

Carbonic anhydrase in the adult mosquito midgut

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Summary

We have previously demonstrated the involvement of carbonic anhydrase (CA) in larval mosquito midgut physiology. In this study, we used Hansson's histochemistry to examine the distribution of the enzyme in the midgut of *Aedes aegypti*, *Aedes albopictus*, *Culex quinquefasciatus*, *Culex nigripalpus*, *Ochlerotatus taeniorhynchus*, *Anopheles albimanus* and *Anopheles quadrimaculatus* adult mosquitoes. Additionally, we quantitated CA content in the anterior and posterior midgut of adult males and females from these species using the ¹⁸O isotope exchange method coupled to mass spectrometry. We also tested the effect of CA inhibitors

such as methazolamide and acetazolamide in the alkalization of the midgut in females from these species. Our results indicate that CA is present in the midgut of adults from the species studied and that it appears to be preferentially associated with the posterior midgut in both males and females. CA inhibitors appear to have a profound effect on midgut pH indicating that this enzyme might play a key role in the maintenance of this pH.

Key words: mosquito, carbonic anhydrase, histochemistry, midgut pH, carbonic anhydrase inhibitor.

Introduction

Mosquitoes reproduce by laying eggs. While in some species both the male and female adults feed on nectar, only the female requires a blood meal in order to produce viable progeny. Upon feeding, the blood meal is stored in the midgut while meals of nectar are diverted to the crop (Clements, 1992). Not surprisingly, the midguts of adult females and males in these species present differences. The male midgut is smaller than the female midgut, although in both cases it consists of a narrow tube-like anterior region and a flask-shaped posterior portion surrounded by a delicate network of circular and longitudinal muscle cells along with trachea. Male and female midguts are composed of a single-cell epithelium consisting of three types of cells. Most of these cells are 'columnar cells', with smaller numbers of undifferentiated 'regenerative cells' and endocrine cells (Nuttall and Shipley, 1903; Thompson, 1905; Christophers, 1960). In terms of function, most of the attention has focused on the female midgut. Its function has been associated with storage and digestion of the blood meal, secretion of digestive enzymes and synthesis and secretion of the peritrophic membrane, nutrient absorption and diuresis (Clements, 1992). Blood feeding in turn, is linked to the transmission of viruses as well as *Plasmodium* and other parasites.

Although the pH inside the larval midgut has been described in detail (Boudko et al., 2001; Charles and de Barjac, 1981; Dadd, 1975; Ramsay, 1950), a comprehensive study on the pH inside the adult mosquito midgut has not been presented. The importance of maintaining pH for physiological processes that occur inside the larval midgut and in survival of the larvae has been described recently (Boudko et al., 2001; Corena et al., 2002).

Little is known about pH maintenance inside the adult mosquito midgut. Although the presence of trypsin and aminopeptidase, as well as esterases and chitinase, has been demonstrated in the adult mosquito midgut (Lemos et al., 1996; Mourya et al., 2003; Okuda et al., 2002; Villalon et al., 2003), there are no reports on the presence of CA and the role of this enzyme in the maintenance of pH inside the midgut. We have previously localized, cloned and measured CA content in the midgut of larval *Ae. aegypti* (Corena et al., 2002) as well as in other species of mosquito larvae (Corena et al., 2004). We have found differences in CA distribution among different species of larvae. However, mosquito larvae are morphologically and physiologically different from adult mosquitoes. The purpose of this study was to determine the pH, localize and measure CA content in the anterior and posterior midgut of adult male

and female mosquitoes, and to determine if CA plays a role in the maintenance of this pH. Additionally, this study aimed to determine if CA distribution between male and female midguts, as well as between the anterior and posterior regions of the midgut, was different. We used Hansson's histochemical method (Hansson, 1967) and the ^{18}O -isotope exchange method coupled to mass spectrometry (Silverman and Tu, 1986) for this purpose. We have used these techniques to measure CA activity in larval mosquitoes and found significant differences between the anterior and posterior regions in several species of larvae (Corena et al., 2004).

Here we present for the first time a detailed study of pH inside the adult mosquito midgut and the localization and measurement of CA activity in the anterior and posterior regions of female and male midguts from seven different species (*Ae. aegypti*, *Ae. albopictus*, *An. albimanus*, *An. quadrimaculatus*, *Cx. nigripalpus*, *Cx. quinquefasciatus* and *Oc. taeniorhynchus*). We also present data on the importance of CA in the maintenance of pH inside the midgut.

Materials and methods

Mosquito species

Mosquito larvae from different species were used in this study based on availability. *Ae. aegypti* and *Ae. albopictus* (family *Culicidae*, subfamily *Culicinae*, tribe *Culicini*, genus *Aedes*, subgenus *Stegomyia*), *Cx. quinquefasciatus* and *Cx. nigripalpus* (family *Culicidae*, subfamily *Culicinae*, tribe *Culicini*, genus *Culex*, subgenus *Culex*), *An. albimanus* (family *Culicidae*, subfamily *Anophelinae*, genus *Anopheles*, subgenus *Nyssorhynchus*), *An. quadrimaculatus* (family *Culicidae*, subfamily *Anophelinae*, genus *Anopheles*, subgenus *Anopheles*) and *Oc. taeniorhynchus* (family *Culicidae*, subfamily *Culicinae*, tribe *Culicini*, genus *Ochlerotatus*, subgenus *Ochlerotatus*).

Larvae were raised from eggs obtained from colonies maintained at the United States Department of Agriculture (USDA) in Gainesville, Florida. Larvae were reared in tapwater or 50% seawater (in the case of *Oc. taeniorhynchus*) at $25\pm 1^\circ\text{C}$. Adults were allowed to emerge and were separated before mating occurred. Both males and females were maintained at $25\pm 1^\circ\text{C}$ and fed with 10% sucrose in water.

Solutions and test formulations

Hemolymph substitute solution (HSS) was prepared according to Clark et al. (1999). The solution consisted of 42.5 mmol l^{-1} NaCl, 3.0 mmol l^{-1} KCl, 0.6 mmol l^{-1} MgSO_4 , 5.0 mmol CaCl_2 , 5.0 mmol l^{-1} NaHCO_3 , 5.0 mmol l^{-1} L-succinic acid, 5.0 mmol l^{-1} L-malic acid, 5.0 mmol l^{-1} L-proline, 9.1 mmol l^{-1} L-glutamine, 8.7 mmol l^{-1} L-histidine, 3.3 mmol l^{-1} L-arginine, 10.0 mmol l^{-1} dextrose, and 25 mmol l^{-1} HEPES, adjusted to pH 7.0 with NaOH.

Artificial seawater (100%) was prepared freshly every time. Final concentrations were 411.04 mmol l^{-1} NaCl, 9.94 mmol l^{-1} KCl, 10.25 mmol l^{-1} CaCl_2 , 53.6 mmol l^{-1} MgCl_2 , 28.24 mmol l^{-1} Na_2SO_4 ; the pH was adjusted to

8.1–8.3 with NaOH. This stock solution was diluted to prepare 50% artificial seawater, used as a culture medium.

