The Journal of Experimental Biology 212, 1662-1671
Published by The Company of Biologists 2009
doi:10.1242/jeb.028084

Review

Carbonic anhydrases and anion transport in mosquito midgut pH regulation

Paul J. Linser*, Kristin E. Smith, Terri J. Seron and Marco Neira Oviedo
The University of Florida Whitney Laboratory, 9505 Ocean Shore Boulevard, St Augustine, FL 32080, USA
*Author for correspondence (e-mail: pjl@whitney.ufl.edu)

Accepted 3 February 2009

Summary

Mosquito larvae use a digestive strategy that is relatively rare in nature. The anterior half of the larval mosquito midgut has a luminal pH that ranges between 10.5 and 11.5. Most other organisms, both large and small, initiate digestion in an acid medium. The relative uniqueness of the highly alkaline digestive strategy has been a long-standing research focus in larval lepidopterans. More recently, the disease vector potential of mosquitoes has fueled specific interest in larval mosquito biology and the alkaline digestive environment in the midgut. The probable principle anion influencing the highly alkaline gut lumen is bicarbonate/carbonate. Bicarbonate/carbonate is regulated at least in part by the activity of carbonic anhydrases. Hence, we have focused attention on the carbonic anhydrases of the mosquito larva. *Anopheles gambiae*, the major malaria mosquito of Africa, is an organism with a published genome which has facilitated molecular analyses of the 12 carbonic anhydrase genes annotated for this mosquito. Microarray expression analyses, tissue-specific quantitative RT-PCR, and antibody localization have been used to generate a picture of carbonic anhydrase distribution in the larval mosquito. Cytoplasmic, GPI-linked extracellular membrane-bound and soluble extracellular carbonic anhydrases have been located in the midgut and hindgut. The distribution of the enzymes is consistent with an anion regulatory system in which carbonic anhydrases provide a continuous source of bicarbonate/carbonate from the intracellular compartments of certain epithelial cells to the ectoperitrophic space between the epithelial cells and the acellular membrane separating the food bolus from the gut cells and finally into the gut lumen. Carbonic anhydrase in specialized cells of the hindgut (rectum) probably plays a final role in excretion of bicarbonate/carbonate into the aquatic environment of the larva. Detection and characterization of classic anion exchangers of the SLC4A family in the midgut has been problematic. The distribution of carbonic anhydrases in the system may obviate the requirement for such transporters, making the system more dependent on simple carbon dioxide diffusion and ionization *via* the activity of the enzyme.

Key words: carbonic anhydrase, mosquito larva, alkalization, midgut, pH.

Introduction

Mosquitoes predate us by several hundred million years and have been a source of human misery and mortality for presumably as long as our specie has been extant on earth. Hematophagy (taking of a blood meal, the mosquito characteristic that leads to disease transmission) has evolved in arthropods well over a dozen times and long before human beings first stepped out on the veldt in Africa (Black and Kondratieff, 2005). The relationship between mosquitoes, specific disease organisms including viruses and various parasites, and human hosts is also very highly evolved. Since the turn of the 20th century, malaria alone has killed probably over 100 million people and infected billions.

Mosquitoes are holometabolous insects. This term describes the fact that mosquitoes have distinct structural and physiological forms during the development from zygote to reproductive adult. Among the four forms (embryo, larva, pupa, imago) the two best recognized by the non-scientist are the larval stage and the adult (imago). Adult female mosquitoes, because they are the vectors of disease for many vertebrate animals, are a focus of control strategies. These strategies are frequently targeted in some way to the winged, flying adult and its behavior and habitats. Largely because adult female mosquitoes are the vector of disease, much research focuses on analyses of their biology. In reality, larval stage mosquitoes are much more abundant than adults and quite distinct in form and function. Larval mosquitoes are aquatic organisms and contrast dramatically with the blood or nectar consuming adult form. In many ways, larval biology is very different from the adult. In circumstances in which larval populations are readily located, larval control strategies are very effective in population reduction (e.g. Killeen et al., 2002). Never the less, relatively little is known about larval biology as might be revealed by modern, state-of-the art molecular physiological analyses. An in-depth understanding of larval biology has the potential to provide new and environmentally safe control strategies.

A specific aspect of larval mosquito biology that has the potential for producing new directions in control and population management is food ingestion, digestion and nutrient absorption. Larval insects spend the majority of their time eating, with the digestive tract being a major portion of the organism's body mass. Larval mosquitoes, similar to certain other dipteran and lepidopteran larvae, utilize an initial digestive strategy that is contrary to most other complex organisms on earth: the anterior portion of the larval mosquito midgut (stomach) harbors a luminal pH as high as 10.5–11 (Fig. 1) (Dadd, 1975; Dow, 1984; Zhuang et al., 1999). Most other organisms utilize a gut luminal pH in the acidic range to initiate food digestion. Aspects of how this pH is generated and related questions of food digestion and ionic homeostasis in larval mosquitoes are the main subjects of this review.

The general layout of the midgut is depicted in Fig. 1 and a few key points should be made. First, the alimentary canal of the larval

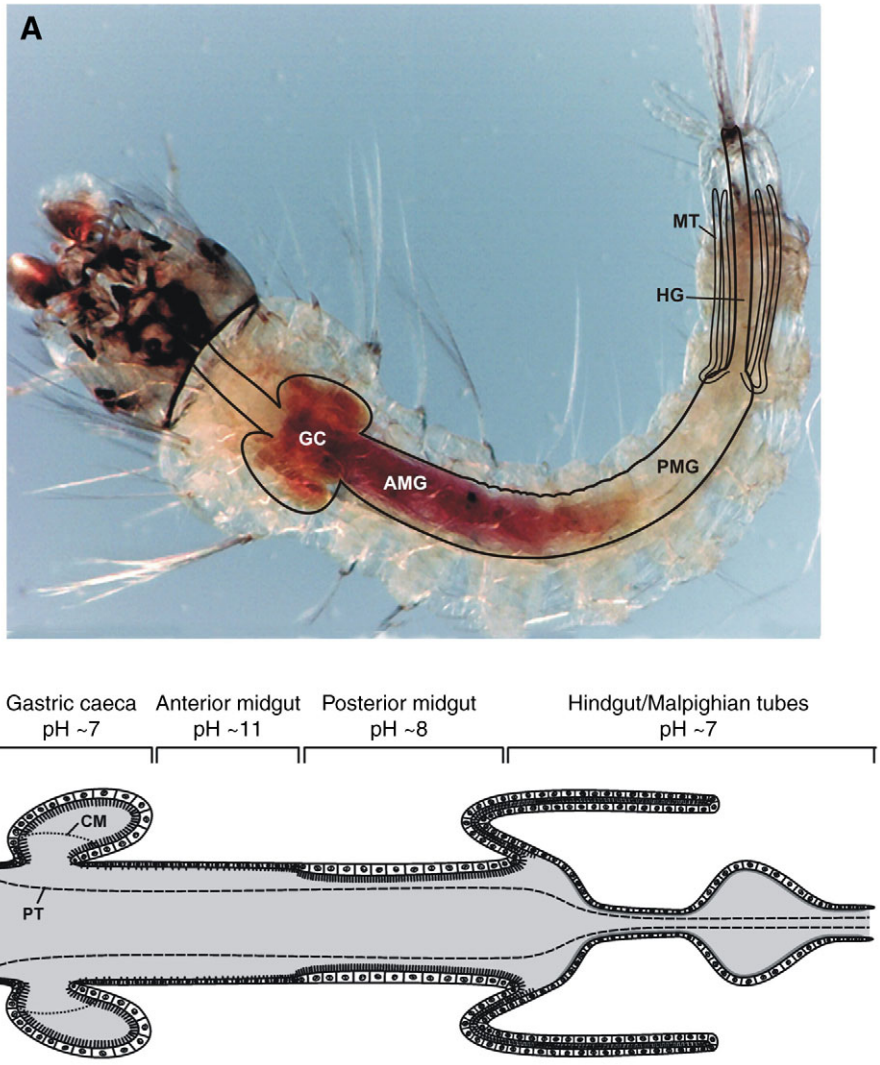

Fig. 1. (A) A living fourth instar *Anopheles gambiae* larva that had been fed pH sensitive dye (*m*-cresol purple) to highlight the gut luminal pH gradient. The structure and compartments of the alimentary canal are outlined in black. (B) A stylized diagram of the gut with the anterior to the left. The brackets above indicate the extent of each region, and the approximate pH of each is indicated (modified from Neira Oviedo et al., 2008). GC, gastric caeca; AMG, anterior midgut; PMG, posterior midgut; HG, hindgut; MT, Malpighian tubules; CM, caecal membrane; PT, peritrophic matrix.

