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RESEARCH ARTICLE

pH control in the midgut of *Aedes aegypti* under different nutritional conditions

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ABSTRACT

Aedes aegypti is one of the most important disease vectors in the world. Because their gut is the first site of interaction with pathogens, it is important to understand *A. aegypti* gut physiology. In this study, we investigated the mechanisms of pH control in the midgut of *A. aegypti* females under different nutritional conditions. We found that unfed females have an acidic midgut (pH ~6). The midgut of unfed insects is actively maintained at pH 6 regardless of the ingestion of either alkaline or acidic buffered solutions. V-ATPases are responsible for acidification after ingestion of alkaline solutions. In blood-fed females, the abdominal midgut becomes alkaline (pH 7.54), and the luminal pH decreases slightly throughout blood digestion. Only ingested proteins were able to trigger this abrupt increase in abdominal pH. The ingestion of amino acids, even at high concentrations, did not induce alkalinisation. During blood digestion, the thoracic midgut remains acidic, becoming a suitable compartment for carbohydrate digestion, which is in accordance with the higher alpha-glucolytic activity detected in this compartment. Ingestion of blood releases alkalising hormones in the haemolymph, which induce alkalinisation in *ex vivo* preparations. This study shows that adult *A. aegypti* females have a very similar gut physiology to that previously described for *Lutzomyia longipalpis*. It is likely that all haematophagous Nematocera exhibit the same type of physiological behaviour.

KEY WORDS: Diptera, Nematocera, Mosquitoes, Haematophagous insects, Digestion

INTRODUCTION

Many haematophagous dipterans belong to the suborder Nematocera and are included in the families Culicidae, Psychodidae, Simuliidae, Corethrellidae and Ceratopogonidae. Some of these insects, such as mosquitoes (*Aedes*, *Anopheles* and *Culex*) and phlebotomine sand flies (*Phlebotomus* and *Lutzomyia*), are important vectors of tropical diseases (Marshal, 2012).

Among haematophagous nematocerans, only females must feed on blood, for ovary development and oocyte maturation. However, males and females constantly ingest carbohydrate-rich solutions such as plant sap, nectar and honeydew from aphids or coccids (Molyneux et al., 1991; McCreadie et al., 1994; Foster, 1995; Stewart and Kline, 1999). Because these insects are phylogenetically related and have similar feeding behaviour, it is reasonable to infer that they share many similarities in the anatomy and physiology of their digestive systems.

The digestive system of adult nematoceran insects is divided into three compartments: foregut, with a diverticulum that stores carbohydrates; midgut, which is divided into a thin thoracic and an enlarged abdominal midgut; and hindgut, which starts at the pylorus and extends to the anus. The digestion of ingested food occurs in the midgut. When ingested, sucrose-rich solutions are initially stored in the diverticulum, from where they are gradually released into the midgut to be processed. In some species, salivary carbohydrases are present and may participate in carbohydrate digestion in the diverticulum (Ribeiro et al., 2000). However, most carbohydrate digestion occurs in the midgut by the action of an alpha-glucosidase linked to the enterocytes' microvilli, as observed in phlebotomine sand flies and *Aedes aegypti* (Gontijo et al., 1998; Ferreira et al., 2010). When females ingest a blood meal, the blood is driven directly to the abdominal midgut, where it is stored and digested, surrounded by a type II peritrophic membrane that is secreted by the midgut cells around the ingested food (Lehane, 1997).

Digestive enzymes require stringent physiological conditions to reach maximum activity, and the hydrogen ion concentration (pH) within the gut is one of the most important factors affecting their function. To date, there is a very limited understanding of intestinal pH regulation in adult haematophagous nematocerans. Most studies of pH control in the midgut of nematoceran insects have been performed in mosquito larvae (Onken and Moffett, 2009) and, so far, there are only five published studies on gut pH control in adults (Gontijo et al., 1998; Billker et al., 2000; del Pilar Corena et al., 2005; Santos et al., 2008, 2011). Our research group has been studying this aspect of digestion in the phlebotomine sand fly *Lutzomyia longipalpis* (Diptera, Psychodidae). In the midgut of non-blood-fed female *L. longipalpis*, the pH is actively maintained at ~6, even when these insects ingest highly buffered solutions at different pH values. After feeding on blood, the mechanism responsible for maintaining this acidic pH in the abdominal midgut is somehow switched off, and, at the same time, a still not entirely characterised alkalinisation mechanism is activated in order to increase pH values to around 8.15, which is adequate for protein digestion (Santos et al., 2008, 2011).

So far, two mechanisms likely to be involved in *L. longipalpis* gut pH alkalinisation have been identified. The first has been better characterised and promotes alkalinisation by releasing CO_2 present in the ingested blood. It is a phenomenon similar to respiratory alkalosis (Santos et al., 2008). The second mechanism depends on hormonal activation of the midgut and subsequent production of HCO_3^- from CO_2 hydration; HCO_3^- is then transported through the enterocyte membranes of the abdominal midgut (Santos et al., 2008, 2011). Other transport systems may also be involved.

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Received 7 March 2017; Accepted 10 July 2017

Journal of Experimental Biology (2017) 220, 3355-3362 doi:10.1242/jeb.158956

Despite the importance of pH control in the midgut of adult insects, little information about this subject has been published for bloodsucking nematoceran insects other than *L. longipalpis*. Using pH-sensitive microelectrodes, Billker et al. (2000) showed in *Anopheles stephensi* that pH values inside the abdominal midgut reached an average of 7.84 in the first 10 h after blood feeding and remained high even 24 h after the digestive process had started, whereas in *A. aegypti*, pH values reached an average of 7.52 but decreased slowly throughout digestion. In another study, del Pilar Corena et al. (2005) used indicator dyes to evaluate the intestinal pH in *A. aegypti* females and observed an alkaline pH in the abdominal midgut. However, the solutions ingested by those insects contained fetal bovine serum, which has proteins and amino acids. We now know that any ingested proteins (even those not usually present in blood, such as egg lysozyme and casein) are able to trigger abdominal midgut alkalinisation in *L. longipalpis* (Santos et al., 2011), and a similar process could occur with mosquitoes, which probably have an acidic midgut before protein ingestion.