CA inhibitors [methazolamide (MTZ) and acetazolamide (ACZ)], dimethylsulfoxide (DMSO), adenosine triphosphate (ATP), sodium bicarbonate, Cresol Red, Phenol Red, Neutral Red, Thymol Blue and Dulbecco's phosphate buffered saline were obtained from Sigma-Aldrich Corp. (St Louis, MO, USA). Solutions of the inhibitors were prepared in DMSO to final concentrations of either 10^{-2} mol l^{-1} or 10^{-3} mol l^{-1} and used as stock solutions to prepare those of lower concentration by diluting aliquot samples of these stocks in distilled water. Protease inhibitor cocktail was obtained from Sigma-Aldrich Corp. The cocktail contained 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin and aprotinin, with no metal chelators. Fetal bovine serum (FBS) was obtained from Gemini (Biotech, Alachua, FL, USA), minimum essential medium (MEM) from Gibco (Grand Island, NY, USA) and non-essential amino acids (Cellgro) from Mediatech, Inc. (Herndon, VA, USA).

pH measurements using indicators

Female mosquitoes were fed meals containing pH indicator in 'media mixture' (see below) for pH measurements inside the midgut. In some cases meals contained 10^{-4} mol l^{-1} of MTZ or/and ACZ for pH measurements in the presence of CA inhibitors. The mosquitoes were allowed to feed on the mixture at 37°C using a Parafilm[®] membrane on a glass feeder. After 30 min, based on an assumed normal feeding time of 2.5–19.5 min (Reisen and Emory, 1976), mosquitoes were anesthetized at 4°C for 10 min, placed on ice, dissected and photographed at room temperature. Each meal was prepared using 20% FBS, 2 \times MEM, 0.02% pH indicator prepared in Dulbecco's phosphate buffer saline, 1 mmol l^{-1} ATP as phagostimulant, 1 \times non-essential amino acids, and 0.25% sodium bicarbonate, at a final pH of 7.2–7.4, as 'media mixture'. DMSO control included media mixture, 0.02% pH indicator and 0.001% DMSO. Acid control was prepared using SfII900 (pH=5.6) media instead of MEM. Finally, a positive control included a mixture of 10^{-4} mol l^{-1} MTZ and ACZ prepared in DMSO.

Midguts of mosquitoes fed on meals containing Cresol Red and CA inhibitors (or no inhibitor) were dissected and observed under a stereoscopic microscope. Images were obtained using the Picture Frame v 1.01 software set in the monochromatic mode also. Images were saved at TIF-8 bit format to be analyzed using the toolbox mode of the ImageQuant TL software from Amersham-Biosciences[®]. The grey TIF-8 bit pictures from the midguts were used for image analysis. Graph and statistical analysis were made using GraphPad[®] software from Prism[®].

CA histochemistry

Histochemistry was performed by the same method used to localize CA activity in mosquito larvae (Corena et al., 2002). Adult mosquitoes (males and females separately) were cold anesthetized and dissected. 15–25 whole midguts from males

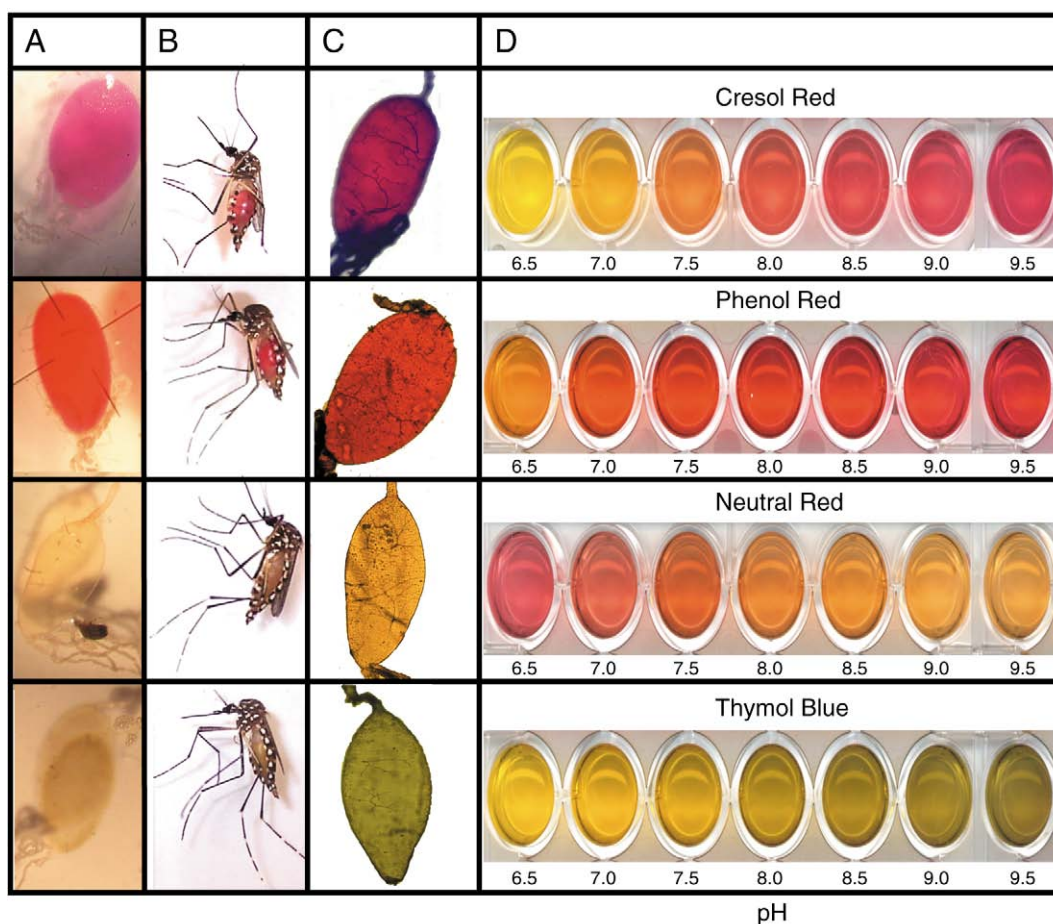


Fig. 1. The pH inside the adult *Ae. aegypti* mosquito midgut. Adult females were fed a mixture containing 0.02% pH indicator. (A) Picture of the dissected mosquito midguts under a stereoscopic microscope. (B) *Ae. aegypti* females immediately after feeding on the indicator mixture. (C) Dissected midguts of engorged mosquitoes photographed at 10 \times . (D) pH standards obtained using Cresol Red, Phenol Red, Neutral Red and Thymol Blue. Our results indicate that the pH inside the posterior midgut in adult females from *Ae. aegypti* ranges between 8.5 and 9.5.