mosquito is complex and can be seen to exist in subdivided domains of structure and function. At the anterior end of the gut is the esophagus (foregut; not shown in Fig. 1). Salivary secretions mix with ingested food particles in the mouth, and the bolus then moves posteriorly as the result of muscular activity and by displacement as new food particles enter from the mouth. The midgut is composed of the cardia (not shown in Fig. 1), the gastric caeca (GC), the anterior midgut (AMG) and the posterior midgut (PMG). The cardia is the region that surrounds the posterior end of the foregut, and a sub-set of its cells secrete the peritrophic matrix (PT), a proteinaceous membrane that continuously surrounds the food bolus from anterior to posterior end of the gut. Between the PMG and the gut epithelium is a compartment called the ectoperitrophic space. The GC are eight lateral bulbous expansions of the gut epithelium that are characterized by a luminal space, which is segregated from the other internal compartments of the gut by an acellular membrane called the caecal membrane (CM). The cells of this region are large and possess very extensive arrays of apical microvilli (reviewed by Clements, 1992; Zhuang et al., 1999). At approximately half the distance from the GC to the termination of the midgut, the epithelial cells change character: the AMG cells (anterior half of the midgut) are distinguished by an apical surface nearly devoid of membrane expansions and/or microvilli; the PMG cells (posterior half of the midgut) exhibit very elaborate amplifications of apical cell surface in the form of vast arrays of microvilli (ibid.). The posterior end of the midgut transitions into the hindgut through a region called the pylorus, followed by the ileum, and then finally the rectum. At the anterior end of the pylorus, lateral cellular tubules (Malpighian tubules) extend from the gut epithelium into the surrounding hemolymph.

The roles of the various cell types within the gut are poorly understood. In general, the GC cells are believed to be involved in the digestion and absorption of proteins and carbohydrates, as well as the secretion of antimicrobial peptides that help keep ingested organisms and pathogens under control. The anterior midgut is devoted mainly to the digestion and absorption of lipids, as well as the detoxification of dietary xenobiotics. The PMG is involved in the metabolism and absorption of carbohydrates and proteins. The Malpighian tubules are thought to be the main ion regulatory organs of the mosquito, and are also involved in xenobiotic detoxification. The hindgut apparently plays roles in the final steps of digestion, absorption, elimination of waste, and retention of crucial ions (Bradley, 1987; Strange et al., 1982; Wigglesworth, 1932). For a more detailed description of the functional compartmentalization of the larval gut, see the transcriptomic analysis recently published by Neira Oviedo et al. (Neira Oviedo et al., 2008).

In the midst of many concurrent events regulated by the cellular diversity of the larval gut, a pH gradient of phenomenal proportions

is established and maintained without any physical barriers to the different pH zones. The pH in the GC lumena and gut lumen at the region of the GC is 7.5–8 (Dadd, 1975; Zhuang et al., 1999). Immediately posterior to that is the AMG where the luminal pH rises rapidly to 10.5–11. In the PMG, the luminal pH drops to 7–8 again as in the GC (Fig. 1). The pH in the hindgut (specifically the rectum) drops again to 6.5–7.0 (Clark et al., 2007) (K.E.S., unpublished observations).

Carbonic anhydrase and midgut pH

Decades of research have focused on pH regulation in the gut of the lepidopteran caterpillar (larva) of *Manduca sexta*, the tobacco hornworm (e.g. Harvey, 1992; Wieczorek, 1992). In this organism, the luminal pH can reach 12 (ibid.). The transepithelial potential, which, along with epithelial transport processes, supports a luminal pH of 12 and a blood/hemolymph pH of near 7 has been intensely studied in this organism with some remarkable insights (ibid.) (see also Harvey, 2009). The actual buffer at such an extremely alkaline pH is very likely to be carbonate (CO_3^{2-}) and paired with a strong cation such as K^+ or Na^+ (Dadd, 1975; Dow, 1984). Indeed, the logical source of the CO_3^{2-} is metabolic CO_2 and its ionization through the activity of the enzyme carbonic anhydrase (CA). The reaction can be simply represented: $CO_2+H_2O\uparrow HCO_3^-+H^+$. Deprotonization of the bicarbonate ion would then produce CO_3^{2-} which has a pK_a of approximately 10.3. Thus, it seems probable that one or more CAs would be present and involved in the alkalization of the caterpillar gut. In the late 1990s, interest in alkalization mechanisms of the larval mosquito gut rose as a result of the possibility that this biological system might be amenable to the development of new larval control strategies.

Confirmation of CA as a central element of the larval mosquito midgut alkalization system came with the demonstration that H^+ and Cl^- flux across the midgut epithelium of *Aedes aegypti* was CA dependent (Boudko et al., 2001). Using SERIS-LIX (self-referencing ion-selective liquid ion exchanger) microelectrodes in a vibrating mode, ionic fluxes from the basal surface of the midgut were measured as a function of position along the anterior–posterior axis. The net vectorial flux of both H^+ and Cl^- showed remarkable polarity reversal along the length of the midgut, in rough correlation with the pH gradient in the lumen of the tube. When CA inhibitors were applied, flux of H^+ and Cl^- ions was dramatically reduced or eliminated (Boudko et al., 2001) indicating that CA activity was central to ion fluxes that are likely to be central to pH regulation in the midgut.

In 2002, the first characterization of a specific CA from a mosquito (larval *Aedes aegypti*) was published by Corena et al. (Corena et al., 2002). Traditional biochemical and enzymatic methodologies showed that the accumulation of base in the aquatic environment of the living larvae was sensitive to classic inhibitors of the α-CA family, such as methazolamide and acetazolamide (Corena et al., 2002). However, CA inhibition led to a neutralization of the highly alkaline regions of the midgut lumen. This work was performed before the publication of any mosquito genomes and hence a mosquito CA cDNA homology-cloning strategy was pursued based on sequences from several vertebrate CAs and putative CAs from the *Drosophila melanogaster* EST database. Corena et al. (Corena et al., 2002) published the first complete sequence of any insect CA from the yellow fever mosquito, *Aedes aegypti*. Sequence alignments and homology analyses showed this mosquito CA to be an α-CA possessing key elements of the classic active site including the three definitive histidine residues used in the GO (gene ontology; http://www.geneontology.org) database as a defining characteristic.

