Journal of Insect Physiology 120 (2020) 103973

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

The role of LuloPAT amino acid/proton symporters in midgut alkalinization in the sandfly *Lutzomyia longipalpis* (Diptera – Psychodidae)

Denise Barguil Nepomuceno, Rafaela Magalhães Macedo Paim, Ricardo Nascimento Araújo, Marcos Horácio Pereira, Grasielle Caldas D'Ávila Pessoa, Leonardo Barbosa Koerich, Mauricio Roberto Viana Sant'Anna, Nelder Figueiredo Gontijo*

Laboratório de Fisiologia de Insetos Hematófagos, Departamento de Parasitologia/ICB, Universidade Federal de Minas Gerais, Caixa Postal 486, 31270-901 Belo Horizonte, MG, Brazil

ARTICLE INFO

Keywords:
Lutzomyia longipalpis
Amino acid transporters
H^+ symport
Midgut pH control
Amino acids carriers

ABSTRACT

In *Lutzomyia longipalpis* females, which are the main vectors of *Leishmania infantum* in the Americas, hematophagy is crucial for ovary development. The control of pH in the midgut during blood digestion is important to the functioning of the digestive enzymes, which release amino acids in the luminal compartment that are then transported through the enterocytes to the hemolymph for delivery to the ovary and other organs. In the present work, we investigated transport systems known as LuloPATs that are present in the midgut of *L. longipalpis* but not in other organs. These transporters achieve symport of amino acids with H^+ ions, and one of them (LuloPAT1) is orthologous to a transporter described in *Aedes aegypti*. According to our results, the transcription levels of LuloPAT1 increased significantly immediately after a blood meal. Based on the variation of the fluorescence of fluorescein with the pH of the medium, we developed a technique that shows the acidification of the cytoplasm of gut cells when amino acids are cotransported with H^+ from the lumen into the enterocytes. In our experiments, the midguts of the sandflies were dissected and opened longitudinally so that added amino acids could enter the enterocytes via the lumen (PAT carriers are apical). LuloPAT1 transporters are part of a complex of mechanisms that act synergistically to promote gut alkalinization as soon as blood intake by the vector occurs. In dissected but not longitudinally opened midguts, added amino acids could only enter through the basolateral region of enterocytes. However, alkalinization of the lumen was observed because the entry of some amino acids into the cytoplasm of enterocytes triggers a luminal alkalinization mechanism independent of LuloPATs. These findings provide new perspectives that will enable the characterization of the set of signaling pathways involved in pH regulation within the *L. longipalpis* midgut.

1. Introduction

Phlebotomines are Diptera of the family Psychodidae (subfamily Phlebotominae) that are important in public health because they are vectors of different species of *Leishmania*. *Lutzomyia longipalpis* is the main vector of these protozoans in the Americas and is currently the most-studied phlebotomine species. Both males and females feed on carbohydrate solutions that they obtain by ingesting sap, nectar or even the honeydew secreted by aphids. However, females are also hematophagous because they need to ingest blood for ovarian development (Killick-Kendrick et al., 1977; Dillon and Lane, 1993; Dillon and El-Kordy, 1997).

Sugars as well as blood need to be digested by enzymes in the midgut. Since pH is one of the main factors that influences the function of digestive enzymes, there are fine mechanisms that regulate the concentration of H^+ in the midgut lumen (Gontijo et al., 1998; Santos, 2008). In females fed only with sugar, the pH of the midgut (thoracic and abdominal parts) is actively maintained at 6.0, which is ideal for the functioning of the enzymes responsible for sugar digestion. When females ingest a buffered alkaline solution, V-ATPases inserted in the apical region of the enterocytes are activated to reacidify the midgut to pH 6 (Santos et al., 2011). Sucrose, the principal sugar ingested, is digested by the enzyme α-glucosidase during a process that takes place mainly in the thoracic midgut, where this enzyme is bound to the microvilli of the enterocytes (Gontijo et al., 1998; Santos, 2008).

When females ingest blood, it goes straight to the abdominal midgut. The perception of proteins and amino acids inside the lumen causes endocrine cells scattered throughout the midgut to release

* Corresponding author at: Departamento de Parasitologia, Instituto de Ciências Biológicas, UFMG, Belo Horizonte, MG, Brazil.
E-mail addresses: marcoshp@icb.ufmg.br (M.H. Pereira), nelder@icb.ufmg.br (N.F. Gontijo).

https://doi.org/10.1016/j.jinsphys.2019.103973
Received 27 May 2019; Received in revised form 7 November 2019; Accepted 8 November 2019
Available online 09 November 2019
0022-1910/

alkalinizing hormones (Santos et al., 2011). These hormones act on receptors present in the basolateral region of the enterocytes, turning off the mechanism responsible for maintaining an acidic pH and turning on the alkalinization of the abdominal midgut (Santos et al., 2011), which is crucial for proper blood digestion. The thoracic midgut remains acidic and permits the normal functioning of α-glycosidase for sucrose digestion (Santos, 2008).