Table 1. Conditions used to measure pH inside the midgut of adult mosquitoes using pH indicators and membrane feeding

Treatment	Media mixture*	0.02% indicator	0.001% DMSO	10 ⁻⁴ mol l ⁻¹ Methazolamide	10 ⁻⁴ mol l ⁻¹ Acetazolamide
Control medium	+	+	-	-	-
Control + DMSO	+	+	+	-	-
Metazolamide + DMSO	+	+	+	+	+
Metazolamide + acetazolamide + DMSO	+	+	+	+	+

*For further explanation, see text.

and females from each species were dissected in HSS. The midguts were incubated overnight at 4°C in 3% glutaraldehyde in 0.1 mol l⁻¹ sodium phosphate buffer, pH 7.3. The following day, the midguts were rinsed three times with 0.1 mol l⁻¹ sodium phosphate buffer (pH 7.3) followed by a 5 min incubation in a solution made by combining 17 ml of solution A (1 ml 0.1 mol l⁻¹ solution of CoSO₄ mixed with 6 ml 0.5 mol l⁻¹ H₂SO₄ and 10 ml 0.066 mol l⁻¹ KH₂PO₄) with 40 ml of solution B (0.75 g NaHCO₃ in 40 ml distilled water). After incubation, the guts were rinsed again in sodium

phosphate buffer and incubated in 0.5% (NH₄)₂S for 2 min followed by a rinse with distilled water. The midguts were placed on depression slides and digitally imaged using a Zeiss Axiovert 135 TV inverted microscope (Carl Zeiss Inc., Thornwood, NY, USA) equipped with a CCD camera.

Total protein concentration

Determination of total protein in each sample was essential to compare the percentage of CA vs total protein in the tissue. The concentration was determined using Coomassie Plus

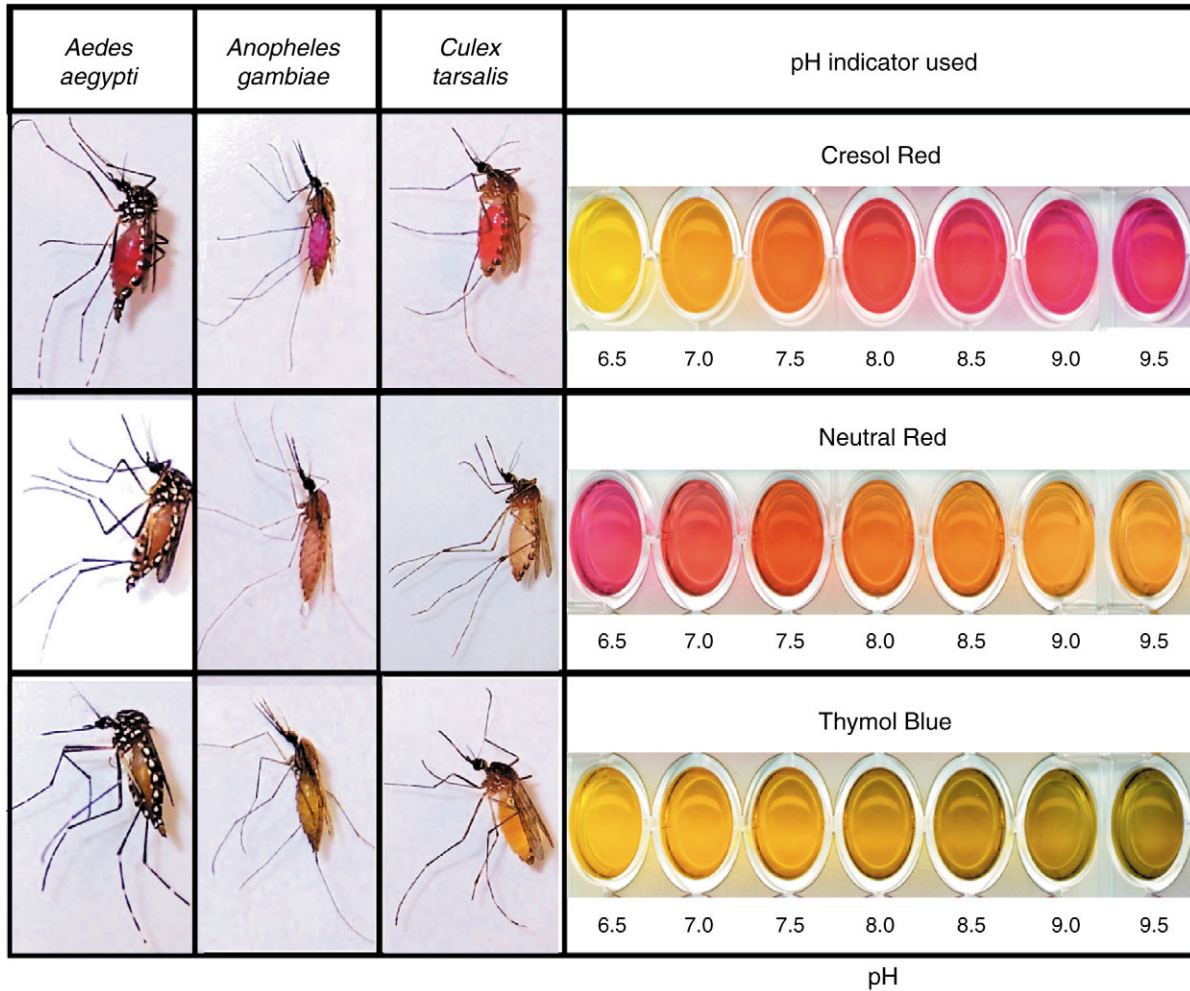


Fig. 2. pH measurements inside the posterior midgut of adult *Ae. aegypti*, *An. gambiae* and *Cx. tarsalis* females indicate that the range is between 8.5 to 9.5. From left to right the columns show the three species, respectively, and the pH standards obtained using Cresol Red, Neutral Red and Thymol Blue.

Protein Assay Reagent Kit from Pierce Biotechnology (Rockford, IL, USA), following a modification of Bradford's method (Bradford, 1976) and using bovine serum albumin as reference protein. Tissue homogenates (in duplicates) from CA activity assays (50 μ l) were diluted appropriately in distilled water according to the manufacturer's instructions. Absorbance was measured at 595 nm using an Ultrospec 4050 spectrophotometer (LKB Biochrom, Ltd., Cambridge, England). Total protein concentration was calculated using Microsoft Excel 2000 and corrected for the addition of protease inhibitor cocktail.

¹⁸O exchange method to measure CA activity

Individual measurements of CA activity were performed using anterior midgut and posterior midgut tissue homogenates separately and in duplicate. Immediately after the midguts were dissected, the Malpighian tubules, rectum and ovaries were removed. Each one of the gut sections was placed in either 100 or 150 μ l of 0.1 mol l⁻¹ ice-cold Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer

(pH 7.6) containing protease inhibitors (1:1000). The tissue was then sonicated in this solution using a sonicator (W-220; Heat Systems-Ultrasonics, Inc., Farmingale, NY, USA) equipped with a microtip. The resulting homogenate was centrifuged at 12 000 *g* at 4°C for 1 min. The supernatant was used to measure CA activity using the ¹⁸O exchange method. Individual measurements of CA activity were performed in duplicate with tissue homogenates corresponding to gastric caeca, anterior midgut and posterior midgut. The reaction medium was 10 mmol l⁻¹ ¹⁸O-labeled NaHCO₃ in 0.1 mol l⁻¹ Hepes buffer, pH 7.6, at 9.5°C. Each experiment was initiated by placing 950 μ l or 925 μ l of this solution in a membrane inlet vessel and allowing it to reach chemical equilibrium for 1–2 min. At this time, the uncatalyzed ¹⁸O-exchange rate was measured. The disappearance of ¹⁸O isotopes from CO₂ and/or bicarbonate upon addition of the enzyme preparations was monitored by mass spectrometry using a gas-permeable probe. Enzyme preparations contained 50 or 75 μ l of mosquito midgut homogenate added sequentially while the temperature and pH were maintained constant. Mosquito homogenate

