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The physiology of the midgut of *Lutzomyia longipalpis* (Lutz and Neiva 1912): pH in different physiological conditions and mechanisms involved in its control

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SUMMARY

Nutrient digestion and absorption after blood feeding are important events for *Lutzomyia longipalpis*, which uses these nutrients to produce eggs. In this context, the pH inside the digestive tract is an important physiological feature as it can markedly influence the digestive process as well as interfere with *Leishmania* development in infected phlebotomines. It was described previously that unfed females have an acidic midgut (pH 6). In this study, the pH inside the midgut of blood-fed females was measured. The abdominal midgut (AM) pH varied from 8.15±0.31 in the first 10 h post-blood meal to 7.7±0.17 after 24 h. While the AM was alkaline during blood digestion, the pH in the thoracic midgut (TM) remained acidic (5.5–6.0). In agreement with these findings, the enzyme α-glucosidase, which has an optimum pH of 5.8, is mainly encountered in the acidic TM. The capacity of unfed females to maintain the acidic intestinal pH was also evaluated. Our results showed the presence of an efficient mechanism that maintains the pH almost constant at about 6 in the midgut, but not in the crop. This mechanism is promptly interrupted in the AM by blood ingestion. RT-PCR results indicated the presence of carbonic anhydrase in the midgut cells, which apparently is required to maintain the pH at 6 in the midgut of unfed females. Investigations on the phenomenon of alkalization observed after blood ingestion indicated that two mechanisms are involved: in addition to the alkalization promoted by CO_2 volatilization there is a minor contribution from a second mechanism not yet characterized. Some inferences concerning *Leishmania* development and pH in the digestive tube are presented.

Key words: *Lutzomyia longipalpis*, midgut pH, pH control mechanisms, acidification, alkalization, *Leishmania* development.

INTRODUCTION

Lutzomyia longipalpis (Lutz and Neiva 1912) is the main phlebotomine vector of *Leishmania infantum* (Nicolle 1908) [syn. *L. chagasi* (Cunha and Chagas 1937)], the etiologic agent of the visceral leishmaniasis in the Americas (Soares and Turco, 2003). As for any other haemathophagous insects, nutrient digestion and absorption after blood feeding is one of the most important events for *L. longipalpis*, which uses these nutrients to produce eggs.

Since the digestive processes are essentially enzymatic and enzyme activities are influenced by the hydrogen ion concentration (pH) in the intestinal environment, studies of pH and the mechanisms involved in pH control become extremely relevant. This importance increases if it is considered that *Leishmania* parasites ingested through an infective blood meal develop exclusively in the phlebotomine gut, from where they are transmitted to a vertebrate host by biting (Bates and Rogers, 2004). In fact, pH could be one of the most important factors in *Leishmania* development within the vector, as well as for the normal functioning of the gut. It has been shown that *Leishmania* promastigotes cultivated at pH 5.5 differentiate into metacyclic forms in much larger numbers than those cultivated at pH 7.6 (Bates and Tetley, 1993; Zakai et al., 1998). Acidification of the medium seems to be one of the main stimuli that determines the differentiation of *Leishmania in vitro* and probably plays a similar role during their development in the sand fly gut (Gontijo et al., 1998).

Although unknown in mammals, a high gut pH (above 9.0) is common in some insects, especially in larvae from the orders Lepidoptera and Diptera (suborder Nematocera only) (Terra et al., 1996). Some models have been proposed in order to explain the high physiological pH observed in these insects. All of them include the participation of the enzyme carbonic anhydrase and the hydration of CO_2 molecules (Boudko et al., 2001a; Boudko et al., 2001b). In adult females of phlebotomine sand flies and mosquitoes (Nematocera), the physiology of the midgut is different. These insects go from a diet composed basically of carbohydrates to one of blood. This implies great modifications in the midgut physiology especially in the production of digestive enzymes and the promotion of a slightly alkaline pH necessary for their action.

Studying the pH of the blood bolus inside the midgut of some blood-fed mosquitoes, Billker and colleagues proposed that the alkalization observed [pH 7.4 to 7.52 and 7.58 in *Aedes aegypti* (Linnaeus 1762) and *Anopheles stephensi* (Liston 1901), respectively] might be attributed to the phenomenon of CO_2 volatilization (Billker et al., 2000). The loss of CO_2 from blood equilibrating with air leads to a reduction in H^+ concentration in accordance with the equation: $CO_2+H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$. The authors showed that the pH of 2–3 μl of blood reached a value of 8.0 when exposed to the air for 10 min.