The tissue distribution of the first CA characterized from an insect turned out to be a bit of a surprise. α-CAs of vertebrate systems have been identified that can be located in one of five different compartments, reflected by gene, transcript and protein structure: soluble cytoplasmic CAs, integral transmembrane CAs, secreted soluble CAs, mitochondrial CAs, peripheral GPI-linked membrane CAs (Hewett-Emmett and Tashian, 1996). The newly cloned CA from *Aedes aegypti* was shown to be of the final type, a GPI-linked peripheral membrane protein (Seron et al., 2004). Publication of the genome of a species of mosquito, *Anophleles gambiae* (Holt et al., 2002), provided the material for ‘in silico’ analyses which confirmed the presence of an orthologous CA in *Anophleles* with the characteristic signal peptide of secreted GPI-linked proteins (Seron et al., 2004). A polyclonal antiserum to a peptide sequence unique to this CA, but conserved between the two orthologous mosquito CAs, was used to confirm a cell surface location. Surprisingly, the localization studies showed this CA to be predominantly present on the basal membranes of a specific subset of midgut muscles (Fig. 2). It is well known that the midgut of mosquito larvae (and most probably all larval insects) is invested with a tubular arrangement of striated muscle fiber bundles that extend both longitudinally and circumferentially along the basal aspect of the gut tubular epithelium (e.g. Jones, 1960). These muscles are hypothetically involved in the movement of food through the length of the gut tube *via* peristaltic and antistaltic waves of contraction (Jones, 1960). Immunolabeling for the mosquito CA showed it to be on the muscle cell surface (Seron at al., 2004). It also demonstrated that the musculature of the gut had at least two distinguishable sub-domains beyond the recognized division into longitudinal and circumferential. CA immunostaining and confocal microscopy showed that on the lateral sides of the gut tube, a subset of muscles were labeled, whereas other muscles that overlapped with these on the lateral sides and those running purely on dorsal and ventral sides did not possess the CA (Fig. 2). The physiological implications of this localization pattern and the newly identified complexity of muscle distribution in the midgut remain purely a matter for speculation at this point. The character of this specific CA was analyzed by molecular cloning with the signal/GPI linkage sequence removed. The expressed protein exhibited high activity characteristics similar to the high activity human form CAII (Fisher et al., 2006). Attempts to crystallize the protein were unsuccessful but in silico comparisons predict a three-dimensional structure very similar to that of human and mammalian CAs with known structures (ibid.).

The presence of a high activity CA positioned on the basal surface of certain muscles of the midgut integument probably has most significant impact on the balance of CO_2 and HCO_3^- in the hemolymph bathing the internal organs (mosquito larval circulation is an open system). Thus, the CA activity that was implicated in gut pH alkalization is possibly another gene product with a distinct localization. The annotation of the *Anopheles gambiae* genome currently indicates the presence of 11 α-CA and one β-CA gene sequences. β-CAs are from a different evolutionary origin than the α-CAs and are more commonly found in prokaryotes (Hewett-Emmett and Tashian, 1996). The presence of a β-CA gene in the mosquito genome is very interesting but as yet CA enzymatic activity has not been demonstrated for this mosquito gene product. Table 1 lists each of these 12 predicted CAs with assigned nomenclature (Smith et al., 2007). Fig. 3 shows a molecular phylogenetic analysis of CA genes from three genomic model

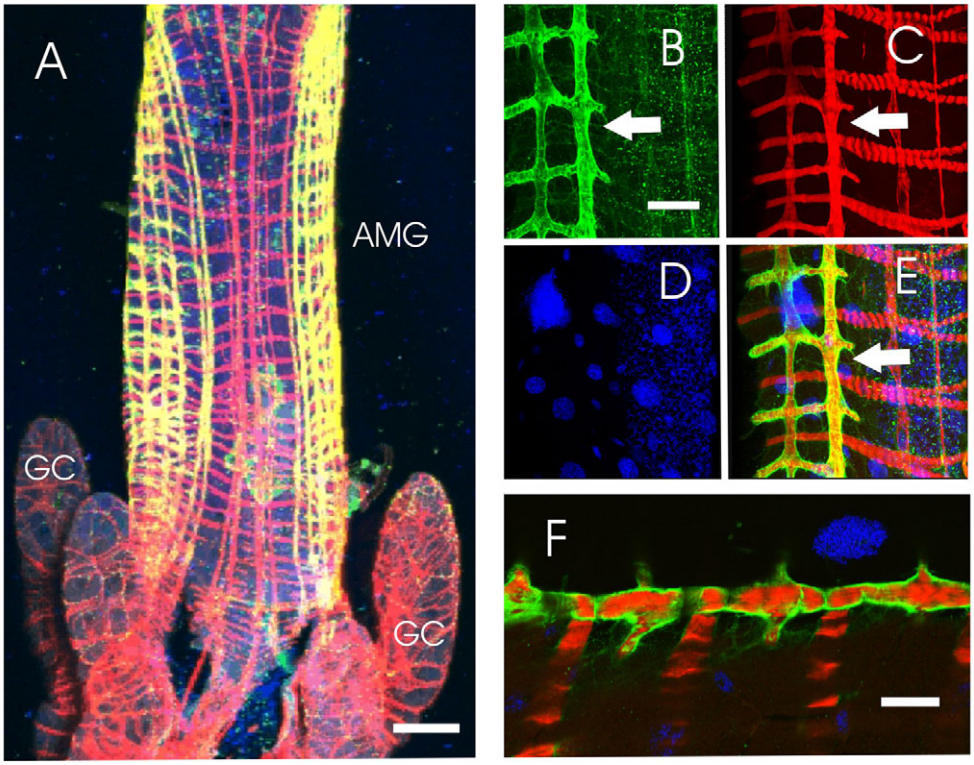

Fig. 2. Confocal immunofluorescence microscopy to identify GPI-linked carbonic anhydrase 10 (CA10) in isolated whole-mount preparations of *Anopheles gambiae* fourth instar larval gut. (A) A low magnification view (maximum projection of a stack of images) of the anterior half of the midgut. CA10 is labeled in green and muscle actin (phalloidin) in red. This panel is an overlay of the two color channels and therefore red indicates peripheral muscles of the gut and yellow indicates simultaneous labeling for CA10 and muscle actin. Note lateral complexes of muscles that are double labeled and central muscles (in this view dorsal) that are labeled for actin only. (B) A single color channel (green; CA10) at high magnification. Arrow indicates specific muscles that label for CA10. (C) The same view but with only the red color channel (phalloidin; muscle) shown. (D) Cell nuclei labeled with DRAQ5 (blue; nuclei). (E) An overlay of all three color channels demonstrating that specific gut muscles are labeled for CA10 and others are not. (F) A single plane of a stack of images at higher magnification, showing the muscle cell surface location of the CA10 labeling (green). GC, gastric caeca; AMG, anterior midgut. Arrows in B,C and E indicate the same point in the CA10-positive muscles (modified from Seron et al., 2004). Scale bars, 80 μm (A); 20 μm (B–E); 30 μm (F).

insects [*Drosophila melanogaster* (red); *Anopheles gambiae* (green); *Aedes aegypti* (blue); and human (black)]. Several observations can be made from the phyolgenetic/genome sequence analyses. First, the majority of insect CAs cluster separately from the human CAs. This probably indicates that gene duplications that led to the range of 12 mosquito CAs and 16 human CAs occurred primarily after the divergence of protostomes and deuterostomes. There are a few notable exceptions to this. Both of the mosquito and the fruit fly genomes possess two CA-related genes that cluster with two human CA-related genes. These genes have been termed CA-RPs (CA-related proteins) in the vertebrate literature (Tashian et al., 2000). They are clearly evolved from an α-CA ancestral gene by virtue of numerous sequence similarities but are in fact not active CA enzymes as they all lack one or more of the crucial active site histidine residues (ibid.). The CA-RPs are very well conserved in vertebrate evolution (ibid.) and the insect analyses show that this conservation goes back even further to a common ancestor to the protostomes and deuterostomes. The function of these conserved gene products remains unresolved.