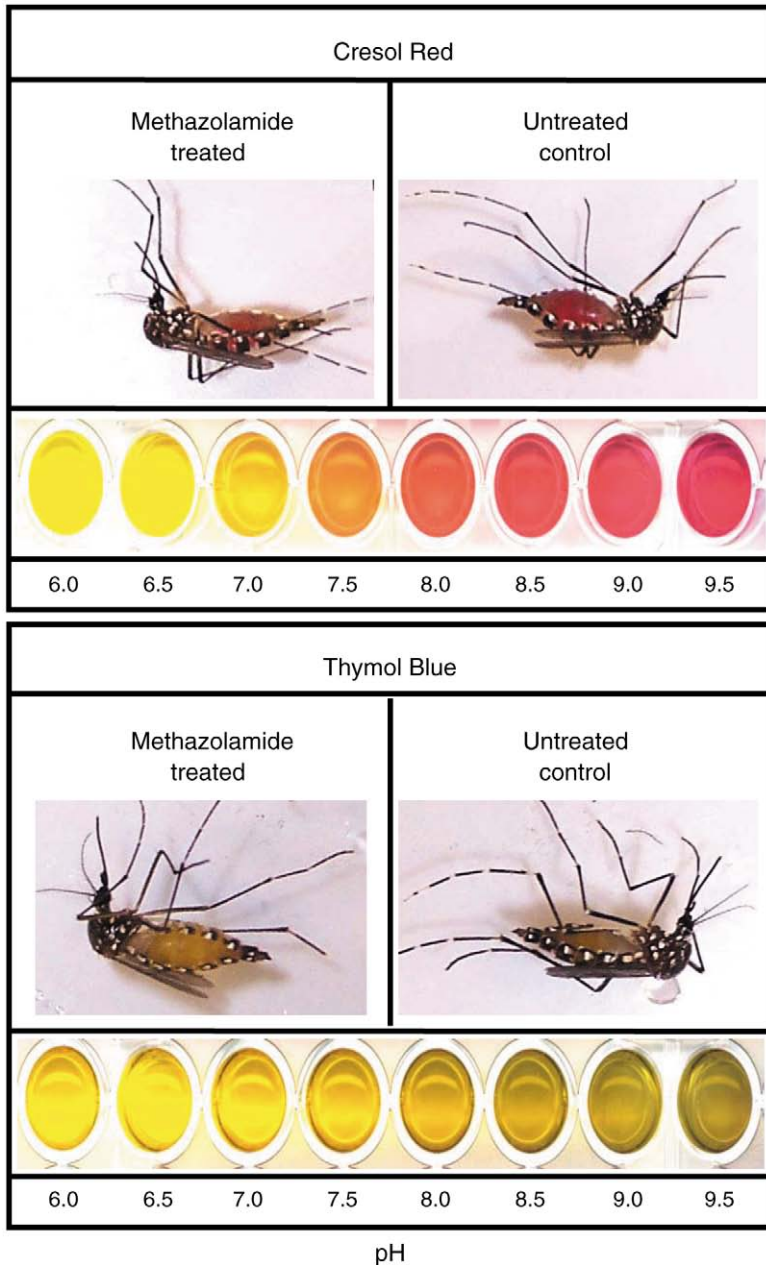


Fig. 3. CA inhibitors decrease the pH inside the posterior midgut in adult *Ae. aegypti* females. (A) Cresol Red added to mosquitoes treated with 10^{-4} mol l $^{-1}$ MTZ revealed a decrease in pH (left) when compared to those not treated (right). (B) Thymol Blue added to mosquitoes treated with 10^{-4} mol l $^{-1}$ MTZ revealed a decrease in pH (left) when compared to those not treated (right). However the effect was not as evident as observed with Cresol Red. Results shown are representative of 10 treated and 10 untreated mosquitoes from 2–4 independent experiments.

to our results we can establish that midgut pH in the adult female *Ae. aegypti* is between 8.5 and 9.5 (Fig. 1) and the pH inside the midgut of *An. gambiae* and *Cx. tarsalis* is between 8.0 and 9.5 (Fig. 2).

Only two pH indicators indicated a change in pH due to the presence of CA inhibitors, Cresol Red and Thymol Blue (Fig. 3). The most noticeable differences were observed with Cresol Red and a mixture of the two CA inhibitors, MTZ and ACZ (Fig. 4). The effect of MTZ was quantitated using color intensity comparisons of 10 treated vs untreated midguts paired in TIF-8 bit grey scale format from two independent experiments (Fig. 5A). Significant differences in color intensity were observed between the methazolamide-treated midguts and the non-treated ones (Fig. 5B).

The presence of DMSO (at 0.001%) in the meal had no effect on either the overall pH of the mixture or the pH inside the mosquito midgut.

Histochemistry of female midguts

Using Hansson's histochemical method, a black precipitate that appears as darkening of the tissue is produced at the sites of CA enzymatic activity. Since this technique shows activity directly in the tissue, the results include both cytosolic and membrane-bound CA activity. Dissected midguts representative of females from each species are shown in Fig. 6. We observed differences among the different species used in this study. Comparison of female midguts

from *Cx. nigripalpus*, *An. quadrimaculatus*, *Ae. albopictus* and *Cx. quinquefasciatus* showed that CA is preferentially localized in the posterior midgut (Fig. 6A,B,C and D, respectively) as indicated by darkening of the tissue. Histochemistry of *Ae. aegypti* and *Oc. taeniorhynchus* female midguts revealed uniform staining throughout the midgut in all the specimens dissected (Fig. 6E,F). Female *Ae. aegypti* midguts appeared darker than the midguts from other species.

Histochemistry of male midguts

In *An. quadrimaculatus* and *Cx. quinquefasciatus* male midguts, the difference in darkening between the anterior and posterior regions of the gut was not clearly marked. However, CA was associated with the posterior midgut in these two

activity was calculated by comparison with the activity of a mutant form of human CA-II and expressed as equivalents of human CA-II (Silverman and Tu, 1986). Samples containing CA activity were inhibited by addition of $10 \mu\text{mol l}^{-1}$ methazolamide to confirm that the activity measured was due to CA (Corena et al., 2002, 2004).