Two independent mechanisms have been described as being responsible for the alkalinization of the abdominal midgut: the release of the CO_2 transported by the ingested blood (Santos, 2008) and the transport of different ions through the plasmatic membrane of the enterocytes. The first mechanism is similar to respiratory alkalosis, and the latter seems to be related to the transport of bicarbonate ions from the enterocytes to the lumen since it is inhibited by the presence of acetazolamide, an inhibitor of the enzyme carbonic anhydrase (Santos, 2008). Together, these mechanisms induce the pH to increase to close to 8.15 (Santos, 2008; Santos et al., 2011). As digestion proceeds, the pH inside the abdominal midgut becomes progressively less alkaline, and at the end of the process, it returns to pH 6. This pattern of pH control in the midgut may be common to all hematophagous insects classified in suborder Nematocera. A similar mechanism is observed in the mosquito *Aedes aegypti* (Nepomuceno et al., 2017).

These events take on greater relevance considering that *L. longipalpis* is the main vector of *Leishmania infantum* in the Americas, and the gut pH plays a key role in the development of this protozoan during and after the digestion of blood in the gut lumen (Molyneux et al., 1975; Bates and Tetley, 1993; Gontijo et al. 1998). In addition, one or more unidentified low-molecular-weight substances produced by *L. infantum* may interfere with the mechanisms of pH regulation to favor its development (Santos et al., 2014).

Among insects with similar feeding behavior, blood digestion provides a large amount of amino acids. These amino acids are transported into the midgut cells and then to the hemolymph, through which they are distributed among the organs of the insect and are used in several vital processes, especially in vitellogenesis (Clements, 1992; Evans et al., 2009; Hansen et al., 2011). Considering that amino acids cannot cross lipid membranes, there is a demand for efficient transporters to pass amino acids through the cell membranes and intracellular compartments (Taylor, 2014).

Based on sequence homology, amino acid transporters are organized into six major families included in the solute carrier superfamily (SLC): SLC1, SLC6, SLC7, SLC36, SLC38, and SLC43. These transporters can be classified according to their substrate selectivity and ionic dependence (Foltz et al., 2004). It is important to consider that one cell type might express various transporters with overlapping specificities and that different transport systems can function simultaneously (Wolfersberger, 2000; Taylor, 2014). Amino acid transporters usually function coupled to the transport of ions such as Na^+, H^+, K^+ and even Cl^-. Transport systems in which an ion is transported accompanying a transported amino acid are referred to as symporters. When the ion is transported in the reverse direction, the system is referred to as an antiporter. In uniport systems, no ion is coupled with amino acid transport. In this case, amino acids are translocated only in response to a concentration gradient (Fredriksson et al., 2008; Fan and Goberdhan, 2018).

The characteristics of some transporters and their importance for insect homeostasis have been studied in mosquitoes. For example, AaCAT1 from *A. aegypti* is highly specific for L-histidine and is related to the development of eggs (Hansen et al., 2011). AaSlif from *A. aegypti* exhibits a dual affinity for neutral and cationic amino acids and is upregulated after a blood meal, which is crucial for the activation of the TOR signaling pathway (Boudko et al., 2015). AgNAT8 from *Anopheles gambiae* larvae displays a narrow substrate preference for essential aromatic amino groups, mediating the absorption of phenol-branched substrates (Meleshkevitch et al., 2006). The PAT amino acid transporters, classified in the SLC 36 family, function as symport systems depending on H^+; consequently, the operation of these carriers leads to pronounced intracellular acidification (Boll et al., 2004). AaePAT1, which was identified in the apical membrane of the midgut of *A. aegypti* and presents increased expression after a blood meal, shows low substrate affinity and specificity and is more active in acidic environments (Evans et al., 2009).

Since PAT transporters can remove H^+ ions from the lumen of the midgut, we hypothesize that they might be involved in the alkalinization process observed in the abdominal midgut following a blood meal. Therefore, the objective of this study was to investigate the involvement of PAT transporters in the mechanism of midgut alkalinization during blood digestion in *L. longipalpis* females.

2. Materials and methods

2.1. Insects and gut dissection procedures

All experiments were performed with 3- to 6-day-old *L. longipalpis* females. Sand flies were maintained according to Modi and Tesh (1983). Adult specimens were kept in nylon cages at 25 °C under 70 ± 10% humidity and were fed with 30% sucrose. When required, females were allowed to feed on the blood of hamsters (*Mesocricetus auratus*) anaesthetized with Thiopental® (0.2 mL of 5% Thiopentax per 100 g). All procedures were developed under approval by the Ethics Committee on Animal Experimentation (CEUA-UFMG), protocol number: 141/2018.