On the other hand, del Pilar Corena and colleagues observed a higher alkalization, to pH 8 or more, in the midgut of seven species of adult mosquito after feeding (del Pilar Corena et al., 2005). Taking into account the fact that these mosquitoes were fed not with blood but with a substitute solution containing indicator dyes, the CO_2 volatilization mechanism cannot be considered the only mechanism responsible for the alkalization observed in blood-fed insects. Probably, both the CO_2 volatilization and a second mechanism

contribute to the alkalization observed in mosquitoes and might be involved in the pH change inside the midgut of phlebotomine sand flies after a blood meal.

Considering the importance of pH to *Leishmania* development and to digestive processes, the objectives of this study were to measure the pH inside different parts of the *L. longipalpis* gut during blood digestion in uninfected sand flies and to investigate some midgut properties that could be involved in intestinal pH control in unfed and blood-fed females. Finally, we offer a hypothesis concerning the pH in the phlebotomine gut and how it correlates with *Leishmania* development from blood intake to transmission.

MATERIALS AND METHODS

Insects

All experiments were performed with females from a population of *L. longipalpis* originating from Teresina, state of Piauí, Brazil, and maintained as a closed colony according to the methodology proposed by Modi and Tesh (Modi and Tesh, 1983).

Microelectrode construction and calibration

Glass micropipettes were prepared with capillaries (1 mm diameter) that were pulled on an electropuller (PP-83; Narishige, Tokyo, Japan). The micropipette tips were strengthened by heat in a microforge to tip final diameters of 15–25 μm. These micropipettes were stored under desiccant conditions until microelectrode construction. The construction of single-barrelled turgor-resistant microelectrodes sensitive to H^+ was carried out according to previous studies (Gibbon and Kropf, 1993; Billker et al., 2000) with some modifications. Ionophore–polyvinyl chloride (PVC) mixture was obtained by mixing 3 μl of H^+ ionophore cocktail A (Fluka, Ronkonkoma, NY, USA) with 7 μl of a 0.075% solution of PVC (Fluka, Buchs, Switzerland) dissolved in tetrahydrofuran (Sigma-Aldrich, Milwaukee, WI, USA). H^+-sensitive microelectrodes were prepared by backfilling the pulled micropipettes with about 1–2 μl of the ionophore–PVC mixture prepared just before use. The mixture was introduced into micropipettes with plastic pipette tips pulled in a flame in order to produce hollow and flexible tubes fine enough to penetrate the back side of the micropipettes. In order to evaporate all tetrahydrofuran, microelectrodes were kept for about 7 days in a desiccator under vacuum. According to our experience, the preparation of such large-tip, turgor-resistant microelectrodes does not require a previous silanization step. Just prior to use, these H^+-sensitive microelectrodes were filled with 0.1 mol l^{-1} Mes/Tris-base buffer pH 4.3 also containing 0.1 mol l^{-1} KCl. Reference microelectrodes were filled at the tip side by suction with about 2 μl of 0.2% warmed agarose dissolved in 3 mol l^{-1} KCl solution. The reference electrodes were then backfilled with 3 mol l^{-1} KCl solution. Both the H^+-sensitive and the reference microelectrodes were connected through a Ag–AgCl wire to a high impedance electrometer (pH meter 26; Radiometer, Copenhagen, Denmark). Electrodes were calibrated at four points between pH 7.0 and 8.5 using 0.05 mol l^{-1} standard buffer solutions (potassium phosphate monobasic/NaOH pH 7.0 and 7.5; Tris-base/HCl pH 8.0 and 8.5). Readings were considered only after complete stabilization of the response, which usually occurred in 30–60 s. Microelectrode measurements were rejected when calibration curves before and after the insect impalement differed by more than 3 mV (corresponding to 0.07 pH units).

pH measurements in the abdominal midgut in blood-fed females

Phlebotomine females were allowed to feed for about 20–30 min on golden hamsters anaesthetized with Thiopentax® (Cristália-Produtos Farmacêuticos LTDA, SP, Brazil; 5% in saline; 0.1 ml 100 g^{-1}). Fed insects were maintained at 25°C and 70% relative humidity until use. Engorged females were individually captured from 10 min to 32 h after feeding and briefly washed in a 1% commercial detergent solution and subsequently in 0.9% saline for a few seconds. Each washed female was rapidly transferred to the surface of a 15% polyacrylamide gel embedded with 0.9% saline, placed under a stereo microscope and immediately impaled through the abdominal cuticle (Billker et al., 2000) with both H^+-sensitive and reference electrodes held by micromanipulators. The readings were manually annotated and the measured values (mV) were transformed into pH units using the respective calibration curves.

pH measurements in the thoracic midgut in blood-fed females

pH of the thoracic midgut (TM) in blood-fed females (3–5 days old) was measured with vital pH indicator dyes as already described (Gontijo et al., 1998). Briefly, unbuffered solutions (about 0.1%) of the indicator dyes Bromothymol Blue or Bromocresol Purple were prepared individually by dissolving the dyes in 10% sucrose solution. The Bromothymol Blue and Bromocresol Purple solutions were adjusted to pH 7.0 and 6.5, respectively, and immediately offered, soaked onto pieces of cotton, to recently blood-fed females. Twenty hours later, the females were dissected and the colours in the TM were compared with standard solutions prepared with the same dyes at different pH values covering 0.5 unit intervals.