Results

pH measurements with indicators

Using different pH indicators we were able to obtain an approximation of the pH inside the adult mosquito midgut using *Ae. aegypti*, *An. gambiae* and *Cx. tarsalis* females. Conditions for this study are presented in Table 1. According

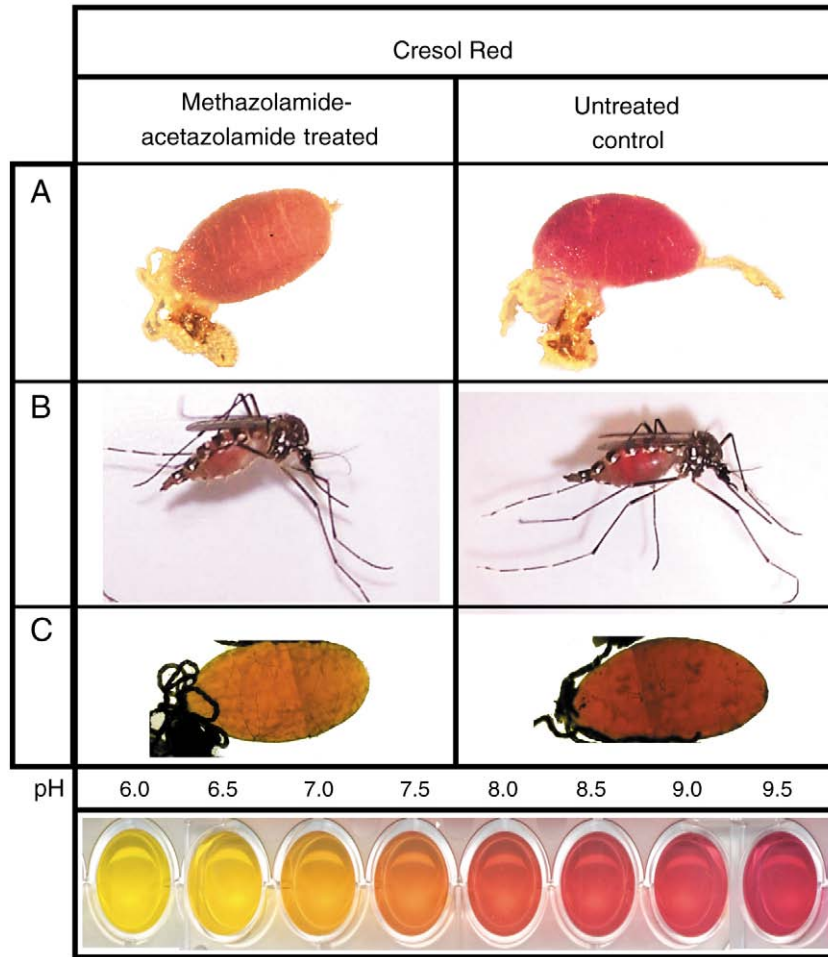


Fig. 4. Treatment of *Ae. aegypti* adult female posterior midgut with MTZ and ACZ combined (10^{-4} mol l $^{-1}$ each) resulted in a significant decrease of pH inside the midgut (left) when compared with those midguts dissected from mosquitoes not treated with these inhibitors (right), when compared with the Cresol Red standards (bottom). (A) Dissected midguts. (B) Intact mosquitoes. (C) Dissected midguts photographed at 10 \times . Results shown are representative of 10–20 treated and untreated individuals from triplicate experiments.

species (Fig. 7A,C). In contrast, in *Ae. albopictus* males, the anterior midgut exhibited clearly more intense darkening when compared to the posterior (Fig. 7B). Dissected *Ae. aegypti* male midguts revealed intense staining throughout the entire midgut. These midguts also appeared darker than the midguts of males from other species (Fig. 7D). In contrast, midguts from *An. albimanus* and *Oc. taeniorhynchus* exhibited less darkening or no darkening (Fig. 7E,F).

Total protein concentration

Total soluble protein for anterior and posterior midgut homogenates was calculated for each species after homogenization and centrifugation of the tissue. The values obtained were corrected for the amount of protease inhibitors added to each individual sample. Although we did not calculate

the amount of protein per midgut, the majority of the species tested exhibited the highest soluble protein content in the posterior midgut (results not shown). Since different species vary in size, a comparison of the protein content from one portion of the gut in one species to the same portion in another species is imprecise.

The highest amount of total soluble protein was found in posterior midgut homogenates of *An. quadrimaculatus* females. The second highest amount was found in anterior midgut homogenates of females of the same species. In the males, the highest total soluble protein content was found in the anterior midgut of *An. quadrimaculatus* followed by the anterior midgut of *Cx. nigripalpus*.

^{18}O exchange method to measure CA activity

Since Hansson's histochemical method provides a qualitative estimate in terms of the total amount of CA activity (both soluble and membrane bound) present in each region of the midgut, a quantitative technique was necessary in order to determine the amount of activity present in each tissue. The ^{18}O isotope exchange method has been used previously to determine CA activity in larval midguts (Corena et al., 2004). Although very sensitive, this method is used only to quantify soluble CA, and discrepancies between Hansson's qualitative method and the ^{18}O isotope exchange technique are expected. These differences are probably the result of the loss of membrane-bound CA in the pellet after centrifugation. Therefore, the amounts of CA activity calculated with the ^{18}O isotope exchange technique in this manuscript refer only to cytosolic CA. We have observed a correlation between Hansson's histochemical method and the ^{18}O isotope exchange method in the larvae but this might not hold true for the

adult mosquito, as adults (flying insects) and larvae (aquatic insects) are very different.

In terms of our findings using the ^{18}O isotope exchange method, CA activity in the adult mosquito midgut seems to be preferentially associated with the posterior midgut in the females of most of the species tested. This also seems to be true for the males of most species. We were able to detect CA activity in the anterior midgut of females in only three species: *Ae. aegypti* (0.6%), *Ae. albopictus* (0.02%) and *Cx. nigripalpus* (0.08%) (Fig. 8A). We were unable to detect CA activity in the posterior midgut of *An. albimanus* and *Cx. nigripalpus* females (Fig. 8B). We were unable to detect CA activity in the anterior midguts of the males of the species tested in this study. We were unable to detect CA in the posterior midgut of *An. quadrimaculatus* and *Ae. aegypti* males (Fig. 8C).

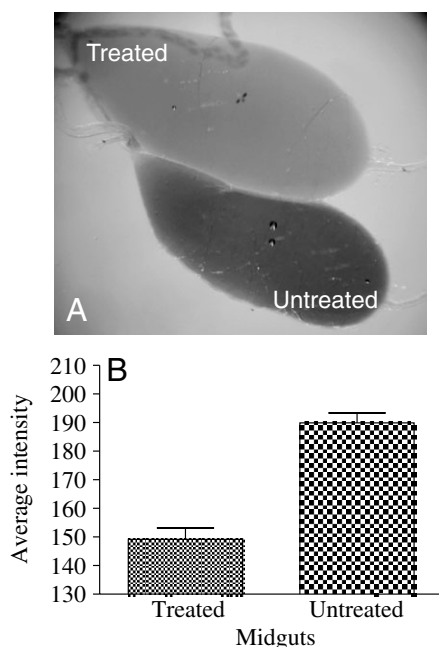


Fig. 5. (A) Grey TIF-8 bit pictures from *Ae. aegypti* midguts were used for image analysis. The top midgut was treated with 10^{-4} mol l $^{-1}$ MTZ and Cresol Red. The lower midgut was treated with Cresol Red in the absence of MTZ. (B) Histogram of average intensity values for treated (10^{-4} mol l $^{-1}$) and untreated midgut images (from A) analyzed with ImageQuant TL software from Amersham Biosciences®. Differences were significant (P value < 0.0001). 20 specimens (10 treated and 10 untreated) were analyzed in triplicate.