The third α-CA of the mosquito that shows an evolutionary relationship with some human CAs is AgCA9 [orthologous to *Aedes aegypti* Ae4930PA and *Drosophila melanogaster* CG7820PA (Ensemble designations)]. AgCA9 clusters with several human CAs including CAVII which is considered to be the human CA most similar to an ancestral gene of origin (Hewett-Emmett and Tashian, 1996). Clustering of three orthologous insect genes with human CAVII is consistent with the hypothesis that this insect CA is also relatively close to the evolutionary origins of the α-CA gene family (Smith et al., 2007). The cDNA for the mRNA of AgCA9 was cloned and its expression analyzed (ibid.). Quantitative PCR, microarray and *in situ* hybridization analyses showed the message to be differentially expressed in the various regions of the larval midgut with highest levels of expression in the GC and the rectum (Smith et al., 2007; Neira Oviedo et al., 2008). To examine the distribution of the actual enzyme in an effort to see if it might play a role in gut alkalization and ion homeostasis, a polyclonal antibody was prepared to a specific unique amino acid sequence of AgCA9 and was used to determine the protein distribution using immunohistochemistry and confocal microscopy (ibid.) (Smith et al., 2008). Fig. 4 shows a summary of the localization studies. In longitudinal paraffin sections of fourth instar larvae, immunofluorescence for AgCA9 is evident in several key locations. First, AgCA9 immunoreactivity is evident in the cells and lumen of the GC. There is also an apparent robust accumulation of the antigen in the ectoperitrophic space (ectoperitrophic fluid) which stands between the PT and the cells of the gut epithelium. Another location of strong expression is in specific cells of the rectum. Malpighian tubules (MTs) also show AgCA9 immunoreactivity in the so-called principal cells (Smith et al., 2007).

Table 1. Carbonic anhydrase genes annotated in the *Anopheles gambiae* genome

	ENSANGP000000	ENSANGT000000	ENSANGG000000	New convention	GenBank accession no.
α-CA	01278	01278	01096	AgCA1	
CA-RP	01574	01574	01335	AgCA-RP2	
α-CA	10017	10017	07528	AgCA3	
α-CA	11013	11013	08524	AgCA4	
CA-RP	11908	11908	09419	AgCA-RP5	
α-CA	12957	12957	10468	AgCA6	DQ518577
α-CA	14919	14919	12430	AgCA7	
α-CA	18999	18999	16510	AgCA8	
α-CA	19212	19212	16723	AgCA9	DQ518576
α-CA	21313	21313	18824	AgCA10	AY280612
α -CA	21739	21739	19250	AgCA11	AY280613
β-CA	29115	28335	15071	AgCAb	EF065522

Two of the α-CAs are CA-related proteins (CA-RPs). One β-CA is also listed. The Ensembl annotation numbers for the coded proteins (second column from left), transcript (third column from left) and gene (fourth column from left) are given along with the new notation for the genes as given by Smith et al. (Smith et al., 2007). The GenBank accession numbers are shown in the final column on the right for each transcript that has been fully cloned by the Linser laboratory.

Identification of the location of AgCA9 provided a new appreciation for the structure and function of the anopheline rectum. Fig. 4 shows whole-mount and longitudinal section preparations of *Anopheles gambiae* larval recta labeled for various proteins. Fig. 4B and C show a single whole-mount rectum labeled for AgCA9 (green) and the α5-subunit of Na^+K^+-ATPase (Smith et al., 2007; Okech et al., 2008). The striking observation made was that the two proteins are expressed in mutual exclusion in two subsets of the rectum epithelium. AgCA9 is found only in a dorsal, anterior grouping of rectum cells (termed DAR cells) and the ATPase is found exclusively in the remaining AgCA9-negative epithelial cells. Fig. 4D shows a longitudinal section at high magnification, again showing AgCA9 in DAR cells (green) and Na^+K^+-ATPase (red) in all other rectal epithelial cells (non-DAR cells). Fig. 4E shows a whole-mount in which DAR cells (AgCA9; green) and the basal surface musculature of the rectum is labeled

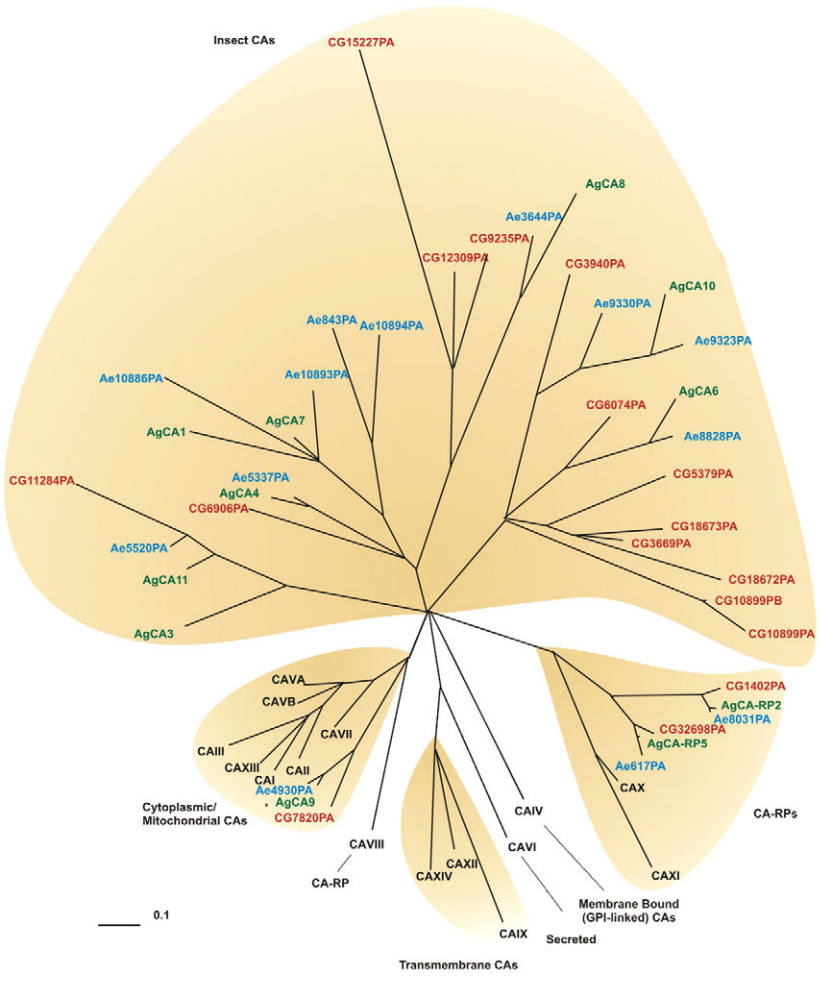

Fig. 3. Molecular phylogenetic analysis of carbonic anhydrase (CA) gene sequences from *Homo sapiens* (black), *Anopheles gambiae* (green), *Aedes aegypti* (blue) and *Drosophila melanogaster* (red) (Smith et al., 2007).