L. longipalpis guts were dissected avoiding cell damage. The integument bristles were removed by stirring the insects in saline containing a drop of detergent. Thereafter, the specimens were immediately transferred to ice-cold saline. For dissection, each insect was transferred to a drop of saline on a microscope slide, and its head was sectioned with a stylet. Holding the thoracic region with a stylet, the terminal portion of the abdomen was pulled back using another stylet, and the gut was removed from the insect's body intact. The Malpighian tubules as well as part of the posterior gut were cut with stylets avoiding tissue damage. We spent about two minutes with this procedure. The thoracic midgut and the posterior gut are very narrow structures in phlebotomine sandflies (Santos, 2008). The guts were cut in very narrow regions of the digestive tract (in the thoracic midgut and posterior gut) so that the lumen of the abdominal midgut was well insulated, preventing insect saline (IS − 119.70 mmol L^{-1} NaCl, 2.68 mmol L^{-1} KCl, 1.36 mmol L^{-1} $CaCl_2$, 0.56 mmol L^{-1} glucose) entrainment through these openings.

Depending on the experiment to be performed, the gut was either opened longitudinally or left unopened. This procedure was performed using two stylets.

2.2. RNA extraction and cDNA synthesis

L. longipalpis females were dissected to isolate the midgut and the carcass (the rest of the body without the midgut). The midguts were obtained from females subjected to different nutritional conditions: non-blood-fed, 0–3 h after blood feeding, and 20–24 h after blood feeding. In all groups, the insects were fed 30% sucrose.

After midgut isolation, RNA extraction was performed from pools of 20 midguts with the Nucleospin RNA Kit (Macherey-Nagel©), whereas RNA from the insect carcass was extracted with TRIzol® according to the manufacturer's instructions. The RNA was treated with DNase, recovered in 20 μL Milli-Q nuclease-free water and quantified at 260 nm in a Nanodrop Lite® (Thermo Scientific); extracted RNA purity was assessed by the 260 nm/280 nm absorbance ratio.

cDNA synthesis was performed with 0.5 μg of RNA, 0.5 μg of random hexamers (Promega®) and an M − MLV reverse transcriptase system (Promega®) in a final volume of 25 μL. The cDNA produced was used for both PCR and qPCR.

2.3. Identification of L. Longipalpis PAT1 – LLOJ004373 and their orthologous

An expressed sequence encoding LLOJ004373 was identified in the Vector Base database (https://www.vectorbase.org/blast) using the *A. aegypti* AaePAT1 (GenBank: ACS96436.1) protein as a template. The obtained sequences were analyzed with Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) and the Transporter Classification Database (TCDB; www.tcdb.org). Primers (for LuloPAT1) were designed with Primer3 software (http://primer3.sourceforge.net/): glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5′-cccttcatcggtctggacta-3′, and reverse, 5′- ttcggagaagacagtgatgg-3′ (150 base pairs); LLOJ004373 forward, 5′-cggcatttgtgattgaattg-3′, and reverse, 5′-tttcatttgttcgtggttgga-3′ (120 base pairs).

Using the neighbor-joining method (Saitou and Nei, 1987), an evolutionary tree was constructed to analyze the relationship between the AaePAT1 sequence and the transporter sequences from *L. longipalpis* belonging to the SLC36 family. The Zuckerkandl and Pauling method was used to compute evolutionary distances (Zuckerkandl and Pauling, 1965). All analyses were conducted with the software MEGA X (Kumar et al., 2018).

2.4. PCR

Reactions were performed in a Veriti® thermal cycler (Thermo Fisher Scientific), with an initial denaturing step at 94 °C for 5 min, followed by 35 cycles consisting of a denaturing step at 94 °C for 45 s, an annealing step at 60 °C for 45 s and an extension step at 72 °C for 45 s, with a final extension step at 72 °C for 7 min. Each reaction was performed with 1 µL of cDNA (10 ng), 200 nmol L^{-1} of specific primers, 200 µmol L^{-1} of dNTPs and 1 U of Taq DNA polymerase in a final volume of 20 µL. For each pair of primers, a negative control was included, in which the cDNA was replaced by Milli-Q. The PCR products were analyzed by 2% agarose gel electrophoresis, stained with GelRed™ (Biotium) and visualized under ultraviolet light.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed using the StepOne Plus real-time quantitative PCR system (Applied Biosystems®). Each reaction was run in triplicate and contained 10 ng of cDNA, 300 nmoL^{-1} of each specific primer and 5 µL of Power Sybr Green PCR Master Mix (Applied Biosystems®) in a 10 µL final volume. A reverse transcriptase negative control and a no cDNA negative control were included for each primer set to confirm the absence of genomic DNA and contamination in the reactions. GAPDH was used as a reference gene (GenBank: ABV60323.1), and the relative amount of the target transcript in each sample was determined by applying the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6. Fluorescence emission by fluorescein at different pH levels

Fluorescein is a dye whose fluorescence emission changes according to the pH of the medium and presents the advantage of not being photosensitive (Martin and Lindqvist, 1975). As a consequence of its relative hydrophobicity, it can cross lipid membranes and penetrate the enterocytes cytoplasm as well as the gut lumen. Before using fluorescein to evaluate the pH in our experiments, we investigated whether the fluorescence intensity was directly proportional to the pH of the medium.