Discussion

Female mosquitoes require a blood meal to obtain protein for ovary development. The requirement for a blood meal usually contributes to the propagation of mosquito-transmitted diseases such as malaria as the mosquito feeds on different hosts. Females store the ingested blood in the posterior midgut (Clements, 1992). When the mosquitoes take a meal different from blood (nectar, sugar solution or artificial meal), the solution is stored in the crop and not the midgut (Clements, 1992). However, storage of this meal in the midgut can be stimulated by the addition of ATP to the food offered (Galun et al., 1988).

Mosquito blood meal pH has been determined *in situ* using ion-selective microelectrode measurements. Using this technique, blood meal pH has been shown to increase from 7.40 to 7.52 in *Ae. aegypti* females and to 7.58 in *An. stephensi* (Billker et al., 2000). Measurements of pH inside the mosquito midgut have been documented in the larvae and the data collected has relied on the use of pH indicators, using particles to assist ingestion (Zhuang et al., 1999). Inside the anterior larval midgut, pH values have been reported to be in the range 9.5–11 (Clements, 1992). Maintenance of this high pH in the anterior larval midgut has subsequently been associated with a high concentration of bicarbonate/carbonate ions and hence presence of CA (Boudko et al., 2001b; Corena et al., 2002, 2004).

Is the pH inside the adult mosquito midgut similar to that of the larvae where alkalization is higher in the anterior region and decreases in the posterior? To answer this question, we determined the pH inside the midgut of female *Ae. aegypti*, *An. gambiae* and *Cx. tarsalis* adult mosquitoes.

Our results using pH indicators suggest that the pH inside the adult posterior midgut in female *Ae. aegypti* is between 8.5 and 9.5. In the other two species it was in the range 8.0–9.5. It is not surprising to find differences in pH inside the midgut of mosquitoes from different species. We have observed such differences in the midgut of larvae from several species and these observations have also been reported by other investigators (Clements, 1992). However, when comparing these results with those previously obtained in the larval midgut, we can conclude that the pH inside the anterior adult female mosquito midgut does not reach the high values found in the anterior midgut in the larvae (pH > 10). In fact, the adult female mosquito midgut presents the complete opposite: a more alkaline posterior midgut and a more acidic anterior midgut. We have focused our observations in the posterior midgut since this is the site of storage of the blood meal.

Does CA play a role in the maintenance of this pH as it has been suggested in the larvae? We have used CA-specific inhibitors to determine if the pH is affected by the absence of CA activity. We must consider that mosquitoes were artificially fed on pH indicator and CA inhibitor at the same time; thus the change on the overall midgut pH reflects only the inhibitory effect that takes place a short time after the meal is ingested. A background effect of CA activity at the time of the meal is to be expected. Treatment with CA inhibitors resulted in a decrease in pH inside the midgut from the initial values (8.5–9.5) to 7.5 and 8.0. Analysis of differences in color intensity of 8-bit grey scale images revealed significant differences ($P > 0.0001$) between the treated and the untreated midguts. We can conclude that CA is important in maintaining the pH inside the adult female mosquito midgut. Little is known about the mechanism of pH maintenance inside the adult mosquito midgut but our observations suggest that CA is crucial in the maintenance of pH within the midgut in all the species tested.

How does MTZ affect alkalization of the midgut? It is possible that this compound inhibits the production of bicarbonate by inhibiting one (or more CA isozymes), therefore interfering with the ion transport processes that occur in the midgut epithelium, ultimately altering the alkalization mechanism and leading to changes in pH.

Is the localization of CA related to the mechanisms of ion regulation in the midgut for each particular species? A detailed analysis of CA isozyme distribution in the midgut for each species and its relationship with ion transport mechanisms will be necessary in order to answer this question. We observed a significant decrease in pH on using a combination of ACZ and MTZ. Since these two compounds inhibit different CA isozymes to various degrees, it is possible that more than one isozyme is present in the adult mosquito midgut. At least 14 CA isozymes have been identified in the *An. gambiae* genome

(Holt et al., 2002). It is possible that multiple CA isozymes are present in the adult mosquito midgut and contributing to the mechanisms of ion transport. However, further studies are necessary to determine the localization and characterization of these isozymes. A correlation between CA localization and ion

transport in the midgut could be made afterwards by using self-referencing ion selective (SERIS) microelectrodes to measure ion fluxes in the midgut, as has been demonstrated in the larvae (Boudko et al., 2001).

Is CA present in the midgut of adult mosquitoes? Are there

any differences between species? CA localization by Hansson's histochemical method revealed that the enzyme is present in the midgut of all the species tested, with the exception of *Oc. taeniorhynchus* males. However, quantitative analysis using the ^{18}O isotope exchange method revealed a small amount of CA activity in *Oc. taeniorhynchus* males. We can conclude that all the species tested exhibited CA activity in the midgut to various degrees.

We have previously observed a correlation between Hansson's histochemical method and the ^{18}O isotope exchange method in the localization of CA in larval *Ae. aegypti*, *Cx. quinquefasciatus*, *Cx. nigripalpus*, *Oc. taeniorhynchus*, *Ae. albopictus* and *An. quadrimaculatus* midgut (Corena et al., 2002, 2004). It is important to clarify that there are at least 14 possible genes that code for CAs in the *An. gambiae* genome and that in this study we only determined total soluble protein in each of the midgut regions. Therefore, the values presented for CA activity account only for cytosolic CA and not membrane-bound CA that could have been lost in the pellet after centrifugation and prior to analysis. To date, there is no direct evidence for the presence of membrane-bound CA in the adult mosquito midgut, although it has been detected in *Ae. aegypti* and *An. gambiae* larval midguts (Seron et al., 2004).

In terms of the CA activity in different species, we observed differences among the mosquito species tested. The highest enzymatic activity quantitated appeared to be associated with the posterior midgut in *Cx. quinquefasciatus* males, followed by the posterior and anterior midgut