α-Glucosidase assay and distribution along the midgut in unfed females

Fifteen 3–4 day old fasted females were dissected in 0.9% saline. Their digestive tracts were separated into TM and abdominal midgut (AM) and were transferred to different microcentrifuge tubes containing 250 or 100 μl of 1% aqueous Triton X-100, respectively. A 50 μl sample of this material containing solubilized enzyme (a volume containing 0.2 TM or 0.5 AM) was transferred to another tube containing 200 μl of 0.25 mol l^{-1} Mes/NaOH buffer pH 6.0. The reaction was initiated by addition of 250 μl of 12 mmol l^{-1} *p*-nitrophenyl α-D-glucopyranoside (Sigma, St Louis, MO, USA), a synthetic substrate for α-glucosidases, dissolved in water (final concentration 6 mmol l^{-1}). The tubes were incubated for 1 h at 30°C and the reactions were stopped by addition of 1 ml of 0.375 mol l^{-1} glycine/NaOH buffer pH 10.5. Readings were taken using a spectrophotometer (Shimadzu UV-1650PC, Columbia, MD, USA) at 400 nm. Blanks were prepared with 50 μl of 1% aqueous Triton X-100 without any midgut material.

Effects of volatilization of CO_2 on AM pH of blood-fed females

In order to investigate the contribution of CO_2 loss to the alkalization observed in blood-fed sand flies, 10 ml of human blood was collected in the presence of heparin and transferred to a 50 ml Erlenmeyer flask, which was exposed for 1 h to atmospheric air under continuous agitation (approximately 60 cycles min^{-1}). During this period, the pH rose from 7.4 to 8.10±0.12 (N=17). After alkalization, the pH was adjusted to 7.4 by careful addition of 1 mmol l^{-1} HCl dissolved in 0.9% NaCl solution and the blood was offered to 3–4 day old fasted females (no sugar was offered to these insects) in an artificial feeding apparatus at 37°C using chick skins (Bastien, 1990). pH measurements were accomplished in the midgut using H^+-sensitive microelectrodes during the initial phase (2–6 h) after ingestion as well as 24–28 h post-ingestion of the 'CO_2-depleted' blood.

Forced feeding

Forced feeding was performed as described previously (Hertig and Mcconnel, 1963; Anez et al., 1997). Microcapillaries were prepared by constricting the extremities of glass capillary tubes, in an alcohol flame, in order to permit the introduction of the insect mouthparts (except the labium) into the narrowed channel obtained. This procedure triggers a reflex that forces the insect to ingest the liquid inside the capillary, probably as it occurs in natural blood-feeding mode. A piece of modelling clay was used as a support for the capillary tube, which could be moved as necessary in order to be finely adjusted to the mouthparts of the insect. During the forced-feeding procedure, each female (3–5 days old) was maintained in an adequate position under a stereo microscope on the tip of a plastic tube (3 mm diameter) covered with a piece of fabric. The insect was kept immobilized by means of continuous suction provided by a vacuum pump connected to the tube (Anez et al., 1997).

pH measurements in the midgut of unfed females challenged with buffered solutions and acetazolamide

The ability of the midgut to maintain its pH (pH 6) in unfed females was investigated by forced ingestion of strongly buffered solutions containing pH indicator dyes. The indicator dyes Bromothymol Blue and Bromocresol Purple were dissolved, to a final concentration of 0.1%, in 0.16 mol l^{-1} Hepes/NaOH pH 7.5 or 0.16 mol l^{-1} Mes/NaOH pH 5.0, respectively. The technique of forced feeding was used to force the females to ingest about 0.5–1 µl of the solutions mentioned above. Immediately after ingestion, females were dissected and the colours inside the diverticulum and midgut compared with standard buffered solutions containing each dye as explained above. The same solutions containing 1 mmol l^{-1} acetazolamide, an inhibitor of carbonic anhydrase (del Pilar Corena, 2005), were also introduced by forced feeding into the midgut of unfed females to evaluate the involvement of this enzyme in the mechanism of pH maintenance.

