Cl^-$ channels (Haley et al., 1998).

For $H^+$, the drop in absorption that occurs when carbonic anhydrase is inhibited by acetazolamide indicates that the bulk of $H^+$ in epithelial cells comes from the conversion

of $CO_2$ and $H_2O$ into free $H^+$ by the enzyme. This $CO_2$ is most likely a waste product

from the oxidative metabolism that provides energy (ATP) to drive transport of ions and

amino acids across the posterior midgut (Briegel, 1985; Zhoe et al., 2004). How $H^+$ is

transported across the basolateral membrane of the posterior midgut is unknown, but

some form of energy-dependent transport is required because the electrochemical

potential for $H^+$ was negative at all time points studied up to 48 hours after the blood

meal. As noted in chapter 3, an amiloride-insensitive sodium:proton exchanger remains a

possibility (Pullikuth et al., 2006).

*Transport across the apical membrane.*

Although the V-type $H^+$-ATPase has been clearly identified in the apical

membrane of the midgut of adult *A. aegypti* (Patrick et al., 2006) there was no effect of

the V-ATPase inhibitor bafilomycin on fluxes of $H^+$, $Na^+$ or $K^+$. Protons may be cycled

across the apical membrane through the coordinated actions of the apical V-ATPase and

the proton:amino acid transporter (Evans et al., 2009). In subsequent studies, perfusion of

the midgut lumen could be used in conjunction with transepithelial potential

measurements to determine if there are conductive pathways for $Na^+$ or $K^+$. SIET could

be used to measure ion transport in midguts dissected so as to expose the luminal surface.

The latter approach has been used to measure $Cd^{2+}$ uptake, for example, from the lumen

side of the dissected midgut of the midge larva *Chironomus riparius* (Leonard et al.,

2009). Such an approach could be used to determine if $H^+$ transport across the apical

membrane is sensitive to the V-ATPase inhibitor bafilomycin applied from the luminal

side.

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