As bloodsucking nematocerans are phylogenetically related, we hypothesised that all haematophagous nematoceran insects have a digestive physiology and midgut pH control mechanism like that described for *L. longipalpis*. Thus, this study aimed to investigate pH control in the midgut of *A. aegypti* females under different nutritional conditions.

MATERIALS AND METHODS

Insects

All experiments were performed with Br-strain *Aedes aegypti* (Linnaeus 1762) females that were 3–6 days old. Adult specimens were maintained in nylon cages at 25±2°C, 70±10% humidity and were fed 10% sucrose. The insects blood-fed on hamsters (*Mesocricetus auratus*) anaesthetised with Thiopentax® (0.2 ml of Thiopentax 5% per 100 g). All procedures involving animals were approved by the Ethics Committee in Animal Experimentation (CEUA-UFMG) under protocol no. 61/2015.

pH measurement by forced feeding

The forced feeding procedure was performed as described by Santos et al. (2008), by introducing the female mosquito mouthparts into capillary tubes containing the solutions to be tested (Fig. 1D). A list of the tested solutions is presented in Table 1. After ingestion of the capillary contents, insects were immediately dissected in insect saline (IS; 119.7 mmol l^{-1} NaCl, 2.68 mmol l^{-1} KCl, 1.36 mmol l^{-1} $CaCl_2$ and 0.56 mmol l^{-1} glucose) (Sunitha et al., 1999). All ingested solutions contained approximately 0.1% Bromothymol Blue (or 0.1% Bromocresol Purple) and 1 mmol l^{-1} ATP as a phagostimulant. The dissected guts were observed under a stereomicroscope. The colours observed in each part of the midgut (thoracic and abdominal) and in the diverticulum were compared with standard buffered solutions containing Bromothymol Blue or Bromocresol Purple (Fig. 1B,C). As BSA (purified or in serum) can potentially interact with Bromothymol Blue to slightly change its colour at different pH, standard buffered solutions were also prepared containing 5% BSA or serum for comparison with colours observed in female guts from mosquitoes that ingested BSA or serum, respectively. pH observations were classified into two groups: pH<6.5 and pH≥6.5.

pH measurement in the abdominal midgut of blood-fed females

Blood-fed females were used for pH measurements after feeding on anaesthetised hamsters as described above. A protocol previously described by Santos et al. (2008) was followed to construct and use pH-sensitive microelectrodes. Micromanipulators were used to insert pH-sensitive and reference electrodes into the abdominal midgut, piercing through the abdominal cuticle of blood-fed females. The pH was measured immediately after blood ingestion and over the next 50 h during blood digestion. Each point on the graph (Fig. 2) corresponds to a different insect.

Measurement of Na^+/K^+-ATPase activity

The measurement of Na^+/K^+-ATPase activity was performed as previously described by Macvicker et al. (1993). *Aedes aegypti* females blood-fed for 30 min, then the midguts were dissected in 50 mmol l^{-1} Tris pH 7.8 and the ingested blood was washed away. Twenty-six dissected midguts free of blood were transferred to a microtube containing 260 µl of 50 mmol l^{-1} Tris pH 7.8 immersed in an ice bath. The microtube was centrifuged at 3000 ***g*** for 1 min, the supernatant was removed and the pellet was suspended in 260 µl of 50 mmol l^{-1} Tris pH 7.8 containing 0.1% Tween-20. The tube was sonicated for 20 s and kept on ice until use. Each assay was performed with a 20 µl sample, the equivalent of two midguts. Stock solutions were added to the assay tubes so that the final concentration was

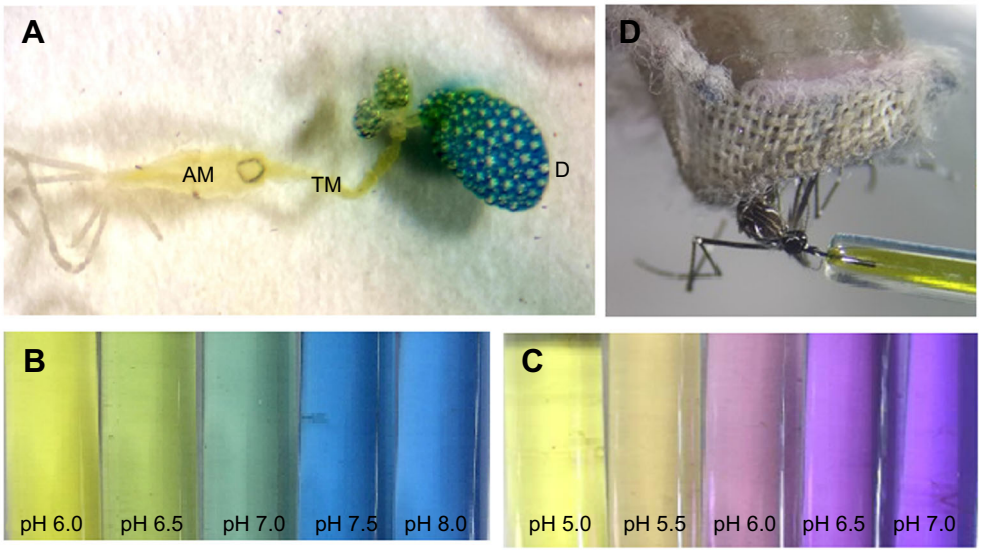

Fig. 1. pH measurements using pH indicator dyes. (A) TM: Thoracic midgut (TM), abdominal midgut (AM) and diverticulum (D) of *Aedes aegypti* females after forced feeding with insect saline (IS)+160 mmol l^{-1} Hepes pH 7.5+0.1% Bromothymol Blue. (B) Buffered standard solutions from pH 6 to pH 8 containing 0.1% Bromothymol Blue (standard solutions containing serum or BSA are not shown). (C) Buffered standard solutions from pH 5 to pH 7 containing 0.1% Bromocresol Purple. (D) Detail of the forced feeding procedure.