Carbonic anhydrase expression in the midgut of *L. longipalpis* – RNA extraction, cDNA synthesis and PCR

To test for the presence of carbonic anhydrase in the midgut cells, seventeen 4 day old females fed only with sucrose were dissected and their midguts collected. Total RNA was extracted using an RNeasy micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA was eluted in 16 µl of Milli-Q water and used for cDNA synthesis with 0.5 µg of oligo-dT primer (Promega, Madison, WI, USA) and the M-MLV reverse transcriptase system (Promega) in a final volume of 25 µl. The nucleotide sequence of the carbonic anhydrase from *A. aegypti* (GenBank accession no. AF395662) and the putative cytoplasmic carbonic anhydrase from *Anopheles gambiae* (Giles 1902) (GenBank accession no. DQ518576) were blasted against The Wellcome Trust Sanger Institute *L. longipalpis* gene database (Hertz-Fowler et al., 2004) in order to find sequences from *L. longipalpis*. Among the blast results, two sequences with probability values higher than 1.0e–25 were chosen, NSFM-137e02.q1k and NSFM-126c10.q1k. Each of these sequences was used to design primer pairs – forward: 5′-gca ttt aac ggt ggt gct tt-3′/reverse: 5′-cat cct tat tgg cca ctg ct-3′; and forward: 5′-tgc tgg aga att gca tct tg-3′/reverse: 5′-tgg tgg acg gta gtt gtt ga-3′. PCR was carried out for 35 cycles (94°C for 30 s, 57°C for 30 s, 72°C for 45 s) with 1 µl of the cDNA in addition to 500 nmol l^{-1} of each primer, 200 µmol l^{-1} of each dNTP and 1 U of Taq Phoneutria® (Phoneutria, Belo Horizonte, MG, Brazil) in a final volume of 20 µl. The products were analysed by 2% agarose gel electrophoresis.

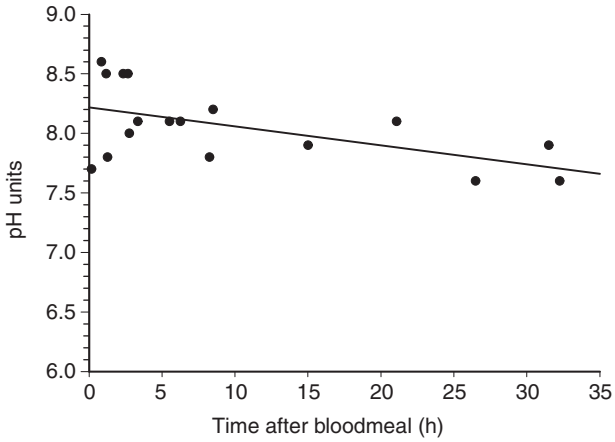

Fig. 1. pH in the abdominal midgut during blood digestion in uninfected females of *L. longipalpis*. Each point is from a separate experiment in which pH was measured using H^+-sensitive microeletrodes.

Statistics

Fisher's test was applied to the experiments in which the normal midgut pH was challenged with buffered solutions to investigate the effect of acetazolamide. For all other occasions, when pertinent, Student's *t*-test was applied. All data are presented as means ± s.d.

RESULTS

In normal conditions, *L. longipalpis* takes about 40–45 h to complete blood digestion. According to the data presented in Fig. 1, in the first 32 h of digestion the pH slowly decreased from 8.15±0.31 (considering the data obtained in the first 10 h post-blood ingestion) to 7.70±0.17 (considering the data obtained at ≥24 h post-blood ingestion; P=0.03) in the AM of blood-fed females.

Despite the alkaline environment inside the AM during blood digestion, the pH in the TM was between 5.5 and 6 at 20 h after feeding (Table 1, Fig. 2). The acidic pH inside the TM is consistent with a site responsible for sucrose hydrolysis. According to Gontijo and colleagues, the α-glucosidases of starved females are membrane-bound enzymes responsible for sucrose digestion in the midgut of *L. longipalpis* with an optimum pH near 5.8 (Gontijo et al., 1998). In agreement with this, the α-glucolytic activity measured in the TM of unfed females was significantly higher (1.806±0.532 OD h^{-1} TM^{-1}) than that of the AM (0.610±0301 OD h^{-1} AM^{-1}; P<0.001).

Table 2 summarizes the results concerning the contribution of CO_2 volatilization to the pH in the AM. The volatilization of CO_2 from 10 ml of blood *in vitro* was enough to alkalize the pH from 7.4 to 8.10±0.12 (N=17) after 1 h of exposure to the air. Longer exposures did not increase the alkalinity of the blood (data not shown). In order to investigate the contribution of CO_2 volatilization to the alkalization observed after a blood meal, females were fed with 'CO_2-depleted' blood, the pH of which was previously adjusted to approximately 7.4 (7.39±0.03). In these females, the pH measured at 2–6 h post-blood ingestion was 7.34±0.48 (N=5). This pH value was not significantly different from pH 7.39 (P=0.75). At 24–28 h post-ingestion, the pH inside the AM was 7.56±0.27 (N=5), significantly higher than pH 7.39 (P=0.04).