Buffered solutions were prepared at different pH levels: for the pH 5.0, 5.5, 6.0 and 6.5 solutions, 10 mmol L^{-1} MES was used (pKa = 6.1), and for the pH 7.0, 7.5 and 8.0 solutions, 10 mmol L^{-1} HEPES was used (pKa = 7.48). With each buffer, a 200 µmol L^{-1} fluorescein (Sigma - F3677) solution was prepared. Each buffered fluorescein solution was then added to the wells of an ELISA plate, and fluorescence was evaluated under an epifluorescence microscope (Leica DM500) coupled to a digital camera. Images were captured using a green light filter (excitation: 450–480 nm; emission: 520 nm) and posteriorly analyzed with ImageJ software (https://imagej.nih.gov/ij/) to provide the relative fluorescence intensity for each pH level. The procedure was performed in triplicate for each fluorescein solution.

2.7. Evaluation of pH changes in the midgut by fluorescein

Fluorescein is an amphoteric molecule that is slightly soluble in water, and its relative hydrophobicity allows fluorescein molecules to cross lipid membranes. Dissected midguts exposed to fluorescein absorb the dye, which changes its fluorescence intensity depending on the pH of the medium.

When the LuloPAT system functions coupled to proton symporters, the uptake of amino acids promotes the acidification of enterocytes. Thus, amino acid uptake by PAT carriers can be visualized by changes in pH.

Females that were 3–6 days old were dissected in insect saline (IS − 119.70 mmol L^{-1} NaCl, 2.68 mmol L^{-1} KCl, 1.36 mmol L^{-1} $CaCl_2$, 0.56 mmol L^{-1} glucose), and once the midgut was removed, it was opened via a longitudinal cut so that the luminal surface was exposed. Each midgut was incubated at room temperature in 200 µmol L^{-1} fluorescein dissolved in buffered IS (IS buffered with 10 mmol L^{-1} HEPES, pH 7.0) for 10 min and then washed in IS to remove the dye that had not penetrated the enterocytes.

The midguts dyed with fluorescein were transferred to an acrylic slide containing a double chamber (Fig. 1a, b). Each chamber was previously filled with 15 µL of 2% agarose gel prepared in IS. These slides were produced to enable the evaluation of two midguts at the same time under an epifluorescence microscope (Leica DM500 - excitation: 450–480 nm; emission: 520 nm) coupled to a digital camera. Subsequently, 1.5 µL of buffered IS containing diluted amino acids

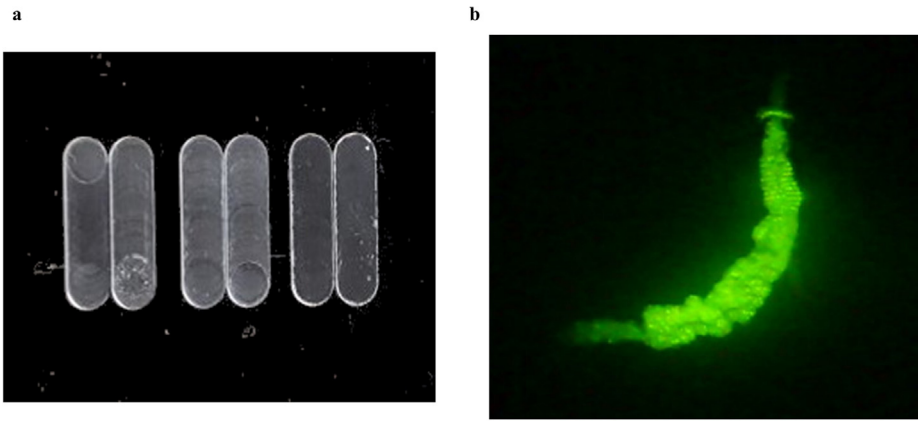

Fig. 1. a, b Evaluation of pH changes in the midguts of *Lutzomyia longipalpis* females: (a) Modified microscopy slide used to simultaneously evaluate fluorescence emission from midguts subjected to different treatments. (b) An opened and washed midgut from a *L. longipalpis* female observed under an epifluorescence microscope after fluorescein staining and before amino acid addition.

(MEM amino acids stock solution – Sigma M5550) was added above one of the midguts (final concentration 5.0 mmol L^{-1}, 7.5 mmol L^{-1} or 10 mmol L^{-1}). The control midgut received the same volume of IS but without amino acids.

The composition of the MEM amino acid stock solution was as follows: 29.928 mmol L^{-1} arginine, 5.160 mmol L^{-1} cysteine, 9.632 mmol L^{-1} histidine, 19.952 mmol L^{-1} isoleucine, 19.952 mmol L^{-1} leucine, 19.952 mmol L^{-1} lysine, 5.160 mmol L^{-1} methionine, 9.976 mmol L^{-1} phenylalanine, 19.952 mmol L^{-1} threonine, 2.408 mmol L^{-1} tryptophan, 9.976 mmol L^{-1} tyrosine and 19.952 mmol L^{-1} valine. The total amino acid concentration in the stock solution was 172 mmol L^{-1}.

The fluorescence emitted by both midguts was recorded for 2 min, and the effects of the different treatments were evaluated by comparing the emitted fluorescence. The videos were defragmented into images and analyzed with ImageJ software. Statistical analysis was performed by counting the number of fluorescent pixels in each midgut before treatment and at 2 min posttreatment.