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Table 1. Solutions ingested by *Aedes aegypti* females through forced feeding

Solution	Description
Control 1	Unbuffered IS with pH adjusted to ~7.4
Control 2	IS buffered with 30 mmol l^{-1} MES pH 6.0
Control 3	IS buffered with 30 mmol l^{-1} Hepes pH 7.4
Control 4	IS buffered with 30 mmol l^{-1} Hepes+DMSO 10% adjusted to pH 7.5
IS pH 7.5	IS buffered with 160 mmol l^{-1} Hepes pH 7.5
IS pH 5.0	IS buffered with 160 mmol l^{-1} MES pH 5.0
Unbuffered human serum	Human serum adjusted to pH 6.0 with HCl
Human serum pH 6.0	Human serum buffered with 30 mmol l^{-1} MES pH 6.0
Unbuffered IS plus amino acids	Unbuffered IS containing 100 mmol l^{-1} free amino acids (Sigma, M-5550) adjusted to pH 6.0 with HCl
Buffered IS plus amino acids	IS buffered with 30 mmol l^{-1} MES containing 100 mmol l^{-1} free amino acids (Sigma, M-5550) adjusted to pH 6.0
IS plus lysozyme	IS containing unbuffered 5% lysozyme (Sigma, L7651) adjusted to pH 6.0
IS plus buffered lysozyme	IS containing 5% lysozyme buffered with 30 mmol l^{-1} MES adjusted to pH 6.0
IS plus BSA	IS containing unbuffered 5% BSA (Sigma, A3059) adjusted to pH 6.0
IS plus buffered BSA	IS containing 5% BSA buffered with 30 mmol l^{-1} MES adjusted to pH 6.0
IS plus ovalbumin	IS containing unbuffered 5% ovalbumin (Sigma, A-5378) adjusted to pH 6.0
IS plus buffered ovalbumin	IS containing 5% ovalbumin buffered with 30 mmol l^{-1} MES adjusted to pH 6.0
Buffered IS plus bafilomycin A1	IS buffered with 30 mmol l^{-1} Hepes+1 µmol l^{-1} bafilomycin A1 (Sigma, B1793)+DMSO 10% adjusted to pH 7.5 (stock solution of bafilomycin was prepared in DMSO)

IS, insect saline. Bromothymol Blue (~0.1%) was present in all ingested solutions except in IS buffered with 160 mmol l^{-1} MES pH 5.0, which contained Bromocresol Purple.

50 mmol l^{-1} Tris pH 7.30, 3 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} NaCl, 15 mmol l^{-1} KCl, 0.5 mmol l^{-1} EGTA, 3 mmol l^{-1} ATP (pH adjusted to 6.5 with Tris-base) and distilled water to a final volume of 100 µl. When present, ouabain (a specific inhibitor of Na^+/K^+-ATPase) was at a final concentration of 1 mmol l^{-1}. The mixture was incubated for 1 h at 30°C. After this period, 70 µl of water was added to each tube, and inorganic phosphate released by ATPases was assayed using a commercial phosphate dosage kit (Labtest® Diagnóstica, Vista Alegre, Brazil). According the manufacturer's instructions, 20 µl of reagent no. 1, 20 µl of reagent no. 2 and 40 µl of reagent no. 3 were added to the tubes to make a final volume of 250 µl. The tubes were immediately mixed after each addition. A 200 µl sample from each tube was transferred to a 96-well plate, which was read at 650 nm. This assay was repeated four times.

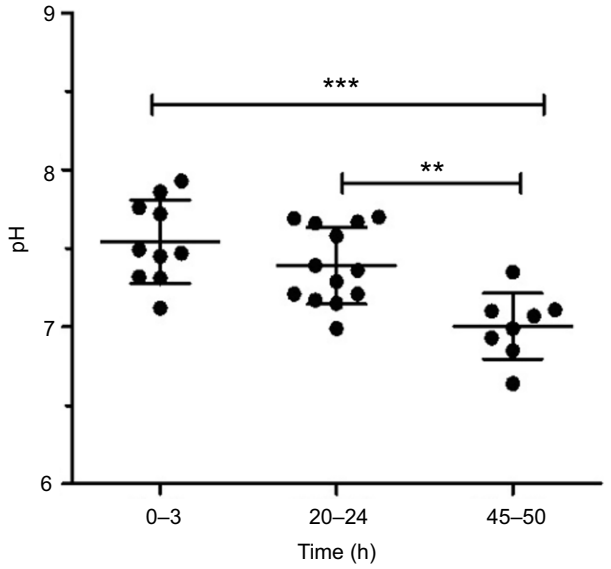

Fig. 2. pH in the abdominal midgut of *A. aegypti* females throughout blood digestion. pH-sensitive microelectrodes were used to measure pH within the abdominal midgut. Each point on the graph corresponds to a different insect. Data are presented as means±s.d. ANOVA, Tukey $^{**}P<0.01$; Tukey $^{***}P<0.001$.

pH measurement in the thoracic midgut from blood-fed females

Three- to six-day-old females fed for 30 min on anaesthetised hamsters and were then separated into two groups. The first group was offered 10% un-buffered sucrose solution containing the vital dye 0.1% Bromothymol Blue at pH 7.5 (pK_a=7.0). The second group was offered the same solution containing 0.1% Bromocresol Purple dye (pK_a=6.3) with the pH adjusted to 6.5. All solutions were given on pieces of soaked cotton wool. After 24 h, females were dissected in IS, and the dye colour observed in the thoracic midgut was compared with 0.1 mol l^{-1} standard buffered solutions containing each dye at different pH values, with 0.5 pH unit intervals (Fig. 1B,C).

