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RESEARCH ARTICLE

Mechanisms of pH control in the midgut of *Lutzomyia longipalpis*: roles for ingested molecules and hormones

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SUMMARY

Control of the midgut pH in *Lutzomyia longipalpis* enables the insect's digestive system to deal with different types of diet. Phlebotomines must be able to suddenly change from a condition adequate to process a sugar diet to one required to digest blood. Prior to blood ingestion, the pH in the midgut is maintained at ~6 *via* an efficient mechanism. In the abdominal midgut, alkalization to a pH of ~8 occurs as a consequence of the loss of CO_2 from blood (CO_2 volatilization) and by a second mechanism that is not yet characterized. The present study aimed to characterize the primary stimuli, present in the blood, that are responsible for shutting down the mechanism that maintains a pH of 6 and switching on that responsible for alkalization. Our results show that any ingested protein could induce alkalization. Free amino acids, at the concentrations found in blood, were ineffective at inducing alkalization, although higher concentrations of amino acids were able to induce alkalization. Aqueous extracts of midgut tissue containing putative hormones from intestinal endocrine cells slightly alkalized the midgut lumen when applied to dissected intestines, as did hemolymph collected from blood-fed females. Serotonin, a hormone that is possibly released in the hemolymph after hematophagy commences, was ineffective at promoting alkalization. The carbonic anhydrase (CA) enzyme seems to be involved in alkalizing the midgut, as co-ingestion of acetazolamide (a CA inhibitor) with proteins impaired alkalization efficiency. A general model of alkalization control is presented.

Key words: midgut pH control, *Lutzomyia longipalpis*, ingested nutrient, insect hormone, midgut alkalization.

INTRODUCTION

Among hematophagous dipterans of the suborder Nematocera, which comprises mosquitoes (Culicidae), black flies (Simuliidae), midges (Ceratopogonidae) and phlebotomine sand flies (Psychodidae), only females ingest blood, because it is necessary for the development of their ovarioles. However, the common food for males and females consists of sugar-rich solutions such as nectar (Alexander and Usma, 1994), honey dew (Cameron et al., 1995) and, at least for phlebotomines, plant sap (Schlein and Warburg, 1986). In these insects, carbohydrates are stored in the diverticulum from where they pass to the midgut to be digested and absorbed as monosaccharides (Gontijo et al., 1998; Tang and Ward, 1998).

The phlebotomine *Lutzomyia longipalpis* is the main vector of *Leishmania infantum* (Cunha and Chagas, 1937), the etiologic agent of visceral leishmaniasis in the Americas (Soares and Turco, 2003). In *L. longipalpis* and probably in all the other hematophagous nematocerans, the physiological parameters of the midgut must quickly change from conditions that are adequate for processing a sugar diet to those that are adequate for processing the ingested blood.

We have previously reported that the pH of the diverticulum in *L. longipalpis* females assumes the value of the ingested food, reflecting an unbuffered condition (Santos et al., 2008). However, the pH in the abdominal midgut is rigorously adjusted according to the physiological state. In unfed or sugar-fed insects, the midgut pH is actively maintained at ~6, even when it is challenged by ingestion of strong buffered solutions such as 0.16 mol l^{-1} HEPES pH 7.5 or 0.16 mol l^{-1} MES pH 5.0 (Santos et al., 2008). The intestinal enzyme α-glucosidase, which digests carbohydrates that are typically found in the sugar diet, requires an acidic pH (Gontijo et al., 1998; Jacobson et al., 2001). When blood is ingested, it is stored in the abdominal midgut, where the mechanism responsible for maintaining the pH at 6 is immediately switched off, allowing alkalization to pH 8.15 (Santos et al., 2008). This switch must occur because an alkaline pH is required for blood digestion by the exoproteases trypsin and quimotrypsin, which are enzymes with alkaline optimum pH values (Terra and Ferreira, 1994). Even when the abdominal midgut is alkaline as a consequence of blood ingestion, carbohydrate digestion in the thoracic midgut continues normally because this region remains acidic (pH 6). This enables action of α-glucosidase, which is concentrated in this region and is bound to the microvilli of the enterocytes (Gontijo et al., 1998; Santos et al., 2008).

Apparently, two mechanisms are involved in the process of alkalization. The first occurs as a consequence of the loss of CO_2 from blood (CO_2 volatilization) according to the equation $CO_2+H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$ (Santos et al., 2008). The second mechanism has not yet been characterized (Santos et al., 2008). It is important to emphasize that CO_2 volatilization, by itself, is insufficient to support alkalization if the mechanism maintaining the pH at 6 is not turned off.

To clarify some aspects of pH control in *L. longipalpis*, the present study aimed to characterize the primary stimulus in the ingested material that is responsible for shutting down the mechanism

maintaining the pH at 6, thereby allowing the uncharacterized mechanism responsible for alkalization to occur.

The midguts of insects, including those of *L. longipalpis* adult females, contain endocrine cells that are dispersed between the enterocytes, the most common cells of the alimentary canal that are responsible for production of digestive enzymes and absorption of digested nutrients (Leite and Evangelista, 2001). It has been proposed that secretions from endocrine cells control some intestinal functions, including the production of digestive enzymes (Dadd, 1961; Lehane et al., 1995; Harshini et al., 2002; Sakai et al., 2006) and regulation of the luminal pH in the midgut, by directly stimulating receptors that are present on the hemolymph side of the enterocytes (Clark et al., 1998; Sunitha et al., 1999; Onken et al., 2008). The last three studies mentioned above (concerning the regulation of luminal pH) were carried out in larvae of three insect species: *Manduca sexta* (Lepidoptera), *Rhynchophorus ferrugineus* (Coleoptera) and *Aedes aegypti* (Diptera), respectively. These studies, concerning the regulation of luminal pH, were carried out in larvae of three insect species: *Manduca sexta* (Lepidoptera) (Clark et al., 1998), *Rhynchophorus ferrugineus* (Coleoptera) (Sunitha et al., 1999) and *Aedes aegypti* (Diptera) (Onken et al., 2008). Considering the possibility that a similar mechanism could be involved in the alkalization observed in the midgut of adult *L. longipalpis*, an aqueous extract of midgut tissue containing putative hormones was tested, as was a solution containing hemolymph obtained from blood-fed females. To our knowledge, these assays are the first to provide evidence of hormonal control of pH in an adult batch digester insect.