Applying the same procedure, we also evaluated unopened midguts, in which fluorescein was present both in the lumen and in the enterocyte cytoplasm. In this case, lumen alkalinization was observed when it occurred. Importantly, in the closed gut, amino acids could only enter the enterocytes through the basolateral region of the enterocytes and not through the apical region where PAT carriers are located.

We also investigated the effect of individual amino acids on opened and closed guts. To investigate the effect of individual amino acids, buffered IS solutions (pH 7) were prepared with 10 mmol L^{-1} lysine, arginine, serine or leucine, and the experiments were performed as explained.

To investigate whether the guts were damaged, we repeated this experiment using buffered IS at pH 6 containing arginine (IS buffered with 10 mmol L^{-1} MES, pH 6.0). Damage to the guts would allow the acid solution to enter and acidify the lumen. If there were no holes in the gut, we would expect the lumen to become alkaline (in the cytoplasm, arginine triggers the PAT-independent alkalinization mechanism described in the Introduction section).

2.8. Statistical analysis

Data were analyzed using Graph Pad Prism 7.0 (San Diego, CA, USA). The Kolmogorov-Smirnov test was applied to evaluate the normality of the data. Data were analyzed with Student's *t*-test or the Kruskal-Wallis test, followed by Dunn's posttest (for nonnormal distribution). The results were considered significant when $p < 0.05$.

3. Results

3.1. The LuloPAT1 symporter is expressed in the L. Longipalpis midgut

Based on the AaePAT1 sequence of *A. aegypti*, it was possible to identify six related sequences from *L. longipalpis* (Fig. 2). Comparison between the proteins from *L. longipalpis* and the AaePAT1 transporter showed that the *L. longipalpis* orthologous sequence belonged to the LLOJ004373-RA gene, which will be referred to as LuloPAT1 (Fig. 2). The LuloPAT1 transporter exhibits 70.4% identity to the AaePAT1 carrier (Figure Supplementary S1). This gene encodes a proton-coupled amino acid transporter from the SLC36 family.

AaePAT1, LuloPAT1 (LLJ004373), LLJ008316 and LLJ001092 have very similar signatures. The transmembrane, cytoplasmic and extracellular sequences are in good agreement according to prediction performed by the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM-2.0/). These sequences include 447–485 amino acids with potential glycosylation signals predicted in a region near the amino terminal sequence predicted by the NetOGlyc 4.0 server (http://www.cbs.dtu.dk/services/NetOGlyc/). The other three sequences (LLJ007545, LLJ002457 and LLJ006275) present a different signature, although they share at least 42.6% identity.

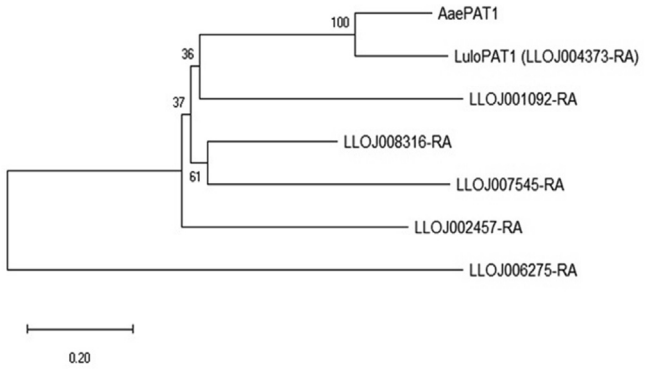

Fig. 2. Phylogeny of LuloPAT carriers. Phylogenetic analysis of proteins related to amino acid carriers of the SLC36 family. Based on the AaePAT1 sequence of *A. aegypti*, it was possible to identify six related sequences from *L. longipalpis*.

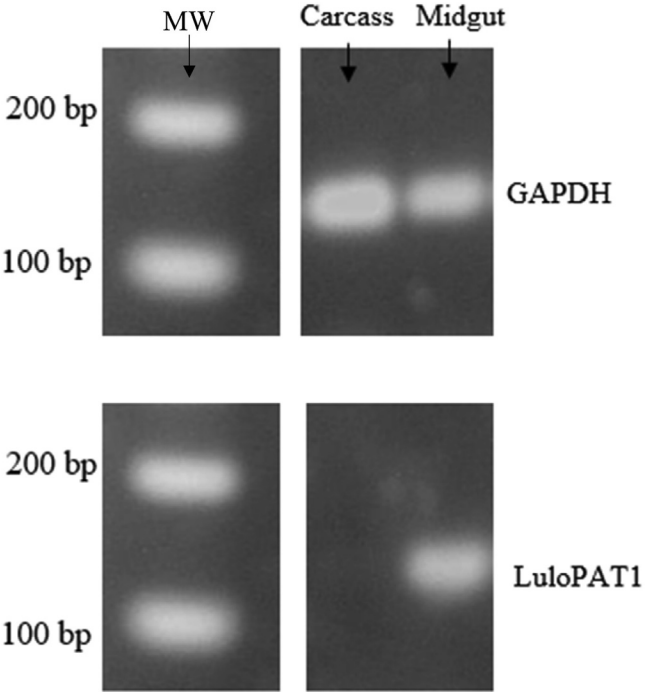

Fig. 3. LuloPAT1 expression evaluated in the midguts and in carcasses (the rest of the body without the midgut) of *L. longipalpis* females. LuloPAT1 is expressed only in the midgut of *L. longipalpis* females and not in the carcass. GAPDH was used as a reference gene (MW = molecular weight marker, bp = base pairs).