Effect of haemolymph from blood-fed females on midgut pH

Following the methodology previously described by Santos et al. (2011) to evaluate whether a hormonal response is involved in the pH control of the midgut, female mosquitoes were blood-fed on anaesthetised hamsters for 30 min. The integument of two females was disrupted with stylets (taking care to avoid midgut rupture) in 1 µl of IS containing 2 mmol l^{-1} EDTA to release and mix the insect's haemolymph with this solution. Immediately, 1 µl of the solution containing the haemolymph was applied to dissected guts obtained from females that had previously ingested 10% sucrose with ~0.1% Bromothymol Blue. Only acidic midguts (pH≤6) were used in the experiments. To avoid desiccation, guts were first distended on a piece of agarose gel prepared with IS. After haemolymph treatment, the midguts were observed for 5 min under a stereomicroscope to evaluate the luminal pH. The colour observed inside the midgut was compared with standard solutions as explained above. We used haemolymph obtained from non-blood-fed females as a control. Each part of the midgut (thoracic or abdominal midgut) was classified as pH<6.5 or pH≥6.5.

Distribution of α-glucosidase activity in the midgut of unfed females

Twenty fasted females (3–6 days old) were dissected in IS, and their midguts were removed. Thoracic and abdominal midguts from these females were separated and transferred individually to different microcentrifuge tubes containing 200 µl of aqueous 1% Triton

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X-100. These samples were sonicated for 15 s and 50 µl (corresponding to 25% of a midgut) from each tube were transferred to tubes containing 100 µl distilled water and 100 µl 0.5 mol l^{-1} MES/NaOH buffer (pH 6). To each tube, we added 250 µl 12 mmol l^{-1} *p*-nitrophenyl α-D-glucopyranoside (Sigma, N-1377) dissolved in water (6 mmol l^{-1} final concentration). The tubes were incubated for 40 min at 30°C. After incubation, 1 ml of 0.375 mol l^{-1} glycine/NaOH buffer (pH 10.5) was added to each tube to stop the reaction. Blanks were prepared with 50 µl 1% Triton X-100. The absorbance was measured in 1 ml cuvettes at 400 nm using a Shimadzu UV-1650PC spectrophotometer. The activity was calculated using the *p*-nitrophenol molar extinction coefficient at 400 nm (18.200 l mol^{-1} cm^{-1}).

Statistical analysis

For pH measurements using microelectrodes, we applied the D'Agostino–Pearson test for normality, followed by ANOVA and Tukey's test. For α-glucosidase assays, we applied the D'Agostino–Pearson's test, followed by Wilcoxon's test. To analyse ATPase activity, we used Student's *t*-test. For the remaining experiments, we used Fisher's exact test. Results were considered statistically significant when $P<0.05$.

RESULTS

pH in the midgut of non-blood-fed female *A. aegypti* is actively regulated to ~pH 6

When *A. aegypti* females were forced to ingest unbuffered IS containing Bromothymol Blue, the colour of the dye in the thoracic as well as the abdominal midguts indicated that their contents were acidic (pH≤6), as previously observed for *L. longipalpis* (Santos et al., 2008). This acidity was maintained at pH≤6 even when highly buffered solutions such as 160 mmol l^{-1} Hepes at pH 7.5 mixed with Bromothymol Blue were ingested (Table 2). As the diverticulum does not interfere with the pH of its contents, the colour inside this compartment resembles the pH of the ingested solution (Table 2). Fig. 1A shows a dissected gut from a non-blood-fed female where it is possible to observe the colour of different anatomical parts after ingestion of the indicator dye Bromothymol Blue.

In contrast, when the insects ingested IS buffered with 160 mmol l^{-1} MES pH 5.0, the abdominal midgut underwent alkalinisation until it reached pH≥6 (Table 3). Together, the results presented in Tables 2 and 3 show that pH in the abdominal midgut is actively maintained at pH 6 by a mechanism strong enough to overcome the highly concentrated buffer solutions used in the study.

The mechanism responsible for restoring pH 6 in the thoracic midgut after ingestion of an acidic solution (160 mmol l^{-1} MES pH 5.0) seems less effective than that in the abdominal midgut (Table 3). Using a less-concentrated buffer would probably allow for a more effective restoration of pH in this part of the midgut.

Table 2. Intestinal pH of non-blood-fed female *A. aegypti* after ingestion of IS buffered to pH 7.5

	N		
pH	TM	AM	D
pH≤6.0	19	24	1
6.0<pH≤6.5	5	0	0
6.5<pH<7.0	0	0	1
pH≥7.0	–	–	12

Females ingested (forced feeding procedure) IS containing 0.1% Bromothymol Blue buffered with 160 mmol l^{-1} Hepes at pH 7.5. pH changes were evaluated by comparing the colour of the dye inside the midgut with standard buffered solutions containing the dye at different pH. *N*, number of observations; TM, thoracic midgut; AM, abdominal midgut; D, diverticulum.

Table 3. Intestinal pH of non-blood-fed female *A. aegypti* after ingestion of IS buffered to pH 5.0

	N		
pH	TM	AM	D
pH≤5.0	6	0	3
5.0<pH<6.0	2	0	0
pH≥6.0	1	10	0

Females ingested (forced feeding procedure) IS containing 0.1% Bromocresol Purple buffered with 160 mmol l^{-1} MES at pH 5.0. pH changes were evaluated by comparing the colour of the dye inside the midgut with standard buffered solutions containing the dye at different pH. *N*, number of observations; TM, thoracic midgut; AM, abdominal midgut; D, diverticulum.