While taking a blood meal, hematophagous insects must rapidly initiate the mechanism responsible for diuresis. This diminishes the volume occupied by the blood ingested in the midgut, thus allowing ingestion of additional blood. It is well known that during hematophagy, serotonin, a hormone involved in diuresis, is released in the hemolymph by the nervous system of *Rhodnius prolixus* (a hematophagous hemipteran) (Maddrell et al., 1991; Orchard, 2006). This process stimulates water absorption by the midgut and urine formation by the Malpighian tubules (Te Brugge et al., 2009). Indirect evidence suggests that serotonin release also occurs in mosquitoes during the hematophagic process (Novak and Rowley, 1994; Novak et al., 1995; Clark et al., 2009). Even among the non-hematophagous larvae of mosquitoes, studies have shown that serotonin acts on the midgut to increase the transepithelial voltage and, consequently, the rate of alkalization (Onken et al., 2008; Onken et al., 2009; Onken and Moffet, 2009). A hormone such as serotonin, which is released in the hemolymph after hematophagy begins, could also be released in the hemolymph of other insects, including *L. longipalpis*. Because this hormone seems to act in the midgut and could be coincidently released during hematophagy, we hypothesized that it could be involved in alkalization in *L. longipalpis*.

In a previous study, we demonstrated that the enzyme carbonic anhydrase participates in the mechanism of pH 6 maintenance in unfed and sugar-fed females (Santos et al., 2008). Thus, in this study, we investigated the possibility that this enzyme is involved in the mechanism of alkalization, perhaps by generating bicarbonate ions to be transported to the lumen.

MATERIALS AND METHODS

Insects

All experiments were performed with 2- to 5-day-old females from a population of *Lutzomyia longipalpis* (Lutz and Neiva 1912) originating from Teresina, state of Piauí, Brazil, that was maintained as a closed colony according to the methodology proposed by Modi and Tesh (Modi and Tesh, 1983).

Forced feeding procedure and pH evaluation

Forced feeding was performed as described previously (Santos et al., 2008) by introducing the insect mouthparts into a glass capillary tube containing the solution to be tested (Fig. 1b). This procedure stimulates the insect suction pump, forcing the insect to ingest the solution. Immediately after ingestion, the insects were 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). The vital indicator dye Bromothymol Blue (pKa 7) was present in all solutions ingested (final concentration ~0.1%) to permit measurement of intestinal pH (Santos et al., 2008).

Screening procedure

The forced feeding procedure was used to evaluate the pH inside the midgut of the females after ingestion of various solutions to determine which was the primary stimulus responsible for alkalization (Table 1). The colors inside the diverticulum, thoracic and abdominal midgut were compared with colors of standard buffered dye solutions at different pH values covering 0.5 unit intervals (pH 6.0 to 8.0) (Fig. 1e). Some experiments required standard solutions prepared by dissolving the dye directly in serum, in 5% bovine serum albumin (BSA) or casein solutions (standard

Table 1. Solutions ingested by *Lutzomyia longipalpis* females *via* the forced feeding technique

Description
Control 1: unbuffered IS solution with pH adjusted to ~7.0
Control 2: IS solution buffered with 30 mmol l^{-1} HEPES/NaOH pH 7.4
Control 3: IS solution buffered with 30 mmol l^{-1} MES/NaOH, pH 6.0
IS buffered with 30 mmol l^{-1} HEPES/NaOH pH 7.4 containing a mixture of 5 mmol l^{-1} of free amino acids purchased from Sigma (code M-5550): 0.87 arginine, 0.15 cysteine, 0.28 histidine, 0.58 isoleucine, 0.58 leucine, 0.58 lysine, 0.15 methionine, 0.29 phenyalanine, 0.58 threonine, 0.07 tryptophan, 0.29 tyrosine and 0.58 valine (concentrations expressed in mmol l^{-1})
Stock solution of amino acids 172 mmol l^{-1} (Sigma; code M-5550) with pH adjusted to ~7.4
Unbuffered human serum with pH adjusted to ~6
Unbuffered human serum with pH adjusted to ~7.4
IS containing 5% BSA not buffered with pH adjusted to ~6
IS containing 5% BSA buffered with 30 mmol l^{-1} MES pH 6.0
Unbuffered IS containing 5% lysozyme with pH adjusted to ~6
IS containing 5% lysozyme buffered with 30 mmol l^{-1} MES/NaOH pH 6.0
Unbuffered IS containing 5% casein with pH adjusted to ~7.4

IS, insect saline (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). Bromothymol Blue (0.1%) was present in all ingested solutions.