To investigate the 'buffering' ability of the *L. longipalpis* midgut, unfed females were challenged by forced ingestion of highly buffered solutions containing pH indicator dyes. In these experiments, females were challenged with 0.16 mol l^{-1} Hepes

Table 1. pH in the thoracic midgut during blood digestion measured with pH indicator dyes

pH range	5.5<pH≤6.0	6.0<pH≤6.5	6.5<pH≤7.0	7.0<pH≤7.5	Total
No. of TM	37	6	5	0	48

Unbuffered solutions (about 0.1%) of the indicator dyes Bromothymol Blue or Bromocresol Purple were prepared in 10% sucrose solution. The Bromothymol Blue and Bromocresol Purple solutions were adjusted to pH 7.0 and 6.5, respectively, and immediately offered, soaked onto pieces of cotton, to recently blood-fed females. Twenty hours later, the females were dissected and the colours in the thoracic midgut (TM) were compared with standard solutions prepared with the same dyes at different pH values covering 0.5 unit intervals.

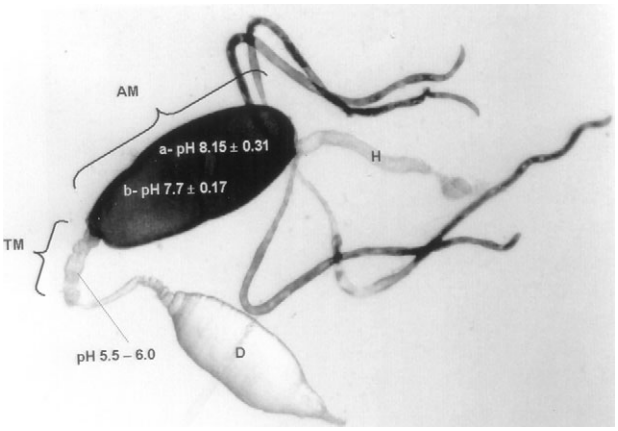

Fig. 2. Anatomy of *L. longipalpis* gut and pH in different parts of the midgut during the first 10 h (a) and 24 h (b) after blood ingestion. TM, thoracic midgut; AM, abdominal midgut; D, diverticulum filled with sugar solution; H, hindgut.

pH 7.5 containing Bromothymol Blue or with 0.16 mol l^{-1} Mes pH 5.0 containing Bromocresol Purple. The results are shown in Tables 3 and 4, respectively. In both cases, the pH inside the TM and AM showed an evident tendency to return to normal (pH 6) despite the presence of the buffers. The presence of acetazolamide, a carbonic anhydrase inhibitor, was significantly effective in diminishing the ability of the TM and AM to return to the normal pH values when challenged with 0.16 mol l^{-1} Hepes pH 7.5. The effect of acetazolamide in the females challenged with 0.16 mol l^{-1} Mes pH 5.0 was less pronounced in the AM but was statistically significant in the TM. In contrast, the pH inside the diverticulum was the same as that of the ingested solutions and was not affected by acetazolamide.

In some insects, the intracellular enzyme carbonic anhydrase is involved in CO_2 hydration and consequently in the production of H^+ and HCO_3^- ions that could be used by the midgut cells for midgut pH control (del Pilar Corena et al., 2004; del Pilar Corena et al., 2005). To test for the presence of transcripts of carbonic anhydrase, RT-PCR was performed using mRNA from the midgut of unfed *L. longipalpis* females. The primers used for PCR were designed based on two mRNA sequences of *L. longipalpis* with 68% homology to an *A. gambiae* carbonic anhydrase and 70% homology to an *A. aegypti* cytoplasmic carbonic anhydrase. The sequence alignment, in addition to the RT-PCR results presented in Fig. 3, suggest that the *L. longipalpis* midgut could produce more than one isoform of carbonic anhydrase-like transcript. However, these data should be confirmed by further experiments.

DISCUSSION

In 2000, Billker and colleagues observed that one drop of blood exposed to the air rapidly reaches pH 8 as the CO_2 volatilizes and equilibrates to the atmospheric concentration of CO_2 (Billker et al., 2000). In their study, the CO_2 volatilization was considered the sole cause of the alkalization phenomenon in the midgut of two mosquito species *A. aegypti* and *A. stephensi*, in which the pH in the AM 30 min after blood ingestion was 7.52 and 7.58, respectively. In contrast to these results, del Pilar Corena and colleagues observed a remarkable alkalization in the AM of adult mosquitoes after ingestion of a solution prepared with fetal calf serum and culture medium containing pH indicator dyes (del Pilar Corena et al., 2005). Although this method did not allow exact measurement of the pH, it was clear that the AM was alkalized to the range 8–9.5 or 8.5–9.5 depending on the mosquito species. It is important to emphasize that in this case the alkalization could not be attributed to the CO_2 volatilization. The authors also observed that this alkalization process was impaired by the use of acetazolamide and methazolamide, which are carbonic anhydrase inhibitors (del Pilar Corena et al., 2005). It should be noted that the specificity of this kind of inhibitor is not absolute and other activities could also be influenced.