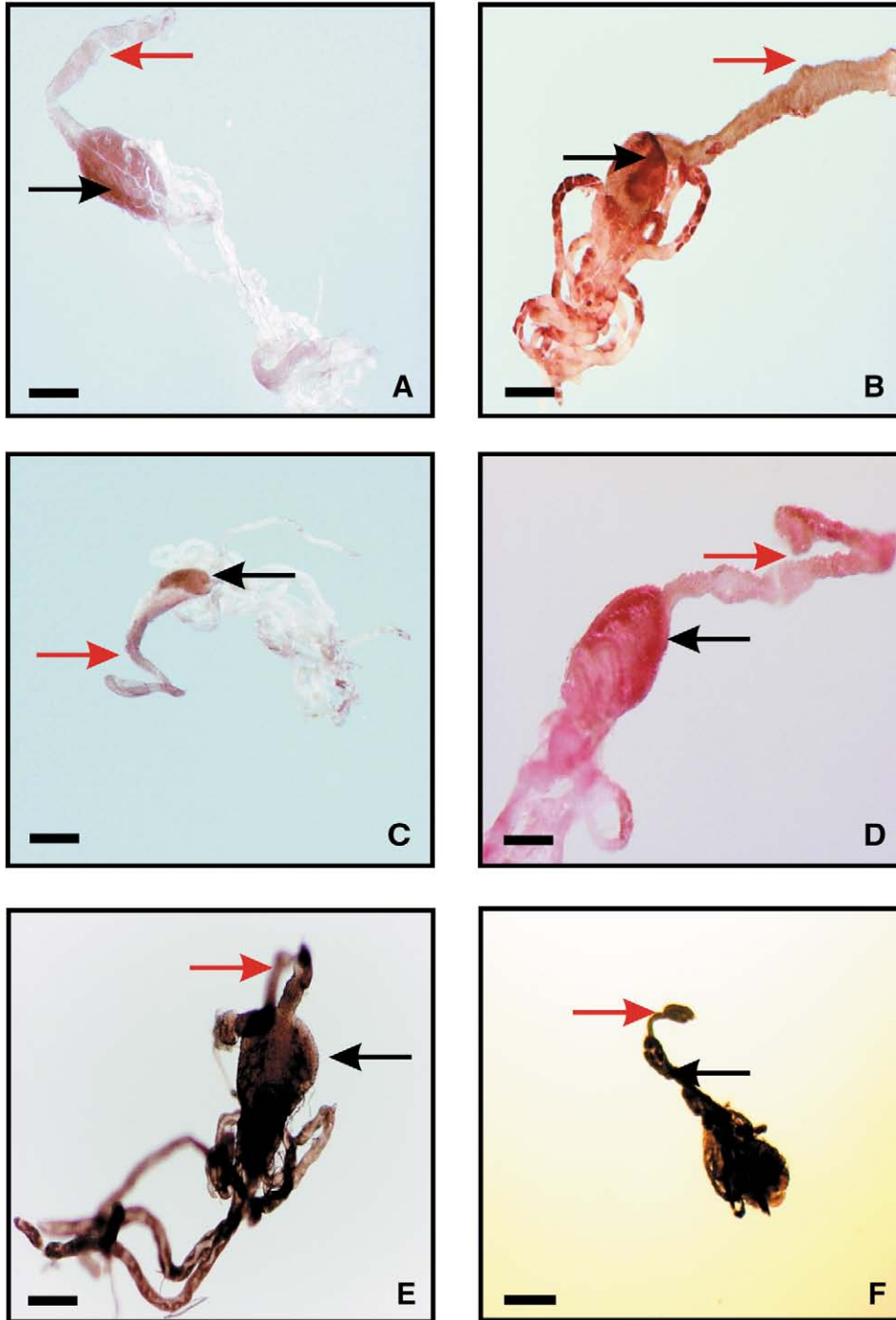


Fig. 6. Hansson's histochemical stain of isolated adult female mosquito midguts. Histochemistry indicates that the enzyme is localized in the midgut of all the species with various degrees of darkening. The enzyme also appears to be associated preferentially with the posterior midgut (black arrows) in several of these species when compared to the anterior midgut (red arrows). (A) *Cx. nigripalpus*, (B) *An. quadrimaculatus*, (C) *Ae. albopictus*, (D) *Cx. quinquefasciatus*, (E) *Ae. aegypti*, (F) *Oc. taeniorhynchus*. Scale bars, 500 μm .

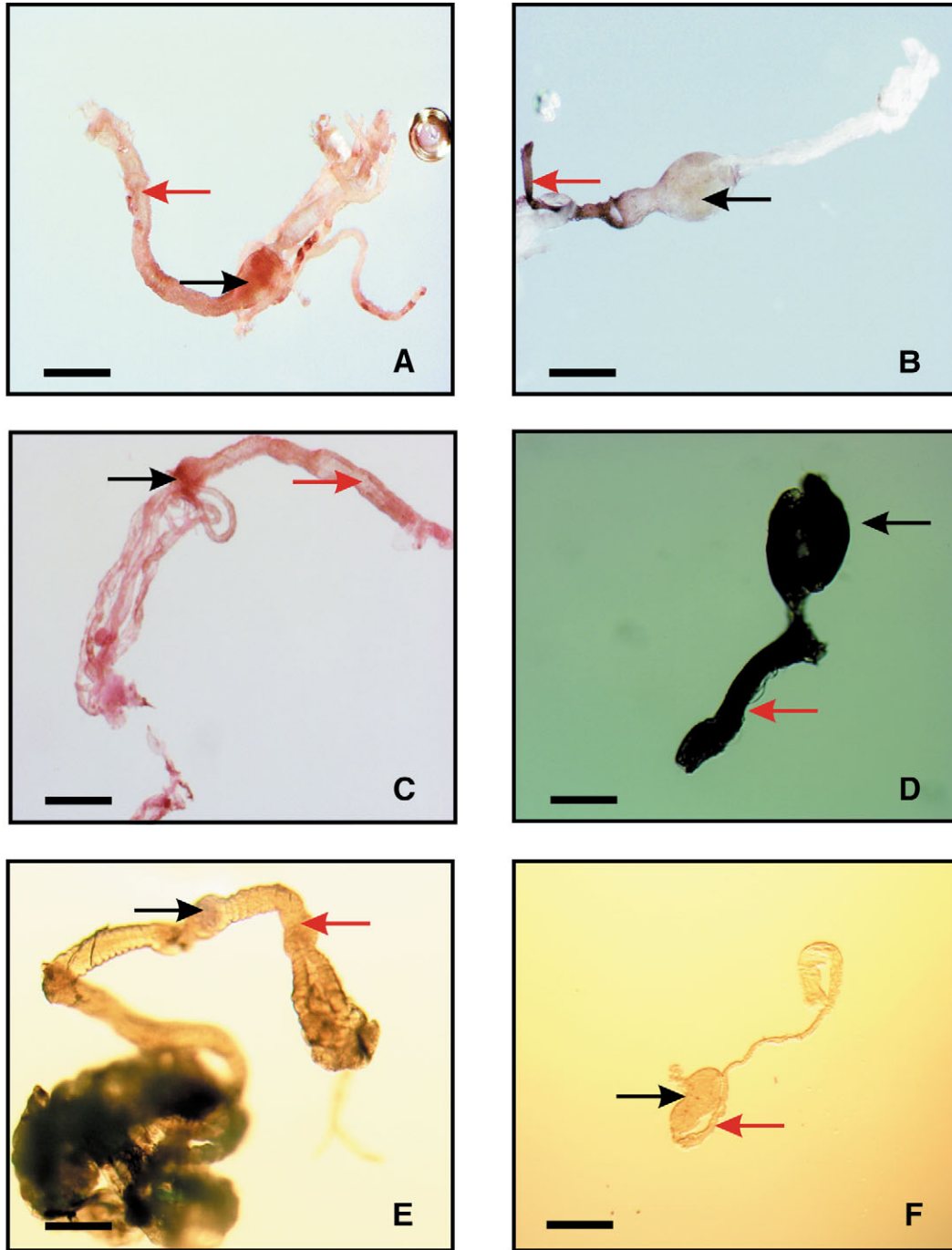


Fig. 7. Hansson's histochemical stain of isolated adult male mosquito midguts indicate that the enzyme is localized in the midgut of all the species with the exception of *Oc. taeniorhynchus*. (A) *An. quadrimaculatus*, (B) *Ae. albopictus*, (C) *Cx. quinquefasciatus*, (D) *Ae. aegypti*, (E) *An. albimanus*, (F) *Oc. taeniorhynchus*. Black arrows, posterior midgut; red arrows, anterior midgut. Scale bars, 500 μm .

in *Ae. aegypti* females, respectively. We are able to conclude that CA distribution in the midgut of all the species of adult mosquitoes tested in this study appears to be species dependent.