The mechanism of midgut acidification is probably driven by V-ATPases

A rapid re-acidification of the midgut after ingestion of alkaline-buffered solutions probably requires an efficient proton-pumping mechanism, usually provided by V-ATPases. V-ATPases are multi-subunit complexes that use ATP to actively pump hydrogen ions through biological membranes (Wieczorek et al., 2009). According to our hypothesis, an intestinal V-ATPase could be responsible for pumping H^+ ions from the cytoplasm of the enterocytes into the midgut lumen when acidification is necessary to keep the gut lumen acidic (pH 6) in unfed females.

To determine whether V-ATPases participate in the midgut acidification mechanism, female *A. aegypti* ingested (forced feeding procedure) solutions buffered at pH 7.5 containing bafilomycin A1, a specific V-ATPase inhibitor. As expected, the presence of bafilomycin A1 hampered the physiological acidification mechanism of the abdominal midgut. However, we did not observe any impairment of the acidification process in the thoracic midgut with the bafilomycin A1 concentration used in this experiment (Table 4).

Na^+/K^+-ATPase appears to be the pump responsible for energising enterocytes

As presented in Table 3, V-ATPases stop functioning when the pH of the intestinal lumen falls below pH 6. While active, V-ATPases can energise enterocyte membranes, but when the luminal pH reaches 6 and interrupts H^+ pumping, the intestine can no longer rely on this form of energisation. Therefore, an additional transport

Table 4. The physiological role of V-ATPases during maintenance of acidic pH (~pH 6) in the midgut of non-blood-fed females

		N		
Treatment	Anatomical localisation	pH<6.5	pH≥6.5	*P*
Control: IS buffered with 30 mmol l^{-1} Hepes pH 7.5 +10% DMSO	TM	15	0	–
	AM	15	0	–
IS buffered with 30 mmol l^{-1} Hepes pH 7.5+1 µmol l^{-1} bafilomycin A1+10% DMSO	TM	10	1	0.4231
	AM	2	13	0.0001

All solutions contained Bromothymol Blue as a pH indicator dye. pH changes were evaluated by comparing the colour of the dye inside the midgut with standards at different pH. The number of observations in each pH interval (*N*) was compared with controls using Fisher's exact test. TM, thoracic midgut; AM, abdominal midgut.

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Table 5. pH in female *A. aegypti* thoracic midgut 24 h after blood feeding

	Bromothymol Blue		Bromocresol Purple	
	pH≤6	pH>6	pH≥6	pH<6
No. of observations in TM	15	0	15	0

pH indicator dyes Bromothymol Blue and Bromocresol Purple were prepared with sucrose and offered to females immediately after blood ingestion. Twenty-four hours later, females were dissected and the colour observed inside their thoracic midgut was compared with standard buffered solutions. *N*, number of observations in each pH interval; TM, thoracic midgut.

system is required to maintain the membrane potential. Instead of a V-ATPase, we found a Na^+/K^+-ATPase to be responsible for most of the ATPase activity in the midgut of *Aedes*; it accounted for 79% of the total ATPase activity in midgut extracts. The total midgut ATPase activity in the presence of Mg^{2+}, Na^+ and K^+ was 1.518±0.342 µmol PO_4^{3-} h^{-1} and the ATPase activity in the presence of Mg^{2+}, Na^+, K^+ and ouabain (total activity minus Na^+/K^+-ATPase activity) was 0.321±0.092 µmol PO_4^{3-} h^{-1}.

pH in the abdominal midgut rises after blood ingestion

We measured the pH of the abdominal midgut in 31 blood-fed *A. aegypti* females using H^+-sensitive microelectrodes (Santos et al., 2008). Measures were taken at various points during the 50 h after blood ingestion. These measurements are presented in Fig. 2. In the first 3 h post-blood feeding, the pH in the abdominal midgut was on average 7.54±0.26, with measurements ranging from pH 7.12 to 7.93. As digestion progressed, the pH decreased slowly, reaching an average of pH 7.39±0.24 at 20–24 h after blood feeding and pH 6.99±0.21 during the last 5 h of the measurement period (45–50 h post-blood feeding). In *A. aegypti*, digestion is fully completed in approximately 72 h.

The thoracic midgut remains acidic after blood ingestion and abdominal midgut alkalinisation

In blood-fed females, the midgut is clearly compartmentalised. While the abdominal midgut became alkaline, the pH in the thoracic midgut remained acidic in all examined females. The pH inside the thoracic midgut was ∼6 according to measurements performed with two indicator dyes: Bromothymol Blue and Bromocresol Purple (Table 5).

Proteins but not amino acids promote shutdown of the acidification mechanism and trigger alkalinisation

To accomplish alkalinisation after blood ingestion, the insect must switch off the mechanism responsible for keeping the midgut acidic and at the same time switch on the alkalinisation mechanism. Because blood intake induces alkalinisation of the abdominal midgut, we decided to investigate the stimulus present in the blood that triggers these physiological responses.

To obtain evidence of alkalinisation mechanisms, we force-fed females normal human serum with pH adjusted to ∼6 and containing Bromothymol Blue. In these conditions, any alkalinisation observed in the midgut would be attributed to intrinsic mechanisms of alkalinisation besides CO_2 release. In fact, in this experiment, the abdominal midgut of *A. aegypti* females became alkaline just after serum ingestion, and the thoracic midgut remained acidic, as observed for blood-fed females. The same result was obtained using serum buffered with 30 mmol l^{-1} MES pH 6.0, reinforcing the existence of an alkalinisation mechanism in the abdominal midgut that could overcome the buffering power of the solution ingested (Table 6).