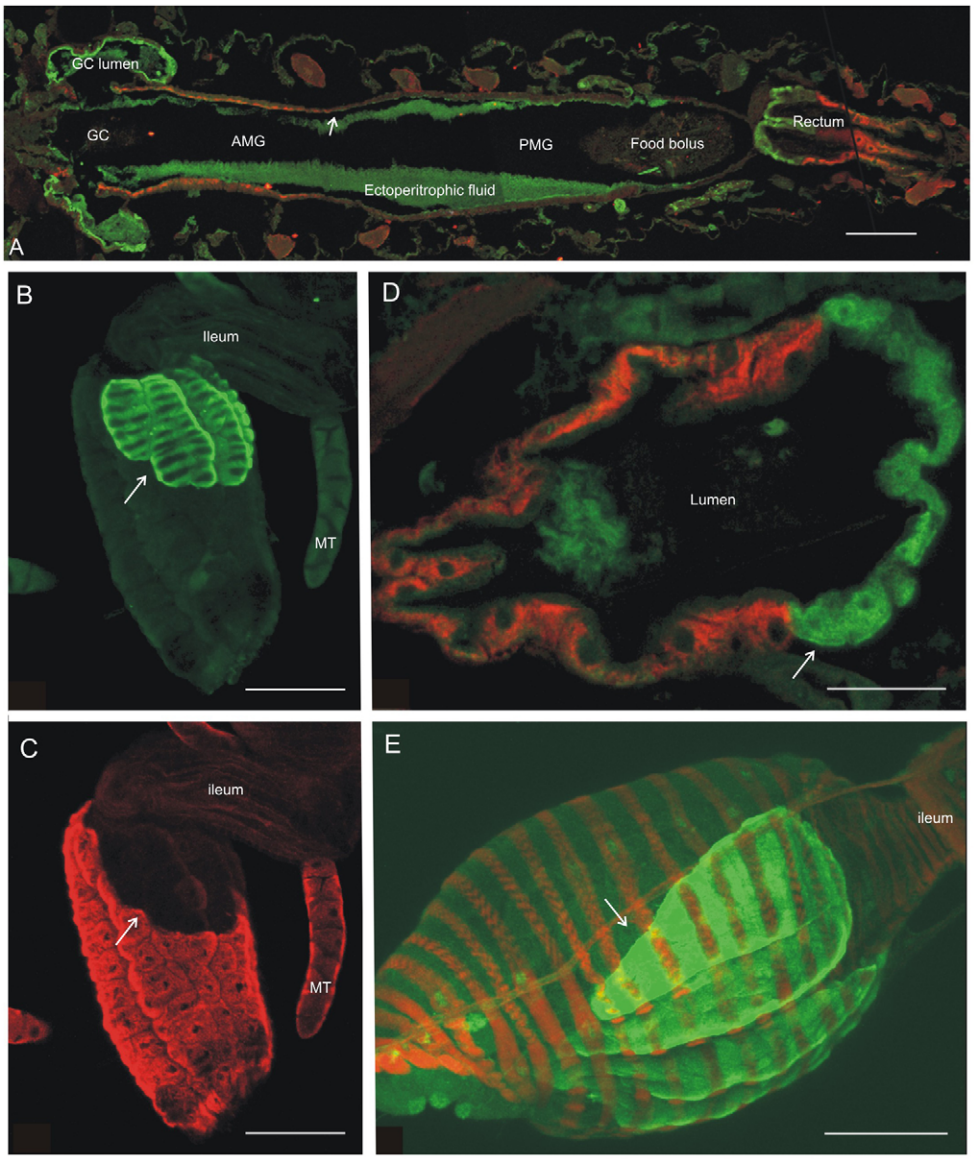

Fig. 4. Confocal immunofluorescence microscopy to identify AgCA9 in sections and whole mounts of *Anopheles gambiae* larval gut tissues. (A) A longitudinal section of a fourth instar larva labeled for AgCA9 (green) and Na^+/K^+-ATPase (red). Note prominent AgCA9 labeling in the cells and lumen of the gastric caeca, the ectoperitrophic fluid and the anterior portion of the rectum. (B,C) Two views of a whole-mount preparation of larval rectum showing AgCA9 (green; B) and Na^+/K^+-ATPase (C). (D) A high magnification cross-section of the rectum with the anterior to the right showing the mutually exclusive labeling for CA-9 (green) and Na^+/K^+-ATPase (red). The dorsal anterior rectum (DAR) cells are indicated by the arrow. (E) A high magnification view of an isolated rectum with the DAR cells (arrow) labeled for AgCA9 and the external rectal musculature labeled in red (phalloidin). GC, gastric caeca; AMG, anterior midgut; PMG, posterior midgut; MT, Malpighian tubules. All arrows indicate the DAR cells. Scale bars, 150 μm (A–C), 75 μm (D,E).

with TRITC-phalloidin (red). These analyses demonstrated a functional differentiation between specific cell types of the anopheline rectum that had not previously been described (Smith et al., 2007).

Ion regulation in mosquito larvae can be a very dynamic function in survival. Many types of mosquito larvae thrive in aquatic environments that can vary greatly in many parameters including pH, osmolarity and ionic milieu. Through the course of larval development, such events as rainfall or evaporation can dramatically change the nature of the osmotic environment. To cope with such changes, mosquito larvae need to adapt to these changes. The rectum is one of the key organs in controlling the final composition of the mosquitoes excretions and hence the composition of the hemolymph. In species of Anopheline mosquitoes that can tolerate wide swings in the osmotic strength of their habitats, protein distribution adjustments take place (Smith et al., 2008). *Anopheles albimanus*, as a specific example, is a salt-tolerant mosquito species. When raised in freshwater, the distribution of AgCA9 and Na^+K^+-ATPase are as described above for *Anopheles gambiae* (see Fig. 4). If, by contrast, the same species is raised in 50% sea water, the Na^+K^+-ATPase primary location shifts from the non-DAR cells into the DAR cells (Smith et al., 2008). The specific physiological implications of shifting the ATPase activity from cells lacking AgCA9 to those that possess the CA is under active investigation but unclear at the time of writing. It seems probable that other enzymes and transporters that play roles in ionic absorption or excretion are also impacted by altering the aquatic environment of the larvae. The full range of important biological systems in this context remains to be studied.

The detection of a soluble CA in the ectoperitrophic space of the gut was unexpected and has implications in the regulation of gut luminal pH. The sequence of AgCA9 has no apparent signal peptide that would be indicative of a secreted protein. CAVI, the human/mammalian salivary CA does indeed possess such a characteristic sequence (Tashian et al., 2000). Certainly, the absence of a signal peptide does not preclude certain types of secretion, such as merocrine exocytosis in insects (Hung et al., 2000). The presence of a soluble CA in the ectoperitrophic space may be central to the pH alkalization paradigm. The presence of high transepithelial potentials and numerous mitochondria in the

gut epithelial cells (Harvey, 1992; Wieczorek, 1992) are both indicative of very high metabolic activity in these cells. Under normal aerobic conditions the final product of such activity will be CO_2. In large, complex organs, metabolic CO_2 is typically converted to carbonic acid and/or bicarbonate intracellularly, which is then transported in a vectorial fashion by an anion exchanger such as AE1 (in human) out of the cell (Romero et al., 2004). In a very small tissue such as a single-cell epithelial tube, the diffusion of gaseous CO_2 into an extracellular environment containing high concentrations of a soluble CA, which would rapidly ionize the catabolite, might obviate the need for specific anion exchange/transport mechanisms. Nevertheless, the mosquito genome does indeed encode proteins whose motif and homology analyses have lead to annotation as putative anion exchangers of the type frequently found in association with CA-containing tissues and cells.

Fig. 5 shows a summary analysis of putative members of the SLC4A family of anion exchangers in the mosquito genome. Three distinct gene sequences of the *Anopheles gambiae* genome have been annotated as members of this family of anion exchangers [AY280611, EU068741 (GenBank) and AGAP006968 (Ensembl)] with one gene predicted to exist as at least two distinct splice variants. There is evidence (M.N.O., unpublished observations) that several splice variants exist in the mRNA pool from gut samples. The predicted transcripts show a high degree of homology with the single insect anion exchanger characterized to date, so-called NDAE1 from *Drosophila melanogaster* (Romero et al., 2000; Sciortino et al., 2001). The *Aedes aegypti* genome contains at least two genes with additional splice variants predicted. Multiple potential splice variants also muddle the analysis of related genes in *Drosophila melanogaster* as well (Fig. 5). Fig. 5 also shows a hydropathy map analysis of the *Anopheles gambiae* gene *AgAE1* compared with that of *Ndae1* from *Drosophila melanogaster*. The structural homology between the two is obvious. This figure also shows a two-dimensional model of the amino acid sequence depicting a hypothetical structure very similar to that of other examples of Cl^-/HCO_3^- exchangers (e.g. Romero et al., 2000; Romero et al., 2004). Cloning of a full-length cDNA of the gene annotated as *AgAE1* has been problematic and we have evidence of numerous splice variants of this anion exchanger. The carboxyl terminus cytoplasmic tail of AgAE1 is highly variable in cloning analyses and the sequence shown is only one of several that have emerged in separate cloning efforts (T.J.S. and M.N.O., unpublished observations). Work in progress will hopefully provide