Are there any differences between male and female adult mosquito midguts? The answer to this question is yes, not only are there differences between male and female mosquito midguts, but the differences are striking. We have observed

differences not only in localization but also in quantitation in male and female midguts. CA localization was consistent in the posterior midgut in females from all species. The amount of CA activity measured using the ^{18}O -isotope exchange method showed that CA is expressed in this tissue at different levels in different species. We were able to detect CA in the anterior midgut only in females from *Ae. aegypti*, *Ae. albopictus* and *Cx. nigripalpus*. We observed interesting

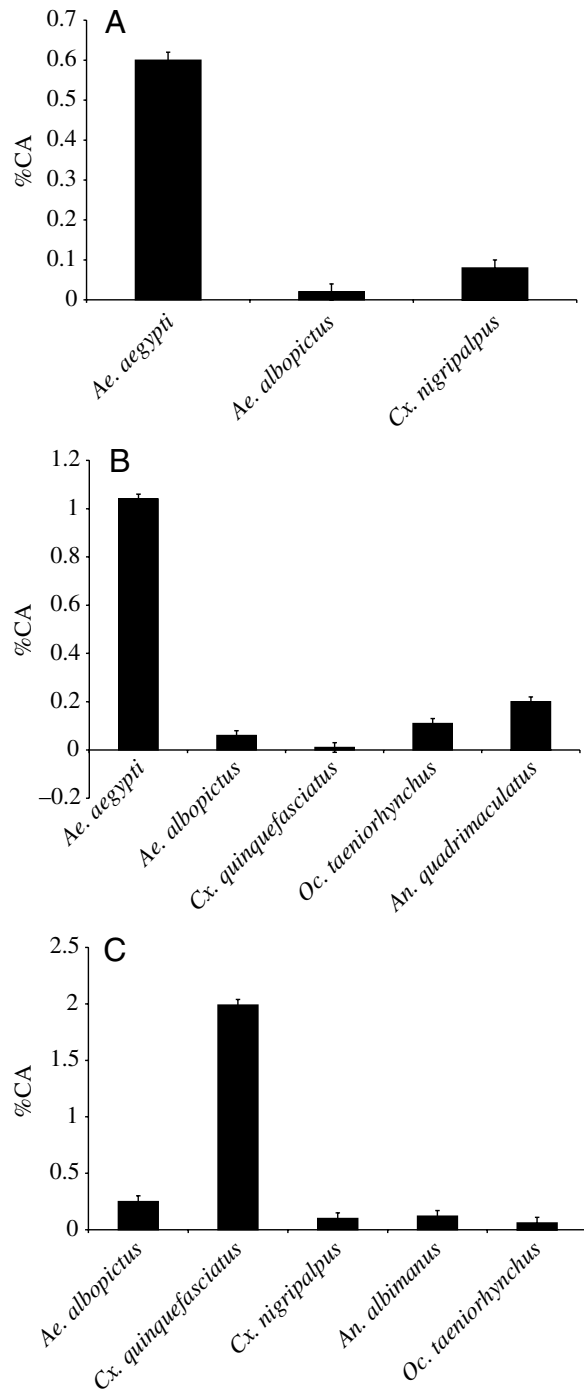


Fig. 8. (A–C) CA activity (as a percentage of total protein) measured in the different regions of the midgut in adult males and females from the species tested revealed that the enzyme is preferentially associated with the posterior midgut in all species, in both males and females. CA activity was detected in anterior (A) and posterior (B) midgut in females and only in the posterior midgut of males (C). (A) CA activity in the anterior midgut of female adult mosquitoes was detected only in *Ae. aegypti* (highest), *Ae. albopictus* and *Cx. nigripalpus* females. (B) CA activity in the female posterior midgut was detected in all species to various degrees. (C) CA activity was detected in the posterior midgut of all species of males tested and was not detected in the anterior midgut of males from any species.

results for males of all the species tested. Although we were able to measure total protein in all of the species, we were unable to detect CA activity in the anterior midgut of the males from the species tested. In contrast, we detected CA activity in the posterior midgut of males from most species with the exception of *An. quadrimaculatus* males.

In terms of CA localization in the anterior and posterior regions of the adult midgut, it is apparent now that all the species tested exhibit CA activity in the posterior midgut, although to various degrees. Since the size of the midgut varies from species to species, we determined the total amount of soluble protein present in the midguts to determine if our data were influenced by the amount of total protein. Analysis of our results demonstrated that there is no apparent relationship between the amount of total protein and the CA activity in the midgut. For example, in *An. quadrimaculatus* females, the total soluble protein content was 164.8 μg in 100 μl of tissue homogenate while the amount of CA activity present in this homogenate was only 0.2% of the total protein. In contrast, the lowest amount of soluble protein found for a female mosquito was that of the anterior midgut of *Ae. aegypti*. However, the amount of CA activity reached 1% of the total protein present in that homogenate. Therefore our results indicate that there is no apparent relationship between the amount of total protein and the amount of CA present in a particular region of the midgut. We have previously made this observation in larvae from different species of mosquitoes (Corena et al., 2004).

As a result of our experiments, we can conclude that the pH inside the adult female mosquito midgut is between 8.0 and 9.5. Furthermore, CA content is not only species dependent but also dependent on the sex of the insect and the portion of the midgut being studied. Inhibition of CA in the adult female mosquito midgut resulted in a pH imbalance with possible inhibition of ion transport processes, which led to a decrease in the alkalinity of the midgut.

The relevance of these studies lies in the importance of maintaining this pH in homeostasis and ion transport mechanisms, which are involved in the rapid digestion of the blood meal, and their role in the malaria infection process. Blood pH is regulated by the equilibrium between dissolved CO_2 and bicarbonate ions. The loss of CO_2 that occurs when infected blood is exposed to ambient conditions has long been known to result in a pH increase large enough to induce malarial gametogenesis *in vitro* (Chorine, 1933; Bishop and Mc Connachie, 1956; Nijhout and Carter, 1978). We believe that the increase in pH observed in the mosquito midgut during digestion of an infected blood meal is the result of a high bicarbonate concentration generated by CA activity. We postulate that this CA activity leads to the conversion of CO_2 into bicarbonate, which in turn contributes to the induction of gametogenesis of *Plasmodium* parasites inside the midgut. We have begun to study the role of CA activity in the development of *Plasmodium* parasites inside the mosquito midgut and the effect of CA inhibition in the infection mechanism. Our results will be published in the near future.

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