To refine our results, we fed the mosquitoes unbuffered solutions containing purified proteins that had previously been adjusted to pH ∼6. We tested solutions containing purified 5% BSA, egg lysozyme or egg albumin, and all of these could stimulate

Table 6. Effect of different solutions ingested by *A. aegypti* females on midgut pH

		N		*P*	
Solution	Anatomical localisation	pH<6.5	pH≥6.5	Control 1	Control 2
Human serum (pH 6.0)	TM	23	2	–	1.0
	AM	0	27	–	<0.0001
Human serum+30 mmol l^{-1} MES (pH 6.0)	TM	10	0	1.0	–
	AM	3	15	<0.0001	–
IS+100 mmol l^{-1} amino acids (pH 6.0)	TM	15	0	–	0.1723
	AM	20	1	–	<0.0001
IS+30 mmol l^{-1} MES+100 mmol l^{-1} amino acids (pH 6.0)	TM	12	0	0.3939	–
	AM	20	0	1.0	–
IS+5% lysozyme (pH 6.0)	TM	15	4	–	0.1723
	AM	0	20	–	<0.0001
IS+5% lysozyme+30 mmol l^{-1} MES (pH 6.0)	TM	20	0	1.0	–
	AM	19	0	1.0	–
Unbuffered IS+5% BSA (pH 6.0)	TM	12	0	–	1.0
	AM	0	16	–	<0.0001
IS+5% BSA+30 mmol l^{-1} MES (pH 6.0)	TM	19	0	1.0	–
	AM	19	0	1.0	–
IS+5% ovalbumin (pH 6.0)	TM	17	0	–	1.0
	AM	4	13	–	<0.0001
IS+5% ovalbumin+30 mmol l^{-1} MES (pH 6.0)	TM	20	0	1.0	–
	AM	20	0	1.0	–
Control 1: IS+30 mmol l^{-1} MES (pH 6.0)	TM	19	0	–	1.0
	AM	20	0	–	1.0
Control 2: unbuffered IS (pH 6.0)	TM	19	0	–	–
	AM	20	0	–	–

Females ingested different solutions (forced feeding procedure) containing 0.1% Bromothymol Blue dye, and pH inside the midgut was evaluated according to the colour of the dye. The number of observations in each pH interval (*N*) was compared with the appropriate control by Fisher's exact test. AM, abdominal midgut; TM, thoracic midgut; *N*, number of observations in each pH category.

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alkalinisation of the abdominal midgut. Interestingly, in some mosquitoes that had ingested unbuffered lysozyme, there was also evidence of thoracic midgut alkalinisation. In our experiments, buffered protein solutions (with 30 mmol l^{-1} MES at pH 6.0) were unable to promote alkalinisation. Most likely, purified proteins provided a suboptimum stimulus when compared with serum. The stimulus of purified proteins was insufficient to overcome the buffering capability of the ingested solution (purified protein+IS buffered with 30 mmol l^{-1} MES at pH 6.0), but a buffered serum solution (serum buffered with 30 mmol l^{-1} MES at pH 6.0) was able to induce alkalinisation (Table 6).

Solutions with high concentrations of amino acids (100 mmol l^{-1}) in buffered (30 mmol l^{-1} MES pH 6.0) and non-buffered solutions also failed to promote alkalinisation. This result contrasts with that observed for *L. longipalpis*, where this treatment promptly promoted alkalinisation (Santos et al., 2011). An overview of these results is presented in Table 6.

Hormones released in the haemolymph participate in abdominal midgut alkalinisation

The presence of proteins inside the abdominal midgut promotes the release of alkalinising hormones in the haemolymph of *A. aegypti* females during blood intake. Indeed, haemolymph collected from females during the first hour after blood ingestion could induce *ex vivo* alkalinisation in isolated guts containing an indicator dye. Accordingly, this alkalinisation did not occur with the haemolymph collected from non-fed females (Table 7).

Digestion of blood and carbohydrates is compartmentalised in the midgut

Proteases (especially trypsin) are the main enzymes involved in blood digestion. In contrast, α-glucosidases are the main carbohydrases associated with digestion of sucrose and other carbohydrates present in sugar meals. Haematophagous insects from the suborder Nematocera usually ingest sugar even during blood digestion. This behaviour presents a problem: the optimum pH values for these enzymes are quite different, so they cannot efficiently work at the same time within the same compartment.

To solve this problem, during blood digestion, the acidic thoracic midgut digests carbohydrates such as sucrose while the alkaline abdominal midgut digests blood. In keeping with this separation, the distribution of α-glucosidase activity is not homogeneous throughout the midgut. Its activity is 2.70-fold higher in the thoracic midgut ($6.95\times10^3\pm5.0\times10^3$ µmol *p*-nitrophenol min^{-1}) compared with the abdominal midgut ($2.55\times10^3\pm2.8\times10^3$ µmol *p*-nitrophenol min^{-1}; $P=0.0011$), even though the abdominal midgut is much larger than the thoracic midgut.

DISCUSSION

Adult insects from the suborder Nematocera regularly feed on carbohydrate-rich liquids, but females from haematophagous species also perform haematophagy to develop their ovaries and prepare for egg laying. Such feeding patterns suggest a necessity for physiological alterations of the abdominal midgut. Initially, this compartment of *L. longipalpis* is slightly acidic, an adequate environment for sucrose digestion. When females ingest blood, their abdominal midgut suddenly becomes alkaline to promote blood digestion.