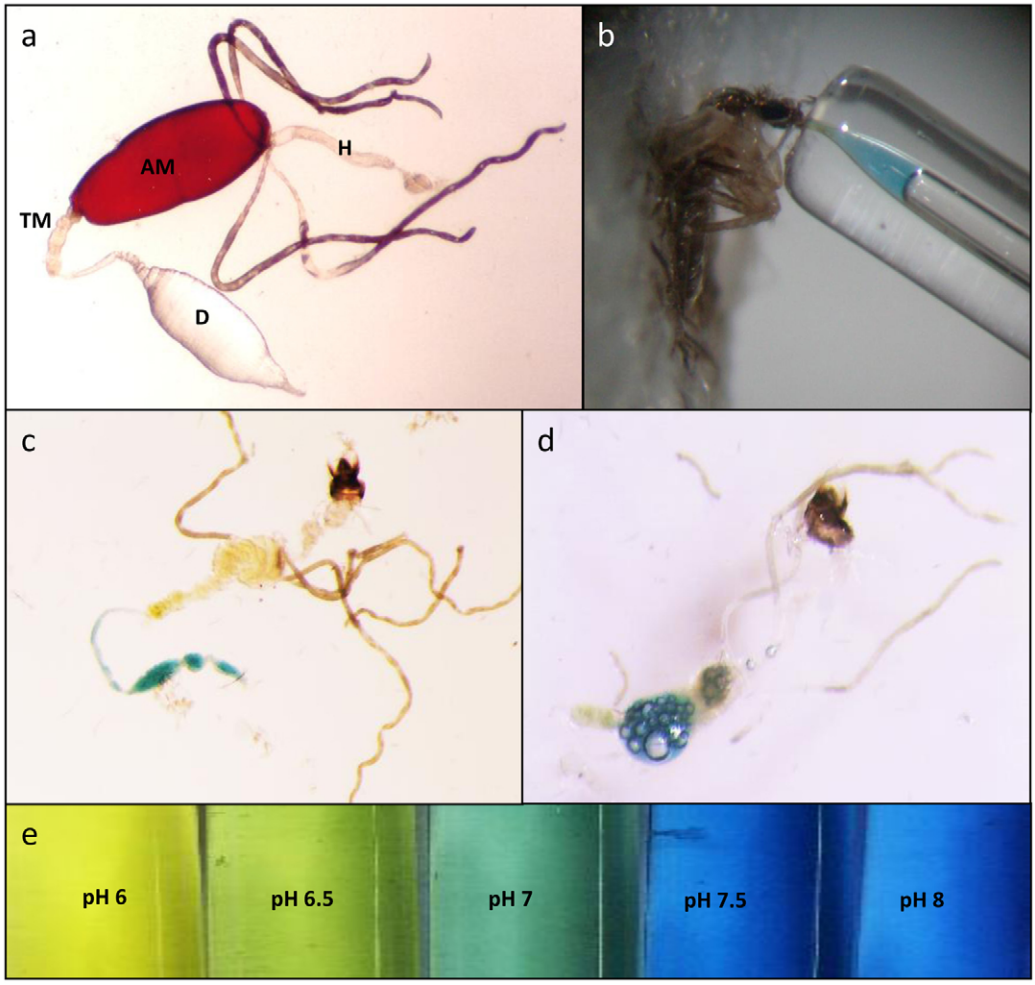

Fig. 1. Anatomy of *Lutzomyia longipalpis* intestine and pH measurement under different physiological conditions. (a) Intestine from a blood-fed female. (b) Forced feeding procedure. (c) Example of an acidified intestine after ingestion of 30 mmol l^{-1} HEPES pH 7.4. (d) Example of an alkalized intestine after ingestion of 30 mmol l^{-1} MES pH 6 containing lysozyme. (e) Standard colors of Bromothymol Blue at different pH levels. AM, abdominal midgut; D, diverticulum filled with sugar solution; H, hindgut; TM, thoracic midgut.

solutions prepared with lysozyme were not necessary). The observations in the thoracic or abdominal midgut were classified as pertaining to one of the following categories: pH<6.5 or ≥6.5. As pH in the diverticulum was not influenced by the insects, it was not considered elsewhere.

Effect of proteins applied directly on the midgut

To evaluate the effects of albumin and lysozyme when in direct contact with the basal side of the midgut epithelia (on the hemolymph side of the midgut), 3 µl of protein solution (5%) was applied directly on dissected preparations of acidic midguts obtained after ingestion of IS containing 0.1% Bromothymol Blue. pH was evaluated after 10 min of exposure. To avoid dehydrating the tissues, all preparations were distended on a 2% agarose gel prepared with IS. As a control, the same volume of IS was applied onto the preparations. The same criterion described above was used to classify the observations in the midgut (categories: pH<6.5 or ≥6.5).

Action of midgut extract, hemolymph of blood-fed insects and serotonin on midgut pH

To study whether intestinal hormones are involved in the midgut alkalization, we prepared a midgut extract using a protocol modified from that proposed by Sunitha et al. (Sunitha et al., 1999). Briefly, 60 midguts of 3-day-old females were dissected in IS solution and transferred to a microcentrifuge tube containing 20 µl cold IS and 2 mmol l^{-1} ethylenediamine-tetraacetic acid (EDTA). EDTA is a metalloprotease inhibitor used to prevent peptide degradation. The mixture containing the equivalent of 3 midguts µl^{-1} was immediately heated to 100°C for 10 min. The material was cooled on ice and sonicated for 10 min to solubilize small peptides such as the midgut hormones. Finally, the sample was homogenized in a vortex for 5 min and centrifuged at 10,000 ***g*** for 10 min at 4°C. The liquid fraction of the midgut extract was collected and stored at –20°C. During the experiments, 1.0 µl of the midgut extract was applied directly on acidic midguts dissected from females fed with 0.1% Bromothymol Blue dissolved in IS. To avoid dehydration of the tissues, all preparations were distended on a piece of 2% agarose gel prepared with IS. The midguts were observed for 10 min, and pH changes were monitored by shifts in the color of the indicator dye (Santos et al., 2008).

To investigate the effect of hormones present in the hemolymph collected from blood-fed females, hemolymph was collected by opening the integument of a blood-fed female in 1 µl IS containing 2 mmol l^{-1} EDTA. The hemolymph was obtained from females 10 to 120 min after a blood meal. This hemolymph solution was immediately applied to acidic midguts dissected from females that had ingested a 10% sucrose solution (12–24 h before) containing 0.1% Bromothymol Blue that was offered to them soaked in a piece of cotton (not by forced feeding). To avoid dehydration, the midgut preparations were distended on a piece of agarose gel as previously described. After addiction of the hemolymph solution to the preparations, the midguts were observed for 10 min, and any modifications of the pH of the lumen were noted. The controls were performed with hemolymph solution collected from sugar-fed

Table 2. Intestinal pH control: summary of the responses of the midgut of *L. longipalpis* to different stimuli