The LuloPAT1 gene was only expressed in the midgut of *L. longipalpis* females and not in the rest of the insect's body (carcass) (Fig. 3).

3.2. LuloPAT1 expression is modulated by the nutritional status of L. Longipalpis females

Quantitative analysis by qPCR demonstrated that the expression pattern of the transporter changed according to the nutritional condition of the insects. In the first 3 h after a blood meal, the expression level of LuloPAT1 was at least 10-fold higher in comparison with its expression in non-blood-fed females. Between 20 and 24 h after blood feeding, the level of gene expression had decreased by approximately 50% (Fig. 4).

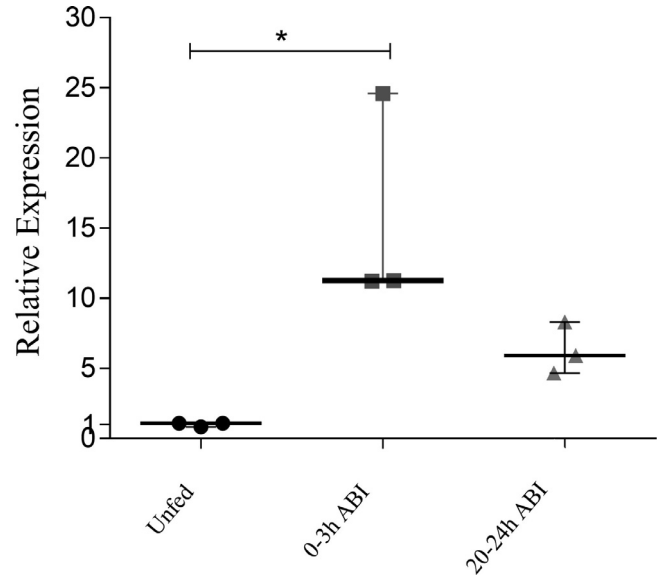

Fig. 4. Expression of LuloPAT1 in the midgut of *L. longipalpis* females under different nutritional conditions. Unfed; 0–3 h after blood ingestion (0–3 h ABI); 20–24 h after blood ingestion (20–24 h ABI). Data are represented as the median ± range from three experiments (pools of 20 midguts were used for each experiment). *, $p < 0.05$; analysis by the Kruskal-Wallis test, followed by Dunn's posttest. (ABI – after blood ingestion).

3.3. The addition of MEM amino acids to opened midguts leads to intracellular acidification

As shown in Fig. 5, fluorescence emission by fluorescein varies linearly according to the pH of the medium. The lower the pH, the lower the fluorescence emitted.

To observe pH changes in the enterocytes treated with the amino acids present in MEM solution, midguts were immersed in a fluorescein solution until the enterocytes had absorbed the dye. After washing away the unabsorbed fluorescein molecules, the midguts were exposed to different concentrations of amino acids. Comparison of the fluorescence intensity emitted before and two minutes after the addition of amino acids revealed H^+ influx into enterocytes. The decrease in fluorescence (acidification of the cytoplasm) was dependent of amino acids concentration (Fig. 6a, b and c). As expected, the control midguts treated only with IS did not undergo cytoplasmic acidification.

As a supplement, we provide a video in which it is possible to observe the decay of fluorescence in the midguts that received the amino acid solution (Supplementary Movie).

3.4. The addition of MEM amino acids to unopened midguts leads to luminal alkalinization

Contrary to the results described above, we observed that in unopened midguts (in which the dye was present inside the enterocytes and, at the same time, in the midgut lumen), the addition of the MEM amino acid solution at 10 mmol L^{-1} led to a significant increase in fluorescence emission (Fig. 7), which was interpreted as the alkalinization of the midgut lumen. However, at 5.0 mmol L^{-1} and 7.5 mmol L^{-1} concentrations, there was no significant difference between the test and control groups (data not shown).

3.5. The addition of individual amino acids to opened midguts leads to intracellular acidification or has no influence on the cytoplasmic pH

Another experiment was performed using individual amino acids (serine, leucine, lysine or arginine) dissolved in buffered IS solutions at pH 7. In opened guts, 10 mmol L^{-1} serine and leucine promoted cytoplasmic acidification, indicating that PAT carriers can transport these amino acids (Fig. 8a, b).

On the other hand, lysine or arginine did not promote cytoplasmic acidification, indicating that carriers other than PATs are involved in transport (Fig. 8c, d).

3.6. The addition of lysine or arginine to unopened guts promotes lumen alkalinization, while serine and leucine have no influence on the luminal pH

In unopened guts, all four amino acids could only enter the enterocytes via basolateral carriers, and these carriers were not PATs (PATs are typically apical proteins).