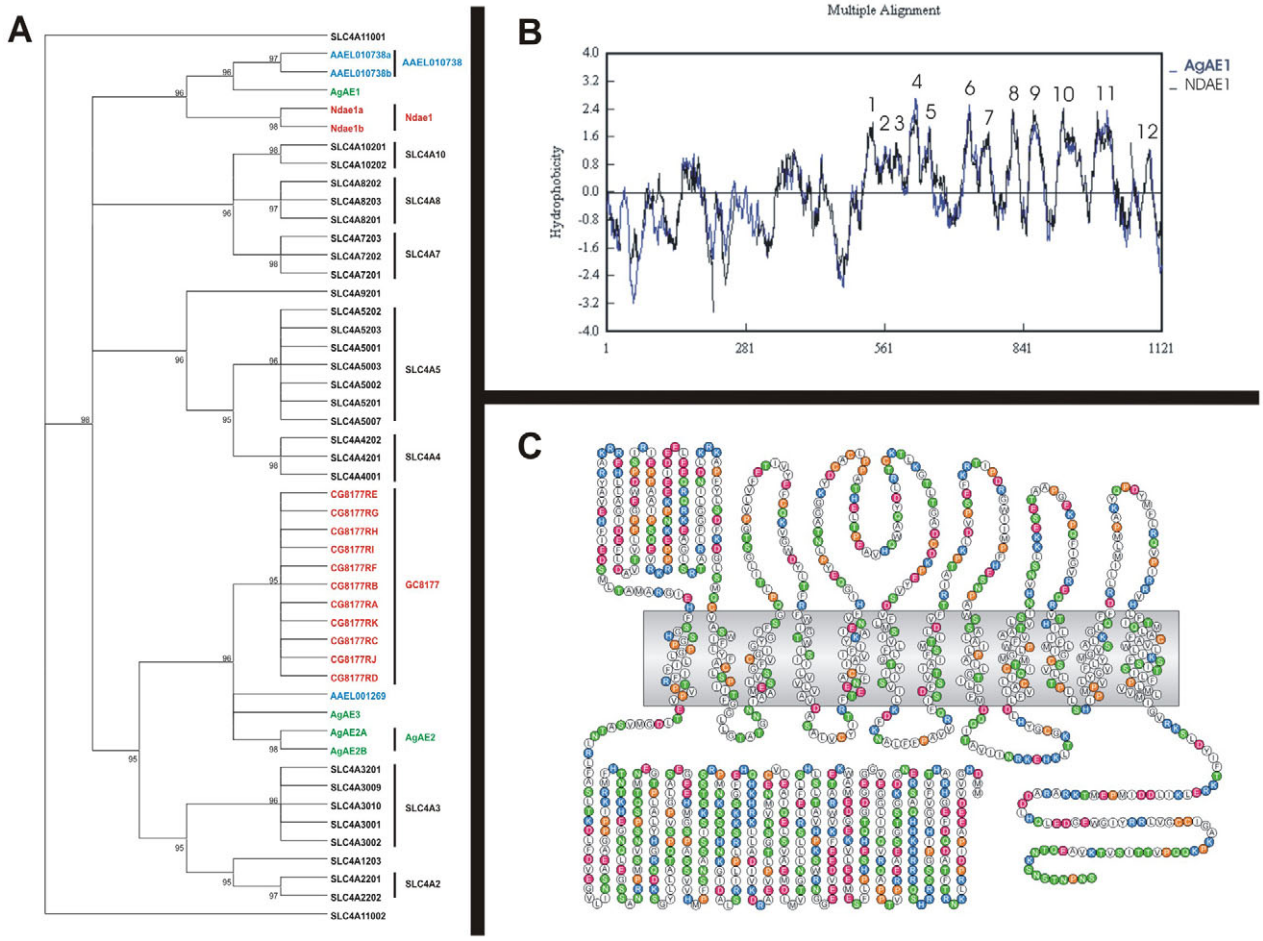

Fig. 5. Analyses of the members of the SLC4A family of anion exchangers/transporters. (A) A molecular phylogenetic tree of the annotated members of this gene family from *Anopheles gambiae* (green), *Aedes aegypti* (blue), *Drosophila melanogaster* (red) and *Homo sapiens* (black). The human genes include putative and confirmed splice variants of several genes. Similarly, several putative splice variants of the *Drosophila* genes are included. The tree was generated using MrBayes, 1.5 million iterations and the JTT amino acid substitution model. All nodes represent at least 95% probability but the branch lengths are arbitrary. (B) Hydropathy plots generated for *Drosophila* NDAE1 (black) and *Anopheles* AgAE1 (blue). (C) The hypothetical secondary structure of one putative splice variant of AgAE1.

both physiological characterization as well as immunolocalization of this potentially important element of acid–base regulation in the larval mosquito gut. Recent microarray-based transcriptome analyses (Neira Oviedo et al., 2008) indicate that at least two out of the three annotated anion exchanger genes in *Anopheles gambiae* larvae exhibit differential expression in the regions of the gut. Fig. 6 shows microarray data for all three of the specific AE-type genes. In this analysis, RNA isolated from dissected regions of the gut (GC, AMG, PMG and HG) was compared to whole gut (W) using Affymetrix microarray (Neira Oviedo et al., 2008). The two AE genes designated as *AE1* (AgAE1 in Fig. 5) and *AE2* (AgAE2 in Fig. 5) showed enrichment in specific gut regions relative to the whole gut sample. *AgAE1* was enriched in the HG sample (which includes the rectum and MT, which are locations of *CA9*) whereas *AgAE2* showed enrichment in the GC sample (an additional site of *AgCA9* expression). Although *AgAE3* showed no specific enrichment in gut regions, it is not safe to interpret the data as indicating that the gene is not expressed in the gut. By the 'present' *versus* 'absent' analysis generated by the Affymetrix methodology, *AE3* is 'absent' from the gut and thus seems to be more abundant in tissues other than the gut (Neira Oviedo et al., 2008). Early physiological analyses indicated that gut alkalization in the mosquito larva is affected by the generalized pharmacological blocker of anion exchangers DIDS (Boudko et al., 2001; Corena et al., 2004). It remains to be seen whether any of the predicted members of the AE gene family in mosquitoes are specifically involved in pH regulation in any or all regions of the larval alimentary canal.

Another family of solute carriers, the SLC26 gene family [multifunctional anion exchangers (e.g. Mount and Romero, 2004)] has potentially important roles in pH balance and anion transport. Indeed, the *Anopheles gambiae* genome has annotated three members of this specific gene family [AGAP010344, AGAP010389 and AGAP010725 (Ensembl)]. Transcripts levels of all three of these genes show differential enrichment when queried against the larval alimentary canal microarray expression profile (Neira Oviedo et al., 2008). Transcripts levels of both AGAP010389 and AGAP010344 are enriched in the hindgut relative to other gut regions, relative to the salivary glands (our unpublished observations) and relative to the whole insect (Neira Oviedo et al., 2008). The transcript level of AGAP010725 is enriched in the salivary glands (our unpublished observations) and the GC (Neira Oviedo et al., 2008). Little is known about this family of SLCs in mosquitoes and we are actively pursuing their characterization.