Treatment	Anatomical localization	*N*		*P*		
		pH<6.5	pH≥6.5	Control 1	Control 2	Control 3
5 mmol l^{-1} amino acids + IS + 30 mmol l^{-1} HEPES pH 7.4	TM	7	4	–	0.09	–
	AM	10	1	–	1.0	–
0.172 mol l^{-1} amino acid aqueous solution, unbuffered, pH ~7.4	TM	1	9	<0.0001	–	–
	AM	0	10	<0.0001	–	–
Human serum unbuffered, adjusted to pH ~6.0	TM	19	2	0.2	–	–
	AM	4	17	<0.0001	–	–
Human serum unbuffered, adjusted to pH ~7.4	TM	22	5	1.0	–	–
	AM	4	22	<0.0001	–	–
5% BSA + IS unbuffered, adjusted to pH ~6	TM	4	8	0.003	–	–
	AM	0	13	<0.0001	–	–
5% BSA + IS + 30 mmol l^{-1} MES pH 6.0	TM	9	13	–	–	0.0003
	AM	1	14	–	–	<0.0001
5% Lysozyme + IS unbuffered, adjusted to pH ~6	TM	15	5	0.5	–	–
	AM	1	20	<0.0001	–	–
5% Lysozyme + IS + 30 mmol l^{-1} MES pH 6.0	TM	17	3			0.3
	AM	3	14			<0.0001
5% Casein + IS unbuffered, adjusted to pH ~7.4	TM	12	2	1.0	–	–
	AM	9	5	0.03	–	–
Control 1: IS unbuffered, adjusted to pH ~7.0	TM	32	7	–	0.3	0.2
	AM	33	3	–	1.0	1.0
Control 2: IS + 30 mmol l^{-1} HEPES pH 7.4	TM	10	0	–	–	–
	AM	10	0	–	–	–
Control 3: IS + 30 mmol l^{-1} MES pH 6.0	TM	14	0	–	–	–
	AM	12	0	–	–	–

Solutions containing bromothymol blue as pH indicator were offered to females by the forced feeding technique. After each treatment, the pH inside midgut was evaluated by the color assumed by the dye. The proportion of midguts in each pH category (pH<6.5 or ≥6.5) was compared with the proportion observed in the respective control (Fisher's exact test). Controls 2 and 3 were also compared with control 1, indicating that buffering does not interfere with the results. AM: abdominal midgut; *N*, number of observations in each pH range; TM: thoracic midgut.

females. Additional controls were performed with IS containing 2 mmol l^{-1} EDTA but without any hemolymph.

The effect of serotonin (5-hydroxytryptamine) on the midgut pH was tested using the same approach described for testing the midgut extract. This test was performed on both acidic and alkalized midguts. IS containing Bromothymol Blue or IS containing the dye and BSA, both ingested by forced feeding, were used to obtain acidic and alkalized midguts, respectively, to be challenged in the assay. In the serotonin assay, 3.0 µl of 100 µmol l^{-1} synthetic hormone dissolved in IS was directly applied on midguts distended on agarose gel. The final concentration of serotonin was estimated to be between 0.3 and 100 µmol l^{-1}. The midguts were observed for 10 min, and changes in pH were noted. In the control groups, the same volume of IS (with or without EDTA) was applied on the midguts.

For evaluation, the results were classified as pH≤6.0 or >6.0 instead of pH<6.5 or ≥6.5, with the exception of results concerning the action of serotonin under alkalized midgets, which were classified as pH<7.5 or ≥7.5.

Effect of acetazolamide on midgut alkalization

We tested the importance of carbonic anhydrase activity in midgut alkalization in females fed with proteins. Sandflies were force-fed a 5% lysozyme solution prepared in IS buffered with 30 mmol l^{-1} MES pH 6.0 containing 1.0 mmol l^{-1} acetazolamide, a carbonic anhydrase inhibitor, and Bromothymol Blue (0.1%) as a pH indicator. Immediately after ingestion, the insect was dissected and pH was measured according to the color acquired by the dye inside each portion of the digestive tube. The control group ingested the same solution without acetazolamide. Based on the more frequent pH observed with lysozyme ingestion (pH>7.0), pH inside the midguts was classified as pertaining to one of the following categories: pH≤7.0 or >7.0.

Statistical analysis

The proportion of midguts in each category of pH in all of the experiments was compared between treatments and controls using Fisher's exact test. Differences were considered significant at $P<0.05$.

RESULTS

In a previous study, we showed that unfed or sugar-fed *L. longipalpis* females maintain an acidic thoracic and abdominal midgut. In this physiological condition, the pH is efficiently maintained at ~6 even after ingestion of highly concentrated buffer solutions (Santos et al., 2008). As shown in Table 2, the same physiological conditions were observed in the present study when the insects were fed with unbuffered IS, IS buffered with 30 mmol l^{-1} HEPES pH 7.4 (Fig. 1c) or IS buffered with 30 mmol l^{-1} MES pH 6.0 (controls 1, 2 or 3, respectively). We observed acidification to pH 6 in most midguts used in controls 1, 2 and 3, although they were included in the pH<6.5 category for analysis.

As batch digesters, *L. longipalpis* females must abruptly change their physiological condition, passing from an acidic midgut to an alkaline abdominal midgut just after blood ingestion, to allow digestion of blood macromolecules (Lehane et al., 1995; Santos et al., 2008). This raises questions concerning the primary stimulus present in the ingested blood that is responsible for turning off the mechanism involved in maintaining a pH of 6 and simultaneously switching on the mechanism responsible for alkalization.

The first hypothesis tested in this study was that the presence and consequent absorption of free amino acids by the abdominal midgut is responsible for the physiological change promoted by blood ingestion. To test this hypothesis, a solution containing a mixture of amino acids and a pH indicator dye was introduced into

Table 3. Action of BSA and lysozyme on the midgut pH of *L. longipalpis* when applied on the hemolymph side of the digestive tube

Treatment	Anatomical localization	*N*		*P*
		pH≤6.0	pH>6.0	
5% BSA dissolved in IS unbuffered (3 μl)	TM	8	3	1.0
	AM	13	0	1.0
5% Lysozyme dissolved in IS unbuffered (3 μl)	TM	14	0	0.03
	AM	14	0	1.0
Control: IS unbuffered (3 μl)	TM	8	4	–
	AM	14	1	–

BSA or lysozyme solutions were applied at the hemolymph side of dissected midguts of insects that ingested IS containing Bromothymol Blue. After the treatment, the proportion of midguts in each pH category (pH≤6.0 or >6.0) was compared with the proportion observed in the control (Fisher's exact test). BSA: bovine serum albumin. *N*, number of observations in each pH range.

the midgut of females by forced feeding. In the first experiment, the concentration of free amino acids (5 mmol l^{-1}) tested was similar to that encountered in human plasma (Adibi and Mercer, 1973; Baertl et al., 1974; Delaporte et al., 1978; Maclean et al., 1983). According to the data presented in Table 2, this treatment was ineffective at promoting alkalization even when buffered with 30 mmol l^{-1} HEPES pH 7.4. However, alkalization was effective when a higher (172 mmol l^{-1}) concentration of free amino acids was ingested (Table 2).