As observed in Fig. 9a–d, lysine and arginine promoted luminal alkalinization, while serine and leucine were unable to do so. It signifies that lysine and arginine act on the cytoplasm of the enterocytes triggering a PAT-independent alkalinization mechanism while serine or leucine mediates a PAT-dependent mechanism but are "not perceived" in the cytoplasm.

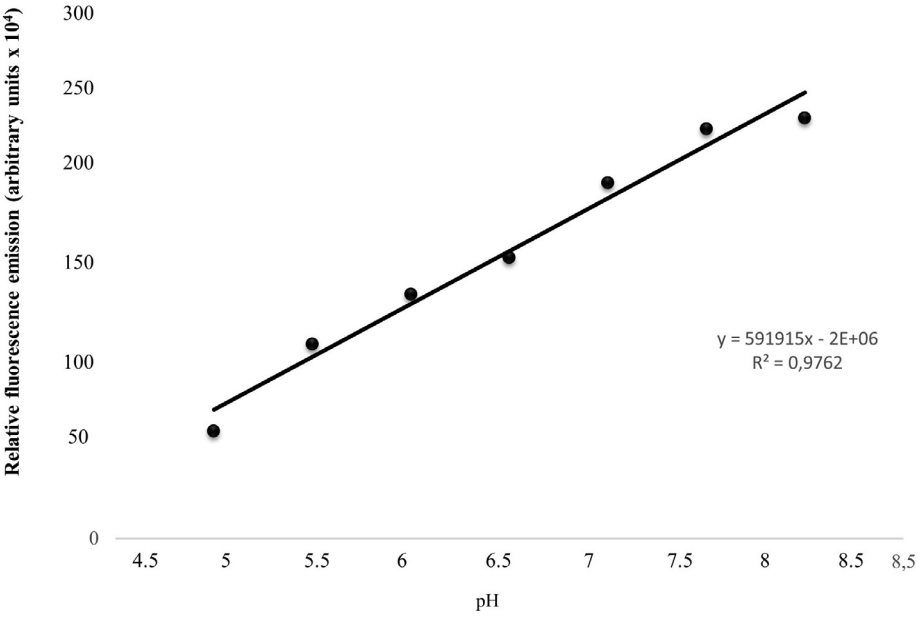

Fig. 5. Relation between fluorescence emission by fluorescein and the pH of the medium. A set of buffered solutions containing the same concentration of fluorescein with different pH levels were exposed inside the wells of an ELISA plate under an epifluorescence microscope (excitation: 450–480 nm; emission: 520 nm). Each well was photographed, and the intensity of the fluorescence was evaluated by counting the fluorescent pixels with ImageJ software.

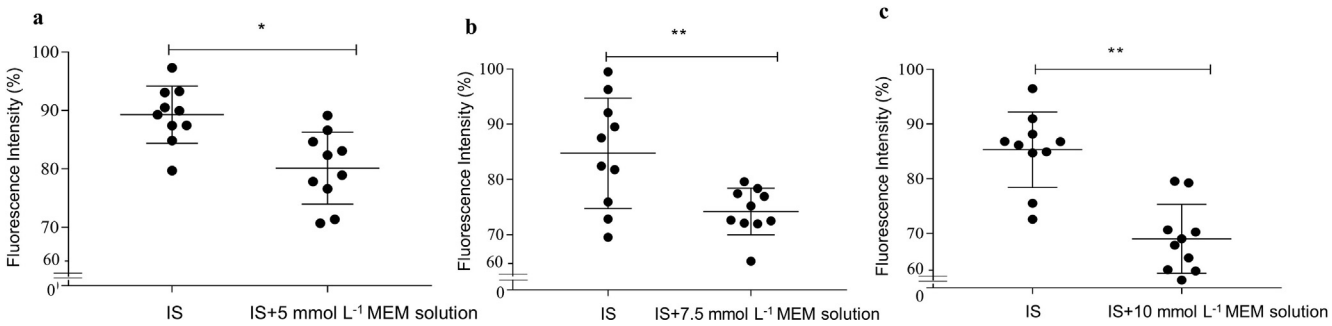

Fig. 6. Fluorescence intensity observed in opened *L. longipalpis* midguts after the addition of the MEM amino acid solution. The MEM amino acid solution was diluted in 10 mmol L^{-1} HEPES-buffered IS, pH 7. The data correspond to the percentage of fluorescence emission two minutes after the treatments were performed (n = 10). (a) Treatment with a 5 mmol L^{-1} MEM amino acid solution; (b) treatment with a 7.5 mmol L^{-1} MEM amino acid solution; (c) treatment with a 10 mmol L^{-1} MEM amino acid solution. The control groups received IS without amino acids. The data were expressed as the mean ± standard deviation. *, $p < 0.05$; ** $p < 0.01$, unpaired Student's *t*-test.