As far as we know, intestinal pH control in different physiological conditions (before and after blood ingestion) was previously only studied in the phlebotomine sand fly *L. longipalpis*. To obtain evidence that this digestion pattern is similar for other haematophagous nematoceran insects, we investigated intestinal pH control in adult female *A. aegypti*.

pH in the midgut of non-blood-fed female *A. aegypti* is actively maintained at pH 6

In keeping with our hypothesis, *A. aegypti* had a similar pattern of intestinal pH control to that of *L. longipalpis*. The *A. aegypti* abdominal midgut was maintained at approximately pH 6 even when the insect ingested highly buffered alkaline solutions such as IS containing 160 mmol l^{-1} Hepes pH 7.5 (Table 2). Similarly, when non-blood-fed insects ingested acidic solutions such as IS containing 160 mmol l^{-1} MES buffer pH 5.0, the midgut underwent alkalinisation to pH 6 (Table 3). The mechanism of acidification (from pH 7.5 to pH 6) seems similarly efficient in the thoracic midgut and the abdominal midgut (Table 2). However, alkalinisation (from pH 5.0 to pH 6) appears to be less efficient in the thoracic midgut (Table 3). A less-concentrated buffer would probably allow for more complete restoration of pH in this part of the midgut.

H^+-driven transporters, such as the Na^+/H^+ (or K^+/H^+) antiporter, may transport H^+ from the lumen to the cytoplasm of enterocytes in exchange for Na^+ or K^+. This transport system could be involved in the alkalinisation (from pH 5 to pH 6) observed when non-blood-fed insects ingest acid-buffered solutions such as 160 mmol l^{-1} MES pH 5.0 prepared in IS (Table 3). The exchange of HCO_3^- with Cl^- may also be involved in the process.

Midgut acidification appears to be driven by V-ATPases, and Na^+/K^+-ATPases appear to energise midgut transport

The mechanism of midgut acidification appears to involve V-ATPases, as bafilomycin A1, a specific inhibitor of these pumps, inhibited gut re-acidification after ingestion of alkaline-buffered solutions (Table 4). Patrick et al. (2006) showed that

Table 7. Action of haemolymph extracted from blood-fed *A. aegypti* females on midgut pH

Treatment	Anatomical localisation	*N*		*P*
		pH<6.5	pH≥6.5	
Control 1: 1 µl of IS containing 2 mmol l^{-1} EDTA	TM	15	1	0.5996
	AM	15	0	0.2241
Control 2: haemolymph from two sugar-fed females collected in 1 µl of IS containing 2 mmol l^{-1} EDTA	TM	13	2	–
	AM	12	3	–
Haemolymph from two blood-fed females collected in 1 µl of IS containing 2 mmol l^{-1} EDTA	TM	15	1	0.5996
	AM	3	12	0.0028

Haemolymph was applied directly to midgut preparations dissected from females that had ingested sucrose solution containing Bromothymol Blue. The pH was inferred from the colour of the dye present in the midgut lumen. The number of observations in each pH interval (*N*) was compared with the appropriate control by Fisher's exact test. Control 1 was performed to show that EDTA does not influence the pH of the midgut. IS, insect saline; TM, thoracic midgut; AM, abdominal midgut.

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V-ATPases are positioned at the apical portion of enterocytes in the midgut of *A. aegypti*. This supports our idea that V-ATPase molecules pump H^+ ions into the intestinal lumen and are activated only when the pH in the abdominal midgut rises beyond pH 6. While active, V-ATPases can energise the membrane, but when the luminal pH reaches pH 6 and interrupts H^+ pumping, the intestine can no longer count on this form of membrane energisation.

Na^+/K^+-ATPases are located on the basolateral side of *A. aegypti* enterocytes (Patrick et al., 2006). According to Pacey and O'Donnell (2014), Na^+/K^+-ATPase is the principal energiser of the midgut of *A. aegypti*, as ouabain interferes more with ionic fluxes in the midgut than bafilomycin A1. To find additional evidence of Na^+/K^+-ATPase activity in the *A. aegypti* midgut, we measured the total ATPase activity in the presence and absence of ouabain. In the presence of ouabain, Na^+/K^+-ATPase was inhibited, eliminating 79% of the total ATPase activity. Such a striking decrease is in accordance with the proposed role of the Na^+/K^+-ATPase as the principal pump involved in membrane energisation.

The midgut of *A. aegypti* possesses a luminal pH sensor

A mechanism that maintains pH at ∼6 implies the existence of a 'pH sensor' able to constantly evaluate the luminal pH such that V-ATPases are turned on when the intestinal lumen incorrectly alkalises without blood ingestion or are turned off when it acidifies below pH 6 by ingestion of acidic solutions. We do not know which molecule is responsible for pH sensing, but this function could be performed by the V-ATPase itself. Previously, the V-ATPase was found to be a pH sensor that could regulate endocytic traffic in intracellular environments (Recchi and Chavrier, 2006). The pH sensor may also be present in the apical portion of intestinal endocrine cells. When activated, the endocrine cells may release alkalinising hormones on their basolateral side in the haemolymph. In this case, the shutdown of the V-ATPases may occur by hormonal action on hormone receptors on the basolateral side of enterocytes. The release of alkalinising hormones triggered by ingestion of acidic solutions should be investigated in the future.

Proteins but not amino acids promote shutdown of the acidification mechanism and trigger alkalinisation

When blood ingestion occurs, it is necessary to turn off the acidification mechanism that would prevent midgut alkalinisation. If this pH-maintenance mechanism is not turned off, even the intake of strongly buffered solutions such as 160 mmol l^{-1} Hepes pH 7.5 cannot promote midgut alkalinisation (Table 2). In the absence of V-ATPase activity (pumping H^+ to the lumen), other transporters may be responsible for the alkalinisation from pH 5 to pH 6 observed in the midgut (Table 3).