After determining that free amino acids, in a concentration similar to that encountered in human plasma, were not the stimulus we were looking for, we tested human serum and purified BSA. Human serum, with a pH that was previously adjusted to either ~7.4 or ~6, promptly turned off the mechanism of pH 6 maintenance and triggered alkalization (Fig. 1d). As observed previously in blood-fed females (Santos et al., 2008), only the abdominal midgut underwent alkalization, whereas the thoracic midgut remained acidic (Table 2). As observed in tests with human serum, unbuffered and buffered BSA solutions (pH 6) induced the same response in the midguts, except that in various preparations, the thoracic midgut also underwent alkalization after BSA ingestion (Table 2).

Because the presence of undigested proteins was capable of promoting alkalization, we wondered whether non-specific proteins stimulate the abdominal midgut. To address this question we designed experiments in which females were fed buffered (pH 6) or unbuffered solutions of white egg lysozyme. As expected for a midgut that is able to respond to non-specific proteins, the lysozyme treatment promptly alkalized the abdominal midgut (Table 2). As observed when the insects had ingested human serum, the pH in the thoracic midgut remained acidic. Although less effective, treatment with casein also promoted significant alkalization of the abdominal midgut (Table 2), reinforcing the hypothesis of non-specific stimulation. Unfortunately, we were unable to test the effect of peptides obtained from digested BSA, lysozyme or casein because they interacted with all the indicators dyes tested, thereby producing a precipitate that was not ingestible.

An effective stimulation of the midgut by proteins was only possible when the insect ingested the proteins, because BSA and lysozyme, when added to the hemolymph side of the preparations, were unable to cause alkalization in the abdominal midgut (Table 3). Alkalization could be promoted by protein binding to receptors located exclusively on the luminal surface of enterocytes or, more likely, on the luminal surface of the endocrine cells present in the midgut. The hypothesis that alkalization could be modulated by hormones released from endocrine cells to the hemolymph was tested. A crude preparation of midgut extract containing putative hormones was applied on the hemolymph side of digestive tubes from females fed with IS. When 1 μl of extract, equivalent to three midguts, was applied to the

Table 4. Action of the midgut extract of *L. longipalpis* on intestinal pH

Treatment	Anatomical localization	*N*		*P*
		pH≤6.0	pH>6.0	
1 μl midgut extract (3 midguts μl^{-1} IS + 2 mmol l^{-1} EDTA)	TM	6	1	0.4
	AM	9	11	0.0002
Control: 1 μl IS + 2 mmol l^{-1} EDTA	TM	10	0	–
	AM	17	0	–

Midgut extract was applied at the hemolymph side of the intestinal preparations of insects that ingested IS containing Bromothymol Blue. The proportion of midguts in each pH category (pH≤6.0 or >6.0) was compared with the proportion observed in the control (Fisher's exact test).

Table 5. Action of hemolymph obtained from blood-fed female *L. longipalpis* on the intestinal pH

Treatment	Anatomical localization	*N*		*P*
		pH≤6.0	pH>6.0	
Hemolymph obtained from one blood-fed female collected in 1 μl IS + 2 mmol l^{-1} EDTA	TM	2	7	0.0003
	AM	6	9	0.027
Control 1: hemolymph obtained from one sugar-fed female collected in 1 μl IS + 2 mmol l^{-1} EDTA	TM	12	0	–
	AM	14	3	–
Control 2: 1 μl IS + 2 mmol l^{-1} EDTA without hemolymph	TM	14	1	–
	AM	13	3	–

Hemolymph was applied on intestinal preparations of insects that ingested 10% sucrose containing 0.1% Bromothymol Blue. The proportion of midguts in each category (pH≤6.0 or >6.0) was compared with the proportion observed in control 1 (Fisher's exact test). *N*, number of observations in each pH range.

Table 6. Action of serotonin on the pH of acidic midguts of *L. longipalpis*

Treatment	Anatomical localization	*N* pH≤6.0	*N* pH>6.0	*P*
Serotonin (0.3–100 μmol l^{-1} serotonin + IS + 2 mmol l^{-1} EDTA)	TM	3	4	0.4
	AM	11	2	0.6
Control (IS + 2 mmol l^{-1} EDTA)	TM	8	4	–
	AM	14	1	–

Serotonin was applied on intestinal preparations of insects that ingested unbuffered IS containing 0.1% Bromothymol Blue (acidic midguts) by forced feeding. The proportion of midguts in each category (pH≤6.0 or >6.0) was compared with the proportion observed in the control (Fisher's exact test). *N*, number of observations in each pH range.

Table 7. Action of serotonin on the pH of alkalized midguts of *L. longipalpis*

Treatment	Anatomical localization	*N* pH<7.5	*N* pH≥7.5	*P*
Serotonin (0.3–100 μmol l^{-1} serotonin + IS + 2 mmol l^{-1} EDTA)	AM	2	11	1.0
Control (IS + 2 mmol l^{-1} EDTA)	AM	2	13	–

Serotonin was applied on intestinal preparations of insects that ingested unbuffered IS containing BSA and 0.1% Bromothymol Blue (alkalized midguts) by forced feeding. Only preparations with initial pH ~7.5 were accepted. The proportion of midguts in each category (pH<7.5 or ≥7.5) was compared with the proportion observed in the control (Fisher's exact test). *N*, number of observations in each pH range.

preparations, a significant (P<0.05) number of abdominal midguts that were originally at pH 6 underwent a slight alkalization (Table 4). A slight alkalization was also observed when dissected midguts were exposed to IS containing hemolymph obtained from blood-fed females (Table 5). In these experiments, it was important to collect the hemolymph just after blood ingestion when the hormones released by the hematophagic process were present in the hemolymph.

Serotonin may be released in phlebotomine hemolymph during blood ingestion and, consequently, it could affect the midgut physiology. Thus, we investigated the effect of this hormone on both acidic and alkalized midguts (N=13 intestines in each case). When 3 μl IS containing 100 μmol l^{-1} serotonin was applied on the hemolymph side of dissected guts, no apparent effect was observed in either the acidic or alkalized midguts (Tables 6 and 7, respectively).