In agreement with the results of del Pilar Corena and colleagues (del Pilar Corena et al., 2005), we found that the pH in *L. longipalpis* AM just after blood ingestion rapidly increased to values above 8 (Fig. 1). On the other hand, this alkalization observed after a blood meal could be attributed principally to the CO_2 volatilization mechanism. A minor contribution could be attributed to a second, unknown mechanism as can be inferred from the data presented in Table 2. Taking the CO_2 out of the blood (*in vitro*) caused an increase in the pH to 8.10±0.12. Before the blood was offered to the females, the pH was adjusted to approximately 7.4 and even without the CO_2 to help the alkalization, the pH of the AM increased significantly from 7.39±0.03 to 7.56±0.27, 24 h after blood ingestion (P=0.04). Evidently, the contribution of this second mechanism to the pH change was lower when compared with that promoted by CO_2 volatilization and was not observed in the first hours after blood ingestion (Table 2). This second mechanism seems similar to that observed by del Pilar Corena and coworkers mentioned above.

Although the buffering system in the midgut of unfed females is very efficient at maintaining pH 6 (Tables 2 and 3), the ingestion of blood is able to switch off this mechanism. This effect seems to be immediate, because in the first 10 min after blood ingestion the pH in the AM increased considerably, as shown in the first data points of Fig. 1. It is possible that free amino acids or even other molecules from the plasma are responsible for shutting down the pH 6 maintenance system and for triggering the alkalization mechanism. This physiological condition is probably maintained while amino acids are absorbed by the intestinal epithelium as a consequence of blood digestion by digestive proteases.

The pH 5.5–6.0, measured in the TM during blood digestion (Fig. 2; Table 1) indicates that the pH 6 maintenance mechanism is switched off in the midgut in a localized manner, i.e. only where the blood is located, such as in the interior of the AM.

Whilst it is important to maintain an alkaline pH in the AM, at the same time it is necessary to keep sucrose digestion working in order to digest and use the ingested sugar meal. In *L. longipalpis*,

Table 2. pH measurements from CO_2-depleted blood before ingestion and in the abdominal midgut of *L. longipalpis* during digestion

CO_2-depleted blood[1]	CO_2-depleted blood after pH correction[2]	CO_2-depleted blood 2–6 h after ingestion[3]	CO_2-depleted blood 24–28 h after ingestion[4]
8.1±0.12 (*N*=17)[a]	7.39±0.03 (*N*=11)[b]	7.34±0.48 (*N*=5)[b]	7.56±0.27 (*N*=5)[c]

[1]pH of the blood measured after CO_2 volatilization by *in vitro* exposure to the air for 1 h; [2]pH of the blood after CO_2 volatilization and adjustment with addition of 1 mmol l^{-1} HCl; [3]pH measured in the abdominal midgut (AM) of *L. longipalpis in vivo* 2–6 h after ingestion of the CO_2-depleted blood with pH previously adjusted to 7.39; [4]pH measured in the AM of *L. longipalpis in vivo* 24–28 h after ingestion of the CO_2-depleted blood with pH previously adjusted to 7.39.
Results marked with different superscript letters are significantly different (P<0.05).

Table 3. pH measurements in the midgut of unfed females of *L. longipalpis* challenged with 160 mmol l^{-1} Hepes pH 7.5-buffered solution with or without acetazolamide

	TM		AM		Diverticulum	
	No ACZ	ACZ	No ACZ	ACZ	No ACZ	ACZ
pH range						
pH≤6.0	22	8	22	5	1	1
6.0<pH≤6.5	15	19	10	14	0	2
6.5<pH≤7.0	0	1	3	7	2	2
7.0<pH≤7.5	0	0	0	0	23	13
Total no.	37	28	35	26	26	18
Statistics	P=0.0230		P=0.0008		P=1.000	

Fisher's test was used to investigate differences provoked by treatment with acetazolamide between the observations presenting pH≤6.0 and a group formed by all other observations (with pH>6.0). P<0.05 was considered significant.
TM, thoracic midgut; AM, abdominal midgut; ACZ, with acetazolamide; No ACZ, without acetazolamide.