Our results obtained with *L. longipalpis* (Santos et al., 2008, 2011) and *A. aegypti* indicate that the midgut of these haematophagous nematocerans can perceive the presence of soluble proteins, a stimulus that promotes midgut alkalinisation in both species. Any ingested protein triggered alkalinisation, including proteins that are not present in the blood, such as lysozyme and ovalbumin (Table 5). Therefore, blood-derived proteins seem to be part of the stimulus responsible for triggering alkalinisation. How the midgut detects the presence of soluble proteins that are so different from each other remains a mystery.

According to Blakemore et al. (1995), the midgut of the haematophagous fly *Stomoxys calcitrans* can detect different soluble proteins and responds with production of trypsin, but insoluble proteins, free amino acids, small peptides or poly L-amino acids do not stimulate trypsin production. These insects probably share the same basic mechanism of protein detection.

Protein detection inside the midgut does not seem to be linked to an increase in intra-intestinal osmotic pressure, as the tested proteins were at low concentrations (0.75 mmol l^{-1} BSA, 3.5 mmol l^{-1} lysozyme, 1.17 mmol l^{-1} ovalbumin). In addition, the buffer Hepes did not induce alkalinisation even at 160 mmol l^{-1}. These unnatural molecules are probably not absorbed by the midgut and instead remain osmotically active in the intestinal lumen after ingestion.

The alkalinisation mechanism seems to be more efficient in *L. longipalpis* than in *A. aegypti*. In *L. longipalpis*, alkalinisation occurs even when the insect ingests purified protein solutions buffered in pH 6.0 (Santos et al., 2011), whereas in *A. aegypti*, buffered protein solutions were unable to promote alkalinisation (Table 6).

Unlike *L. longipalpis*, which responds to highly concentrated amino acid solutions by triggering midgut alkalinisation (Santos et al., 2011), *A. aegypti* only responds to proteins (Table 6). The detection of amino acids in the intracellular compartment is normally performed by the TOR signalling system present in any cell type (Shimobayashi and Hall, 2016; Goberdhan et al., 2016). According to our results, it is reasonable to hypothesise that *L. longipalpis* can perceive the presence of amino acids (in high concentration) by means of the TOR system, which may be involved with V-ATPase inactivation and alkalinisation. Recently, we found direct evidence that the midgut of *L. longipalpis* can detect amino acids (Santos et al., 2014). In that study, we showed that the midgut, in *ex vivo* preparations, responds to the presence of amino acids by producing trypsin. The higher the amino acid concentration, the higher the production of trypsin. The TOR system of *A. aegypti*, despite being able to detect amino acids, does not interfere with the function of V-ATPases but stimulates early trypsin synthesis (Brandon et al., 2008).

The PAT1 symporter, which is responsible for transport of amino acids and H^+ from the lumen to midgut cells and is present in the *A. aegypti* midgut (Evans et al., 2009), may have a role in maintaining an alkaline abdominal midgut during blood digestion. This is possible if the presence of proteins under digestion inside the lumen keeps the V-ATPase off.

Hormones released in the haemolymph participate in abdominal midgut alkalinisation

Protein detection in the midgut may be associated with endocrine cells, which are normally distributed throughout the enterocytes (Brown et al., 1985). Endocrine cells can respond to the presence of proteins by releasing alkalising hormones in the insect's haemolymph. As soon as they are released, these hormones could activate receptors in the basolateral side of the enterocytes to promote the physiological changes observed in the present study. Similar results were previously observed in *L. longipalpis* (Santos et al., 2011). In both studies, the haemolymph taken from blood-fed females can induce alkalinisation in isolated midguts (Table 7). Hormones released by endocrine organs other than the midgut could be involved in the alkalinisation process.

The participation of hormones in the regulation of intestinal pH has already been observed in the larvae of other insects. The normal alkalinisation of the midgut of *Manduca sexta* larvae depends on factors released by the insect's own gut in *ex vivo* preparations (Clark et al., 1998). Indeed, when those factors were washed away, alkalinisation was compromised. The participation of hormones in intestinal pH control was also observed in *Rhynchophorus ferrugineus* larvae. Sunitha et al. (1999) observed in *ex vivo*

preparations that hormones obtained from intestinal extracts restored normal pH in each part of the lumen after they had been altered by the introduction of a buffer solution.

Digestion of blood and carbohydrates is compartmentalised in the midgut

In general, the optimum pH for α-glucosidase activity in insects is slightly acidic (Terra and Ferreira, 1994). This is in keeping with our hypothesis that nematoceran insects have an acidic midgut before blood intake by females. According to our results, *L. longipalpis* (Santos et al., 2011) and *A. aegypti* herein have an acidic thoracic midgut even after blood ingestion. This suggests that sugar and blood digestion occur separately inside the midgut to compartmentalise these digestive processes in accordance with their distinct enzyme pH requirements. Accordingly, α-glucolytic activity is greater in the thoracic portion of *A. aegypti* midgut. The same was observed for *L. longipalpis* (Gontijo et al., 1998).

The present study shows that adult *A. aegypti* females have a very similar gut physiology to that previously described for *L. longipalpis*. It is probable that all haematophagous nematocerans exhibit the same physiological behaviour. An acidic midgut seems to be conserved among all adult Nematocera. It is possible that the alkalinisation mechanism is an adaptation to haematophagy. This mechanism may also exist in non-haematophagous insects to help them digest protein-rich diets other than blood.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: V.C.S., N.F.G.; Methodology: D.B.N., V.C.S., M.H.P., N.F.G.; Validation: D.B.N.; Formal analysis: M.H.P., N.F.G.; Investigation: D.B.N.; Resources: R.N.A., M.R.S., M.H.P., L.A.M., N.F.G.; Data curation: D.B.N.; Writing - original draft: D.B.N., N.F.G.; Writing - review & editing: R.N.A., M.R.S., M.H.P.; Supervision: N.F.G.; Project administration: R.N.A., M.H.P., N.F.G.; Funding acquisition: N.F.G.

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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