The ingestion of acetazolamide, a carbonic anhydrase inhibitor, was used to investigate this enzyme's role in the alkalization process. As shown in Table 8, alkalization of the abdominal midgut induced by lysozyme was significantly compromised by the presence of inhibitor (P<0.05). This suggests that carbonic anhydrase contributes to alkalization.

DISCUSSION

Larvae of phlebotomines, mosquitoes and other nematoceran Diptera ingest food continuously; the food is digested and absorbed along the midgut. Digestion in larvae does not require any abrupt change in the physiological conditions of the gut, except during molting when the larvae must stop feeding (Lehane et al., 1995). However, adult females are batch digesters, changing abruptly from a carbohydrate digestion phase to a blood digestion phase. This requires the abdominal midgut to switch from an acidic (pH 6) to an alkaline environment (pH 8.15) for optimum activity of enzymes responsible for blood digestion (Santos et al., 2008). Such a remarkable change in the physiology of the midgut must be tightly controlled to be completely functional.

We developed a method to investigate the control of pH in the midgut that is simple and reproducible, and can be easily adapted to investigate other physiological parameters such as the production of digestive enzymes under different stimuli.

According to Adibi and Mercer and others (Adibi and Mercer, 1973; Baertl et al., 1974; Delaporte et al., 1978; Maclean et al., 1983), the concentrations of free amino acids in human plasma are 3.2, 9.1, 2.5 and 2.5 mmol l^{-1}, respectively (mean=4.3 mmol l^{-1}). The results obtained in our study clearly showed that free amino acids, at a concentration similar to that in human plasma, were not able to stimulate the process of alkalization (Table 2). An effective concentration, such as 172 mmol l^{-1} (Table 2), could be present in the midgut lumen only several hours after blood digestion had been initiated. Thus, it is reasonable to speculate that higher concentrations of amino acids could participate in maintenance of the alkaline state only after its initialization by contact with proteins. Accordingly, proteins at relatively low concentrations were capable of inducing alkalization of the abdominal midgut (Table 2), and they are

Table 8. Involvement of carbonic anhydrase in alkalization of *L. longipalpis* abdominal midgut

Treatment	Anatomical localization	*N* pH≤7.0	*N* pH>7.0	*P*
5% Lysozyme + IS + 1.0 mmol l^{-1} acetazolamide + 30 mmol l^{-1} MES pH 6.0	TM	16	0	1.0
	AM	12	3	0.02
Control: 5% lysozyme + IS + 30 mmol l^{-1} MES pH 6.0	TM	20	0	–
	AM	6	11	–

Lysozyme solution containing Bromothymol Blue as pH indicator was introduced in the insect midguts by forced feeding. Acetazolamide, a carbonic anhydrase inhibitor, was present in the solution ingested by insects from treated group. The proportion of midguts in each pH category (pH≤7.0 or >7.0) was compared with the proportion observed in the control (Fisher's exact test). *N*, number of observations in each pH range.

probably the primary stimulus responsible for triggering this process after blood intake.

When we were investigating the alkalizing effect of BSA or lysozyme ingestion, these proteins were offered to the insects dissolved in IS containing 30 mmol l^{-1} MES buffer pH 6. In this case, these proteins not only switched off pH 6 maintenance, but also switched on another mechanism that is responsible for alkalization. This mechanism, first suggested in our previous publication (Santos et al., 2008), was strong enough to surpass the buffering capability of the ingested solution (initially at pH 6) and promoted alkalization of the abdominal midgut to pH ≥6.5 (Table 2). Despite the fact that the data were classified in the pH ≥6.5 category, in 80% of insects that ingested BSA or lysozyme at pH 6.0, the pH measured in the abdominal midgut was ≥7 (data not shown). Thus, in a natural situation, CO_2 volatilization and the new alkalizing mechanism, investigated here, share responsibility for alkalization to pH 8.15 after a blood meal.

Initially, we expected that one or more of the blood proteins could specifically trigger alkalization. However, activation obtained with egg white lysozyme or casein has shown that any protein that comes into contact with the midgut lumen could nonspecifically promote this alkalization. This result is difficult to explain, taking into account the existence of receptors. However, a similar pattern of protein recognition was observed in midgut preparations of *Stomoxys calcitrans*, a hematophagous fly. While searching for factors responsible for regulating digestive enzyme production *in vitro*, researchers found that a wide range of proteins could stimulate trypsin secretion, but amino acids, small peptides or poly L-amino acids could not (Blakemore et al., 1995). Intimate contact between proteins and protein-receptors seems a necessary condition to induce a response. For example, soluble proteins stimulate the production of digestive enzymes in *Aedes*, whereas insoluble (denatured) proteins do not (Lehane et al., 1995). We do not know yet whether, in *L. longipalpis*, the alkalization process and the induction of digestive enzymes are controlled by the same mechanism. It is known, however, that they are concomitant events in the *L. longipalpis* midgut.

Our experiments show that osmotic shock can be discarded as a possible stimulus for induction of alkalization, because the alkalization effect was not observed when proteins were applied on the hemolymph side of the midgut. Stretching of the midgut should be also discarded as the primary stimulus for alkalization. Although the mean volume of the ingested solutions was considerable for a phlebotomine (~0.5 µl), this volume was not great enough to stretch the midgut wall. During ingestion, a considerable portion of the water present in the solutions was quickly absorbed by the enterocytes.

Receptors that are sensitive to proteins should be present on midgut cell membranes facing the lumen. This conclusion is based on the fact that proteins induce alkalization when ingested, but not when applied outside the midgut preparations (Tables 2 and 3). This is also consistent with the fact that, *in vivo*, hemolymph proteins are constantly in contact with the surface of the midgut but this does not result in alkalization of the lumen (Table 5, control 1). It is important to emphasize that dissected midguts do not allow proteins to enter the lumen when these proteins are applied outside. This is because, as shown in our experiments, the stomodeum valve seems tightly closed, as does the anal sphincter.