Table 4. pH measurements in the midgut of unfed females of *L. longipalpis* challenged with 160 mmol l^{-1} Mes pH 5.0-buffered solution with or without acetazolamide

	TM		AM		Diverticulum	
	No ACZ	ACZ	No ACZ	ACZ	No ACZ	ACZ
pH range						
≤5.5	0	8	1	4	13	13
5.5<pH≤6.0	34	14	26	12	–	–
6.0<pH≤6.5	1	0	2	3	2	–
6.5<pH≤7.0	0	0	–	–	–	–
Total no.	35	22	29	19	15	13
Statistics	P=0.0002		P=0.0724		P=0.4841	

Fisher's test was used to investigate differences provoked by treatment with acetazolamide between the observations presenting pH≤6.0 and a group formed by all other observations (with pH>6.0). P<0.05 was considered significant.
TM, thoracic midgut; AM, abdominal midgut; ACZ, with acetazolamide; No ACZ, without acetazolamide.

this problem was solved by maintenance of the pH at 5.5–6.0 in the TM, even when the pH in the AM was alkaline. This pH range in the TM is perfect for sucrose digestion by the *L. longipalpis* digestive α-glucosidase, at the optimal pH of 5.8 (Gontijo et al., 1998). In this manner, the simultaneous digestion of proteins and carbohydrates can occur in distinct parts of the midgut with different pH values. The data presented here show that most of the α-glucolytic activity is in the TM.

In *L. longipalpis*, the α-glucosidase is more efficient in slightly acidic conditions, even when the enzyme is obtained from insects that are digesting blood (data not shown). In contrast, in *Phlebotomus langeroni*, the α-glucolytic activity described in blood-fed females has an optimal pH between 7 and 7.5 (Dillon and el-Kordy, 1997). These data indicate important physiological differences between these two phlebotomine species that deserve to be studied in more detail.

Phlebotomus papatasi (and probably other phlebotomine species) can sometimes ingest starch grains when they bite plants to obtain sap (Schlein and Warburg, 1986). These grains are apparently stocked in the diverticulum where the pH is not controlled by the insect. Probably, the salivary α-amylase (Ribeiro et al., 2000), which works well at pH 7 and is ingested with the saliva when the insects ingest sugar (Cavalcante et al., 2006), digests the starch present in the diverticulum, making this carbohydrate an alternative nutrient for the insect.

The presence of transcripts of carbonic anhydrases in the midgut cells and the results obtained with acetazolamide indicate that this enzyme may be involved in the pH control mechanism in unfed *L. longipalpis* females and is probably also involved in pH control in blood-fed ones. The enzyme carbonic anhydrase functions by

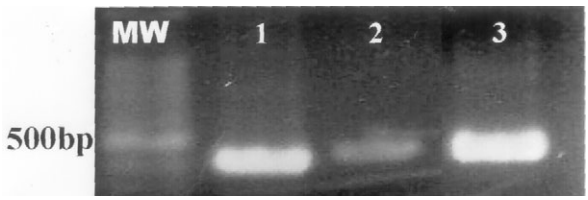

Fig. 3. Carbonic anhydrase expression in the midgut of unfed *L. longipalpis* females. Lanes: MW, relative molecular mass marker; 1, transcript with homology to *A. gambiae* carbonic anhydrase (427 bp); 2, transcript with homology to *A. aegypti* cytoplasmic carbonic anhydrase (455 bp); 3, 18S subunit of rRNA (468 bp).

hydrating CO_2 molecules in order to produce H^+ and HCO_3^- ions. The subsequent destiny of them, determined by different ion pumps, could be responsible for alkalization or acidification of the midgut, depending on the physiological conditions. In fact, it has already been demonstrated that carbonic anhydrase is involved in pH control in the midgut of mosquitoes (adults and larvae) (Corena et al., 2002; del Pilar Corena et al., 2004; del Pilar Corena et al., 2005).

As well as carbonic anhydrase, V-ATPases are also involved in pH regulation in insects. These enzymes, which are important proteins able to pump H^+ using ATP as an energy source, have already been described in *L. longipalpis* midgut cells (Ramalho-Ortigão et al., 2007). It is very likely that they also participate in the mechanism involved in pH control, probably acting differently in unfed and blood-fed females.

Cuprophilic and goblet cells are specialized cells involved in pH regulation in the midgut of Diptera (Muscomorpha) and Lepidoptera larvae, respectively (Terra, 1988; Terra et al., 1988; Lepier et al., 1994). However, morphological studies did not identify any of these cells in the adult midguts of phlebotomine sand flies, which are Diptera (Nematocera) (Billingsley, 1990; Leite and Evangelista, 2001).