Summary, conclusions and the road ahead

Fig. 7 presents a summary of some of the data presented within this review. At the top of the figure is a cartoon of the larval mosquito gut with the luminal pH zones color-coded and the locations of two different α-CAs (AgCA9 and AgCA10) indicated. Although it is clear that CA activity is central to pH regulation in the gut, it is presumed that these two specific CAs play important roles. We know that other CAs are present in the gut but their distribution and specific roles (and the roles of already characterized CAs) remain to be elucidated. The bottom half of Fig. 7 shows the distributions of a few of the known enzymes and transporters that probably play important roles in gut pH regulation. Four stylized cells are shown: GC, AMG, PMG and muscle. The differential distribution of key proteins such as V-ATPase (V), Na^+/K^+-ATPase (Na/K) and the Na^+/H^+ antiporter (NHA) energize the transepithelial potential and provide the driving force for producing extreme pH gradients along the length of the gut (Harvey, 1992; Wieczorek, 1992; Harvey et al., 2009). CA activity rapidly converts metabolic CO_2 into ionic forms that are then vital to buffering the pH ranges of the gut lumen. CAs exist within specific cell types of the gut as well as in extracellular compartments. This wide range of distribution is indicative of the important role that CA plays at all stages of ion and pH regulation in the larval mosquito gut. It should be noted that a recent review (Onken and Moffett, 2009) compared analyses

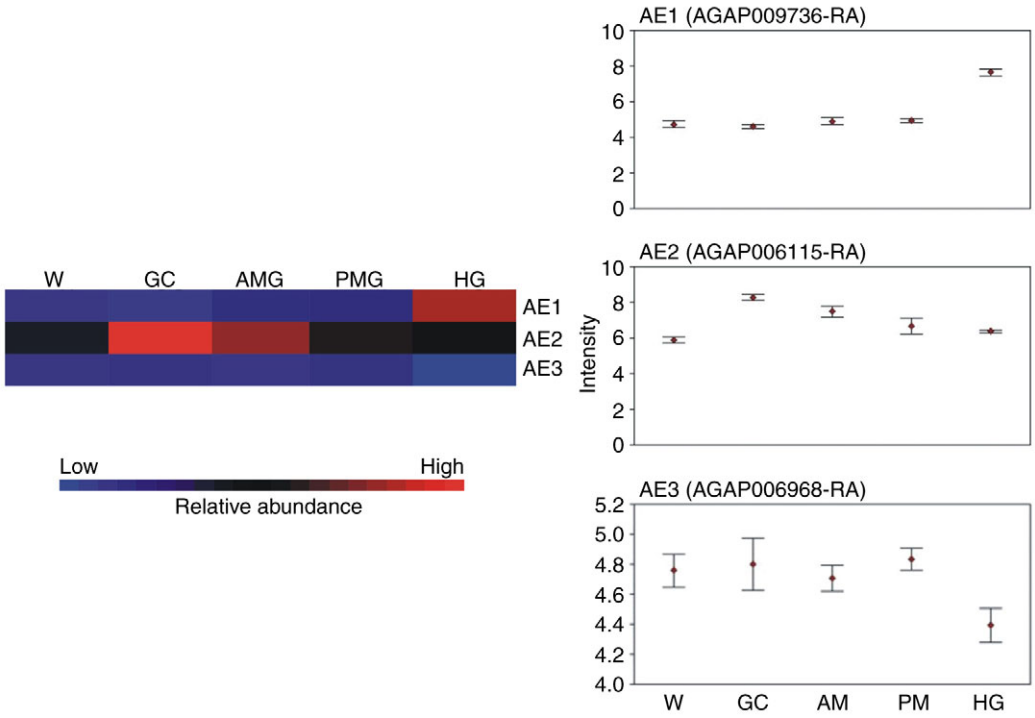

Fig. 6. A DNA microarray-based analysis of regionalized expression of the three annotated SLC4A gene family members in *Anopheles gambiae* larvae. Whole larval gut expression (W) is compared with transcriptome levels in the gastric caeca (GC), anterior midgut (AMG), the posterior midgut (PMG) and the Malpighian tubule/hindgut (HG) (Neira Oviedo et al., 2008). A color scale indicates the transcriptome levels.

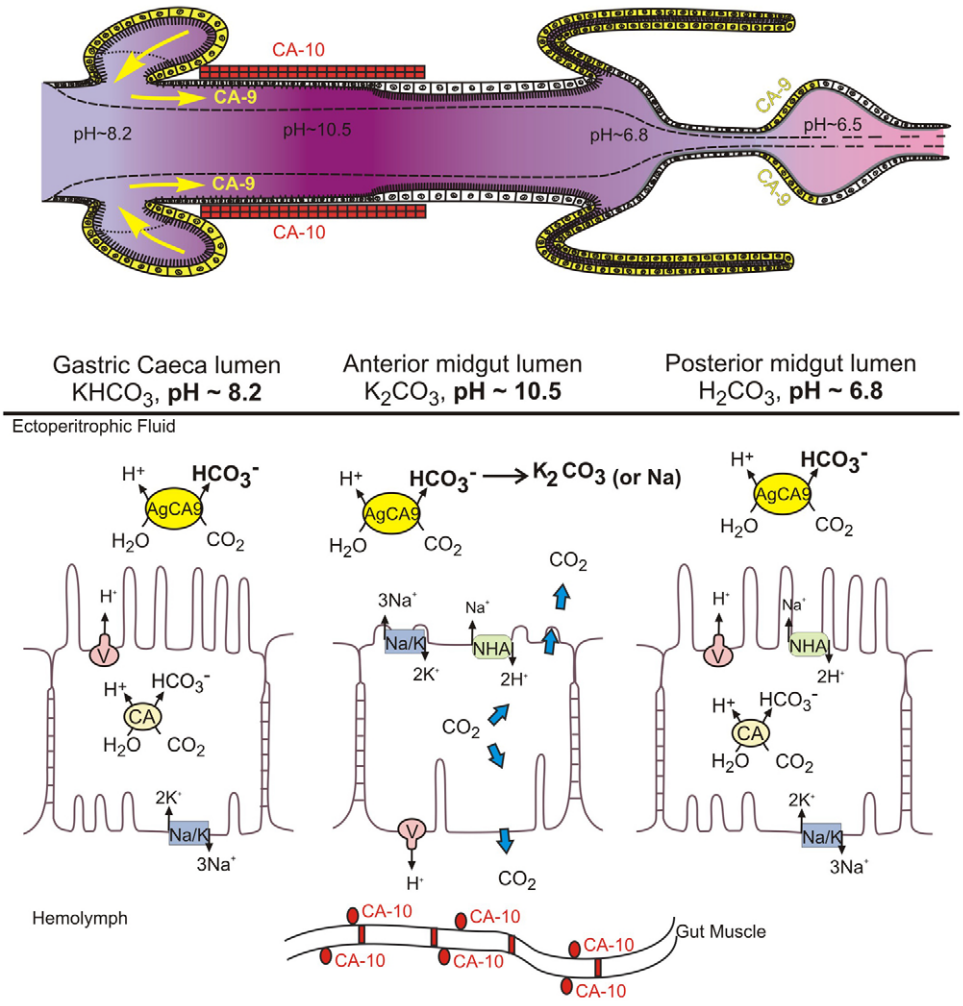

Fig. 7. Partial model of CA distribution in gut (upper drawing) and physiological impact (lower drawing). Blue arrows indicate hypothetical diffusion of metabolic CO_2, only indicated in AMG cells which lack an α-CA. V, V-ATPase; Na/K, Na^+/K^+-ATPase; NHA, Na^+/H^+ antiporter; CA, carbonic anhydrase.

described herein with analyses of the *Aedes aegypti* isolated gut preparation. In this preparation, the anterior portion of the gut tube (i.e. the AMG) was isolated from other organismal elements such as luminal contents, PT, ectoperitrophic fluid and all of the upstream and downstream contributions from other regions of the alimentary canal. The authors discuss both published and 'unpublished' data that infers that isolated AMG can continue to alkalize the gut lumen even in the presence of CA inhibition. The role of a soluble CA in the ectoperitrophic fluid is brought into question. But in this simplified system (i.e. the perfused AMG) it seems probable that the continued presence of a vectorial capacity to move H^+ ions from the apical luminal side to the basal hemolymph side could alkalize the lumen with no apparent need for locally produced HCO_3^-. A basally located V-ATPase (Harvey 1992, Zhuang et al., 1999) and an apical exchange mechanism for cations could produce measurable alkalization of the AMG lumen. Also, the assumed effectiveness of pharmacological inhibitors that have never been well characterized in mosquito systems creates concern. As Onken and Moffett (Onken and Moffett, 2009) state, more is probably unknown than known about this system with many avenues to pursue. Perhaps, until potency and specificity of these pharmacological agents have been demonstrated in the mosquito system, the pursuit of a clear model will be aided by further molecular (genetic and proteomic) characterization of known and potential effectors in this complex biological system. Among our several goals and ongoing investigations is the application of reverse genetic techniques to query the roles of specific gene products in pH and ion balance in the alkaline midgut of the mosquito larva.