From experiments performed with larvae of *Manduca sexta* (Clark et al., 1998), the authors inferred that factors secreted from the basal aspect of the midgut epithelium were necessary for effective alkalization of the lumen. In experiments in which the secreted material was washed away, alkalization did not occur. A more complex response was observed in the midgut of *Rhynchophorus ferrugineus* larvae (Sunitha et al., 1999). When preparations of midgut containing buffer solutions were exposed to midgut epithelial extract, the pH of the lumen returned to its basal value. In *L. longipalpis*, factors present in the hemolymph collected just after blood ingestion were effective at promoting alkalization of the abdominal midgut (Table 5). These data are in accordance with our proposition that hormones are released from the midgut under protein stimulation.

Taking our data into account, we hypothesize that stimulating endocrine cells with proteins could modulate the function of enterocytes, leading to alkalization of the lumen. According to this hypothesis, the receptors involved in protein perception would be located in the apical membrane of the endocrine cells. This type of endocrine cell has been described in the *L. longipalpis* midgut (Leite and Evangelista, 2001); it is anchored in the basal membrane and, following stimulation, it could release hormones to the hemolymph. From the hemolymph, the hormones then would act on receptors located on the basal side of the enterocytes, inducing them to promote alkalization. Hormones released from other endocrine cells of insect body could, in principle, participate in the process.

Hormone preparations applied to the abdominal midgut were less effective at promoting alkalization than protein ingestion. There are several possible explanations for this. First, this could be due to low hormone concentrations in our preparations. Under *in vivo* conditions, the concentration of hormone released in the hemolymph should be considerably higher because of the small volume of hemolymph bathing the midgut at any given time. The concentration of hormone is probably much lower in the 1 µl of midgut extract that we added on the gut preparations *in vitro*. It is also important to consider two other factors: the unknown efficiency of hormone extraction in our process and the action of peptide-degrading peptidases. These peptidases are usually found on the surface of various organs, including the midgut (Isaac et al., 2009), and could quickly decrease our already suboptimal hormone concentrations. Finally, we cannot rule out the possibility that the ingested proteins may also act directly on the enterocytes, by inducing a physiological change in them that could cause midgut alkalization. In this case, the hormones could have a secondary but significant participatory role.

Although serotonin did not affect pH in our experiments (Tables 6 and 7), it is possible that it promotes an increase in membrane potential, as was observed in the gut of *Aedes* larvae (Onken et al., 2009). In *L. longipalpis*, the effects of serotonin on membrane potential could make the maintenance of physiological functions more efficient. This possibility should be further investigated by measuring the membrane potential under different experimental treatments.

In our previous study we demonstrated that the thoracic midgut remains acidic after blood ingestion (Santos et al., 2008). Although in some experiments, BSA promoted alkalization in the thoracic midgut (Table 2), the results of most experiments are in accordance with our previous study. This indicates that the thoracic and abdominal midguts are physiologically distinct entities that enable separate digestion of blood and carbohydrates.

Our results indicate that carbonic anhydrase is involved in the alkalization process in the *L. longipalpis* midgut (Table 8). We previously showed the presence of at least two transcripts of carbonic anhydrase in the midgut of this phlebotomine (Santos et al., 2008). Bicarbonate ions produced in the cytoplasm of the enterocytes are probably exchanged for chloride ions that are present in the lumen

by means of an anion antiport system. Before the intake of proteins, when the midgut is acidic, bicarbonate ions are not expected to be transported to the lumen. The activation of this putative anion antiport system is probably under strict control of the alkalization mechanism. According to our results, the alkalization seems to be a specific characteristic of the abdominal midgut. Similarly, carbonic anhydrase was preferentially present in the abdominal midgut of adult mosquitoes (del Pilar Corena et al., 2005). By using specific inhibitors, these authors observed that this enzyme is involved in the process of alkalization in mosquitoes. Adult mosquitoes' midguts are probably physiologically similar to those of phlebotomines. However, del Pilar Corena et al. presumed that the midgut of adult mosquitoes is permanently alkaline (del Pilar Corena et al., 2005). As they did not offer a free-protein solution to the insects in their experiments, it is possible that they inadvertently activated an alkalization mechanism similar to the one we described here.

Our investigation has shed light on a finely regulated mechanism responsible for regulating the pH in a batch digester insect. It is likely that a similar mechanism operates in all hematophagous Diptera. Further studies will elucidate how different ion transport systems and intracellular signaling pathways are involved in the control of intestinal pH.

LIST OF ABBREVIATIONS

AM	abdominal midgut
BSA	bovine serum albumin
EDTA	ethylenediamine-tetraacetic acid
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulphonic acid
IS	insect saline
MES	2-(N-morpholino) ethanesulphonic acid
TM	thoracic midgut

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REFERENCES

Adibi, S. A. and Mercer, D. W. (1973). Protein digestion in human intestine as reflected in luminal, mucosal, and plasma amino acid concentrations after meals. *J. Clin. Invest.* **52**, 1586-1594.

Alexander, B. and Usma, M. C. (1994). Potential sources of sugar for the phlebotomine sandfly *Lutzomyia youngi* (Diptera: Psychodidae) in a Colombian coffee plantation. *Ann. Trop. Med. Parasitol.* **88**, 543-549.

Baertl, J. M., Placko, R. P. and Graham, G. G. (1974). Serum proteins and plasma free amino acids in severe malnutrition. *Am. J. Clin. Nutr.* **27**, 733-742.

Blakemore, D., Williams, S. and Lehane, M. J. (1995). Protein stimulation of trypsin secretion from the opaque zone midgut cells of *Stomoxys calcitrans*. *Comp. Biochem. Physiol.* **110B**, 301-307.

Cameron, M. M., Pessoa, F. A., Vasconcelos, A. W. and Ward, R. D. (1995). Sugar meal sources for the phlebotomine sandfly *Lutzomyia longipalpis* in Ceará State, Brazil. *Med. Vet. Entomol.* **9**, 263-272.

Clark, T. M., Koch, A. R. and Moffett, D. F. (1998). Alkalinization by *Manduca sexta* anterior midgut in vitro: requirements and characteristics. *Comp. Biochem. Physiol.* **121A**, 181-187.