In phlebotomine sand flies, *Leishmania* parasites live exclusively in the lumen of the gut where amastigotes ingested during blood feeding differentiate into flagellates that are morphologically and biochemically distinct from amastigotes (Bates, 1994; Handman, 2000). These forms, generally known as promastigotes, present morphological and physiological differences throughout their development (Bates and Rogers, 2004) that culminate in the appearance of metacyclic promastigotes able to infect mammalian hosts (Sacks and Perkins, 1984; Sacks, 1989). Since *Leishmania* development occurs exclusively within the midgut of the vectors, many biochemical and physiological characteristics of this environment may decisively influence the development of these parasites and thus the success of transmission. Previously, some factors have been described that are probably capable of influencing *Leishmania* development towards metacyclic forms in culture medium: exhaustion of nutrients (Giannini, 1974; Sacks and Perkins, 1985) as observed in cultures in the stationary phase (Sacks, 1989); higher concentrations of CO_2 in the culture medium when compared with usual cultures (Méndez, 1999); absence or low levels of factors able to inhibit metacyclogenesis such as haemoglobin (Schlein and Jacobson, 1994), haemin (Charlab et al., 1995) and tetrahydrobiopterin (Cunningham et al., 2001); contact with phlebotomine saliva, which only acts in the absence of haemin (Charlab et al., 1995); and, finally, acidification of the medium (Bates and Tetley, 1993).

On the other hand, in apparent contradiction related to the importance of an acidic environment to metacyclogenesis, promastigotes cultivated in different culture media grow better in neutral or slightly alkaline pH (M. N. Melo, personal communication). Furthermore, some enzymes responsible for protein digestion in the sand fly gut, especially proteases similar to trypsin and chymotrypsin, function better in an alkaline pH (Mahmood and Borovsky, 1993; Gontijo et al., 1998). To clarify this apparent contradiction and explain the development of *Leishmania* in infected sand flies, the following hypothesis was proposed (Gontijo et al., 1998): after an infective meal, an alkaline pH and the availability of nutrients during protein digestion would favour the multiplication of *Leishmania*. This *in vivo* phase of development would correspond to the logarithmic growth phase in culture medium. During this period, the alkaline pH and the presence of haemoglobin and haemin would inhibit metacyclogenesis, as haemoglobin (Schlein and Jacobson, 1994) and haemin (Charlab et al., 1995) seem to inhibit differentiation when present. When protein digestion is complete, haemoglobin and haemin should no longer be present and the nutrients would be practically exhausted, including tetrahydrobiopterin. At this moment, the pH of the intestine should decrease, stimulating metacyclogenesis as it occurs *in vitro*. In 1998, Gontijo and colleagues found the midgut pH of unfed *L. longipalpis* females to be 6.0, proving that the gut is acidic when blood is not being digested (Gontijo et al., 1998). In addition to the acidification, the phlebotomine saliva could act on *Leishmania* promastigotes in the absence of haemin, driving them to a differentiation pathway, as proposed by Charlab and colleagues (Charlab et al., 1995). In fact, saliva is regularly ingested during the sugar meal phase after blood digestion (Cavalcante et al., 2006). This post-blood meal stage, characterized by low levels of nutrients in an acidic environment, should correspond (in infected phlebotomines) to the stationary growth phase in the culture medium.

The alkaline pH measured in the *L. longipalpis* AM after blood ingestion (Fig. 1; Table 2) is entirely in accordance with the hypothesis proposed by Gontijo and colleagues described above (Gontijo et al., 1998). Indeed, an alkaline environment is favourable for the multiplication of *Leishmania* promastigote forms in culture, where the parasite presents optimum growth in the pH range 7–8 (M. N. Melo, personal communication). Depending on the *Leishmania* species, good growth rates, from 46% to 85% of the growth rate obtained at the optimum pH, can be reached even at pH 8.5 (M. N. Melo, personal communication). After 24 h, the AM pH in uninfected females remains at around 7.7 (Fig. 1), a pH favourable to the growth of *Leishmania* once it is within the pH range for optimal growth.

At present, there is no information about the pH in the midgut of *Leishmania*-infected insects. However, it is possible that, in females with a regular diet of carbohydrate, the pH is slightly lower than in uninfected insects due to the carbohydrate metabolism from promastigote forms. In fact, the phenomenon, called aerobic fermentation, could generate acid catabolites from an incomplete metabolism of monosaccharides such as glucose or fructose (Cazzulo et al., 1985; Darling et al., 1987).

Further studies are being developed on this subject and we hope soon to be able to propose a more complete model of how the intestinal pH is controlled by phlebotomines and also to obtain information about the pH variation in the midgut of *L. longipalpis* females infected with *L. infantum*. The findings could provide a better understanding of the development of this parasite in its vector.

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