This research was supported by NIH grant number 5RO1AI45098 to P.J.L.
Deposited in PMC for release after 12 months.

References

Black, W. C. and Kondratieff, B. C. (2005). Evolution of arthropod disease vectors. In *Biology of Disease Vectors*, 2nd edn (ed. W. C. Marquardt), pp. 9-23. Burlington, MA: Elsevier.
Boudko, D. Y., Moroz, L. L., Harvey, W. R. and Linser, P. J. (2001). Alkalinization by chloride/bicarbonate pathway in larval mosquito midgut. *Proc. Natl. Acad. Sci. USA* **98**, 15354-15359.
Bradley, T. J. (1987). Physiology of osmoregulation in mosquitoes. *Annu. Rev. Entomol.* **32**, 439-462.
Clark, T. M., Vieira, M. A., Huegel, K. L., Flury, D. and Carper, M. (2007). Strategies for regulation of hemolymph pH in acidic and alkaline water by the larval mosquito *Aedes aegypti* (L.) (Diptera;Culicidae). *J. Exp. Biol.* **210**, 4359-4367.
Clements, A. N. (1992). *The Biology of Mosquitoes*, vol. 1 (ed. A. N. Clements). London: Chapman Hall.
Corena, M. P., Seron, T. J., Lehman, H. K., Ochrietor, J. D., Kohn, A., Tu, C. and Linser, P. J. (2002). Carbonic anhydrase in the midgut of larval *Aedes aegypti*: cloning, localization and inhibition. *J. Exp. Biol.* **205**, 591-602.
Corena, M. P., Fiedler, M. M., VanEkeris, L. and Linser, P. J. (2004). A comparative study of carbonic anhydrase in the midgut of mosquito larvae. *Comp. Biochem. Physiol.* **137**, 207-225.
Dadd, R. H. (1975). Alkalinity within the midgut of mosquito larvae with alkaline-active digestive enzymes. *J. Insect Physiol.* **46**, 1847-1853.
Dow, J. A. T. (1984). Extremelu high pH in the biological systems: a model for carbonate transport. *Am. J. Physiol.* **246**, R633-R635.
Fisher, S. Z., Tariku, I., Case, N. M., Tu, C., Seron, T., Silverman, D. N., Linser, P. J. and McKenna, R. (2006). Expression, purification, kinetic, and structural characterization of an alpha-class carbonic anhydrase from Aedes aegypti (AaCA1). *Biochim. Biophys. Acta* **1764**, 1413-1419.

Harvey, W. R. (1992). Physiology of V-ATPases. *J. Exp. Biol.* **172**, 1-17.
Harvey, W. R. (2009). Voltage coupling of primary H^+ V-ATPases to secondary Na^+- or K^+-dependent transporters. *J. Exp. Biol.* **212**, 1620-1629.
Hewett-Emmett, D. and Tashian, R. E. (1996). Functional diversity, conservation and convergence in the evolution of the alpha-, beta- and gamma-carbonic anhydrase gene families. *Mol. Phylogenet. Evol.* **5**, 50-77.
Holt, R. A., Subramanian, G. M., Halpern, A., Sutton, G. G., Charlab, R., Nusskern, D. R., Wincker, P., Clark, A. G., Ribeiro, J. M., Wides, R. et al. (2002). The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science* **298**, 129-149.
Hung, C., Lin, T. and Lee, W. (2000). Morphology and ultrastructure of the alimentary canal of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera:Tephritidae)(2): The structure of the midgut. *Zool. Stud.* **39**, 387-394.
Jones, J. C. (1960). The anatomy and rhythmical activities of the alimentary canal of *Anopheles* larvae. *Ann. Entomol. Soc. Am.* **53**, 459-474.
Killeen, G. F., Fillinger, U. and Knols, B. G. J. (2002). Advantages of larval control for African malaria vectors: low mobility and behavioural responsiveness of immature mosquito stages allow high effective coverage. *Malar. J.* **1**, 8.
Mount, D. B. and Romero, M. F. (2004). The SLC26 gene family of multifunctional anion exchangers. *Pflugers Arch.* **447**, 710-721.
Neira Oviedo, M., VanEkeris, L., Corena-Mcleod, M. D. and Linser, P. J. (2008). A microarray-based analysis of transcriptional compartmentalization in the alimentary canal of *Anopheles gambiae* (Diptera: Culicidae) larvae. *J. Insect. Mol. Biol.* **17**, 61-72.
Okech, B. A., Boudko, D. Y., Linser, P. J. and Harvey, W. R. (2008). Cationic pathway of pH regulation in larvae of *Anopheles gambiae*. *J. Exp. Biol.* **211**, 957-968.
Onken, H. and Moffett, D. F. (2009). Revisiting the cellular mechanisms of strong luminal alkalinization in the anterior midgut of larval mosquitoes. *J. Exp. Biol.* **212**, 373-377.
Romero, M. F., Henry, D., Nelson, S., Harte, P. J. and Sciortino, C. M. (2000). Cloning and characterization of a Na^+ driven anion exchanger (NDAE1): a new CNS bicarbonate transporter. *J. Biol. Chem.* **275**, 24552-24559.
Romero, M. F., Fulton, C. M. and Boron, W. F. (2004). The SLC4 family of HCO_3^- transporters. *Eur. J. Physiol.* **204**, 495-509.
Sciortino, C. M., Shrode, L. D., Fletcher, B. R., Harte, P. J. and Romero, M. F. (2001). Localization of endogenous and recombinant Na^+-driven anion exchanger protein NDAE1 from *Drosophila melanogaster*. *Am. J. Physiol. Cell Physiol.* **281**, 449-463.
Seron, T. J., Hill, J. and Linser, P. J. (2004). A GPI-linked carbonic anhydrase expressed in the larval mosquito midgut. *J. Exp. Biol.* **207**, 4559-4572.
Smith, K. E., VanEkeris, L. A. and Linser, P. J. (2007). Cloning and characterization of AgCA9, a novel α-carbonic anhydrase from *Anopheles gambiae* Giles *sensu stricto* larvae. *J. Exp. Biol.* **210**, 3919-3930.
Smith, K. E., VanEkeris, L. A., Okech, B. A., Harvey, W. R. and Linser, P. J. (2008). Larval anophleine mosquito recta exhibit a dramatic change in localization patterns of ion transport proteins in response to shifting salinity: a comparison between anopheline and culicine larvae. *J. Exp. Biol.* **211**, 3067-3076.
Strange, K., Phillips, J. E. and Quamme, G. A. (1982). Active HCO_3^- secretion in the rectal salt gland of a mosquito larva inhabiting $NaHCO_3$-CO_3 lakes. *J. Exp. Bol.* **101**, 171-186.
Tashian, R. E., Hewett-Emmett, D., Carter, N. D. and Bergenhem, N. C. (2000). Carbonic anhydrase (CA)-related proteins (CA-RPs), and transmembrane proteins with CA of CA-RP domains. In *The Carbonic Anhydrases: New Horizons* (ed. W. R. Chegwidden, N. D. Carter and Y. H. Edwards), pp. 105-120. Basel: Birkhäuser.
Wieczorek, H. (1992). The insect V-ATPase, a plasma membrane proton pump energizing secondary active transport: molecular analysis of electrogenic potassium transport in the tobacco hornworm midgut. *J. Exp. Biol.* **172**, 335-344.
Wigglesworth, V. B. (1932). On the function of the so called "rectal glands" of insects. *J. Cell Sci.* **75**, 131-150.
Zhuang, Z., Linser, P. J. and Harvey, W. R. (1999). Antibody to H^+ V-ATPase subunit E colocalizes with portasomes in alkaline larval midgut of a freshwater mosquito (*Aedes aegypti*). *J. Exp. Biol.* **202**, 2449-2460.