Clark, T. M., Lawecki, J. L., Shepherd, J. J., Hirschler, A. N. and Samandu, T. R. (2009). Effects of serotonergic agents on survival and hemolymph composition of the larval mosquito *Aedes aegypti* (Diptera: Culicidae, L.) in vivo: does serotonin regulate hemolymph acid-base homeostasis? *J. Exp. Biol.* **212**, 3728-3736.

Dadd, R. H. (1961). Evidence for humoral regulation of digestive secretion in the beetle, *Tenebrio molitor*. *J. Exp. Biol.* **38**, 259-366.

del Pilar Corena, M., VanEkeris, L., Salazar, M. I., Bowers, D., Fiedler, M. M., Silverman, D., Tu, C. and Linser, P. J. (2005). Carbonic anhydrase in the adult mosquito midgut. *J. Exp. Biol.* **208**, 3263-3273.

Delaporte, C., Jean, G. and Broyer, M. (1978). Free plasma and muscle amino acids in uremic children. *Am. J. Clin. Nutr.* **31**, 1647-1651.

Gontijo, N. F., Almeida-Silva, S., Williams, P., Mares-Guia, M. L. and Melo, M. N. (1998). *Lutzomyia longipalpis*: pH in the gut, digestive glycosidases, and their relationship with *Leishmania* development. *Exp. Parasitol.* **90**, 212-219.

Harshini, S., Nachmab, R. J. and Sreekumar, S. (2002). In vitro release of digestive enzymes by FMRF amide related neuropeptides and analogues in the lepidopteran insect *Opisina arenosella* (Walk.). *Peptides* **23**, 1759-1763.

Isaac, R. E., Bland, N. D. and Shirras, A. D. (2009). Neuropeptidases and the metabolic inactivation of insect neuropeptides. *Gen. Comp. Endocrinol.* **162**, 8-17.

Jacobson, R. L., Schlein, Y. and Eisenberger, C. L. (2001). The biological function of sand fly and *Leishmania* glycosidases. *Med. Microbiol. Immunol.* **190**, 51-55.

Lehane, M. J., Blakemore, D., Williams, S. and Moffatt, M. R. (1995). Regulation of digestive enzyme levels in insects. *Comp. Biochem. Physiol.* **110B**, 285-289.

Leite, A. C. R. and Evangelista, L. G. (2001). Ultrastructure of endocrine cells from the abdominal midgut epithelium of *Lutzomyia longipalpis* (Diptera: Psychodidae). *J. Med. Entomol.* **38**, 749-752.

Maclean, W. C., Jr, Graham, G. G., Plako, R. P. and Lopez de Romanã, G. (1983). Plasma free amino acids in children consuming lupin protein with and without methionine supplementation. *J. Nutr.* **113**, 779-785.

Maddrell, S. H., Herman, W. S., Mooney, R. L. and Overton, J. A. (1991). 5-Hydroxytryptamine: a second diuretic hormone in *Rhodnius prolixus*. *J. Exp. Biol.* **156**, 557-566.

Modi, G. B. and Tesh, R. B. (1983). A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. *J. Med. Entomol.* **20**, 568-569.

Novak, M. G. and Rowley, W. A. (1994). Serotonin depletion affects blood-feeding but not host-seeking ability in *Aedes triseriatus* (Diptera: Culicidae). *J. Med. Entomol.* **31**, 600-606.

Novak, M. G., Ribeiro, J. M. and Hildebrand, J. G. (1995). 5-hydroxytryptamine in the salivary glands of adult female *Aedes aegypti* and its role in regulation of salivation. *J. Exp. Biol.* **198**, 167-174.

Onken, H. and Moffet, D. F. (2009). Revisiting the cellular mechanisms of strong luminal alkalinization in the anterior midgut of larval mosquitoes. *J. Exp. Biol.* **212**, 373-377.

Onken, H., Moffett, S. B. and Moffett, D. F. (2008). Alkalinization in the isolated and perfused anterior midgut of the larval mosquito, *Aedes aegypti*. *J. Insect Sci.* **8**, 1-20.

Onken, H., Parks, S. K., Goss, G. G. and Moffett, D. F. (2009). Serotonin-induced high intracellular pH aids in alkali secretion in the anterior midgut of larval yellow fever mosquito *Aedes aegypti* L. *J. Exp. Biol.* **212**, 2571-2578.

Orchard, I. (2006). Serotonin: a coordinator of feeding-related physiological events in the blood-gorging bug, *Rhodnius prolixus*. *Comp. Biochem. Physiol.* **144A**, 316-324.

Sakai, T., Satake, H. and Takeda, M. (2006). Nutrient-induced α-amylase and protease activity is regulated by crustacean cardioactive peptide (CCAP) in the cockroach midgut. *Peptides* **27**, 2157-2164.

Santos, V. C., Araujo, R. N., Machado, L. A., Pereira, M. H. and Gontijo, N. F. (2008). The physiology of the midgut of *Lutzomyia longipalpis* (Lutz and Neiva 1912): pH in different physiological conditions and mechanisms involved in its control. *J. Exp. Biol.* **211**, 2792-2798.

Schlein, Y. and Warburg, A. (1986). Phytophagy and the feeding cycle of *Phlebotomus papatasi* (Diptera: Psychodidae) under experimental conditions. *J. Med. Entomol.* **23**, 11-15.

Soares, R. P. and Turco, S. J. (2003). *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae): a review. *An. Acad. Bras. Cienc.* **75**, 301-330.

Sunitha, V. B., Reena, T., Harshini, S. and Sreekumar, S. (1999). Is gut pH regulated by midgut endocrine system in larvae of *Rhynchophorus ferrugineus* Fab.? *Indian J. Exp. Biol.* **37**, 476-480.

Tang, Y. and Ward, R. D. (1998). Sugar feeding and fluid destination control in the phlebotomine sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae). *Med. Vet. Entomol.* **12**, 13-19.

Te Brugge, V., Ianowski, J. P. and Orchard, I. (2009). Biological activity of diuretic factors on the anterior midgut of the blood-feeding bug, *Rhodnius prolixus*. *Gen. Comp. Endocrinol.* **162**, 105-112.

Terra, W. R. and Ferreira, C. (1994). Insect digestive enzymes: properties, compartmentalization and function. *Comp. Biochem. Physiol.* **109B**, 1-62.