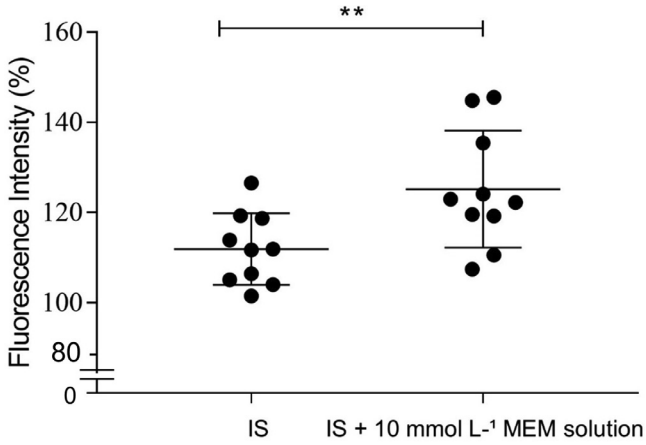

Fig. 7. Fluorescence intensity observed in unopened *L. longipalpis* midguts in response to treatment with the MEM amino acid solution. The MEM amino acid solution was diluted in 10 mmol L^{-1} HEPES-buffered IS, pH 7. The data correspond to the percentage of fluorescence emission two minutes after the treatments were performed (n = 10). The control group received insect saline without amino acids. The data were expressed as the mean ± standard deviation. *, $p < 0.05$; **, $p < 0.01$, unpaired Student's *t*-test.

When the same experiment was performed with arginine at pH 6, the lumen of the unopened gut exhibited alkalinization despite the acidic external environment (Fig. 10), indicating that the guts were not damaged. Damaged guts would allow the acidic solution to enter, promoting lumen acidification.

4. Discussion

L. longipalpis females as well as females of other hematophagous insects belonging to the former suborder Nematocera (including phlebotomines, mosquitoes, midges, black flies and insects from the family Corethrellidae) feed on both sugars and vertebrate blood. Blood meals (due to their particular high protein content Figure Supplementary S1) are crucial for the maturation of the female reproductive system in most of these species (Dillon and Lane, 1993; Dillon and El-Kordy, 1997; Soares and Turco, 2003; Hansen et al., 2004). The digestion of these different nutrients requires strict control of the midgut environment, and the efficient regulation of gut pH is one of the main factors involved. In this work, we describe a new facet of the mechanisms involved in this tightly controlled process.

In 2009, Evans et al. cloned and expressed the *A. aegypti* AaePAT1 transporter in *Xenopus* oocytes to characterize its function using radioactively labeled amino acids. When transporting amino acids, AaePAT1 promoted the movement of charges through the membrane. This electrogenic transport could be attributed to the simultaneous movement of H^+ ions and amino acids when the carrier was active; however, the authors did not directly observe the acidification of the oocyte cytoplasm.

The SLC36 family, to which AaePAT1 and its orthologue LuloPAT1 belong, is a family of well-characterized proton-coupled carriers (Boll et al., 2004; Abbot et al., 2006). In addition to AaePAT1, LuloPAT1 appears to be an amino acid transport system coupled to the symport of protons (Fig. 2).

LuloPAT1 is expressed only in the midgut of *L. longipalpis* and, similar to its orthologue AaePAT1, its expression profile is modulated by the ingestion of blood. The levels of gene transcripts increased rapidly in the first hours after females consumed a blood meal, which probably coincides with the higher amino acid availability due to blood digestion. We believe that the reduction in LuloPAT1 expression verified 20–24 h after blood feeding is related to a decrease in the amount of amino acids to be transported into the midgut cells with the progression of the digestive process (Figs. 3 and 4). Therefore, gene expression would decrease until returning to the levels observed before blood feeding. The expression of the other PATs was not investigated in our study. If active in the midgut, they could participate in the PAT-dependent alkalinization mechanism described here.

Our research group has investigated how *L. longipalpis* controls gut pH (Gontijo et al., 1998; Santos, 2008; Santos et al., 2011). Two mechanisms involved in the process of alkalinization occurring during and after blood ingestion have been described. The first involves the loss of CO_2 from ingested blood (Santos, 2008), and the other mechanism is dependent on ionic transport (probably of bicarbonate) by the midgut epithelium (Santos, 2008; Santos et al., 2011). This ionic transport would be triggered by increasing the concentrations of certain amino acids such as lysine or arginine in the cytoplasm of enterocytes. At the same time, the perception of amino acids in the cytoplasm of enterocytes (probably by the TOR system) promotes the shutdown of the mechanism responsible for the maintenance of a pH of 6 inside the lumen, an event necessary to permit alkalinization.

In the present work, we describe a third mechanism that acts in synergy with the other two. This mechanism contributes to the alkalinization of the gut lumen through the symport of amino acids/H^+ ions from the gut lumen into the cytoplasm of the enterocytes. The acidification of the cytoplasm of the enterocytes stimulated by the functioning of one or more LuloPATs necessarily implies alkalinization of the lumen. At least serine and leucine are involved in the PAT-dependent alkalinization mechanism, but lysine and arginine are not (Fig. 8a–d). For while, we have no information regarding the effects of the other amino acids in the PAT-dependent or independent alkalinization mechanisms.

The similarity between AaePAT1 and LuloPAT1 is very high (70.4% identity). Phylogenetic analysis of the sequences strongly indicated that

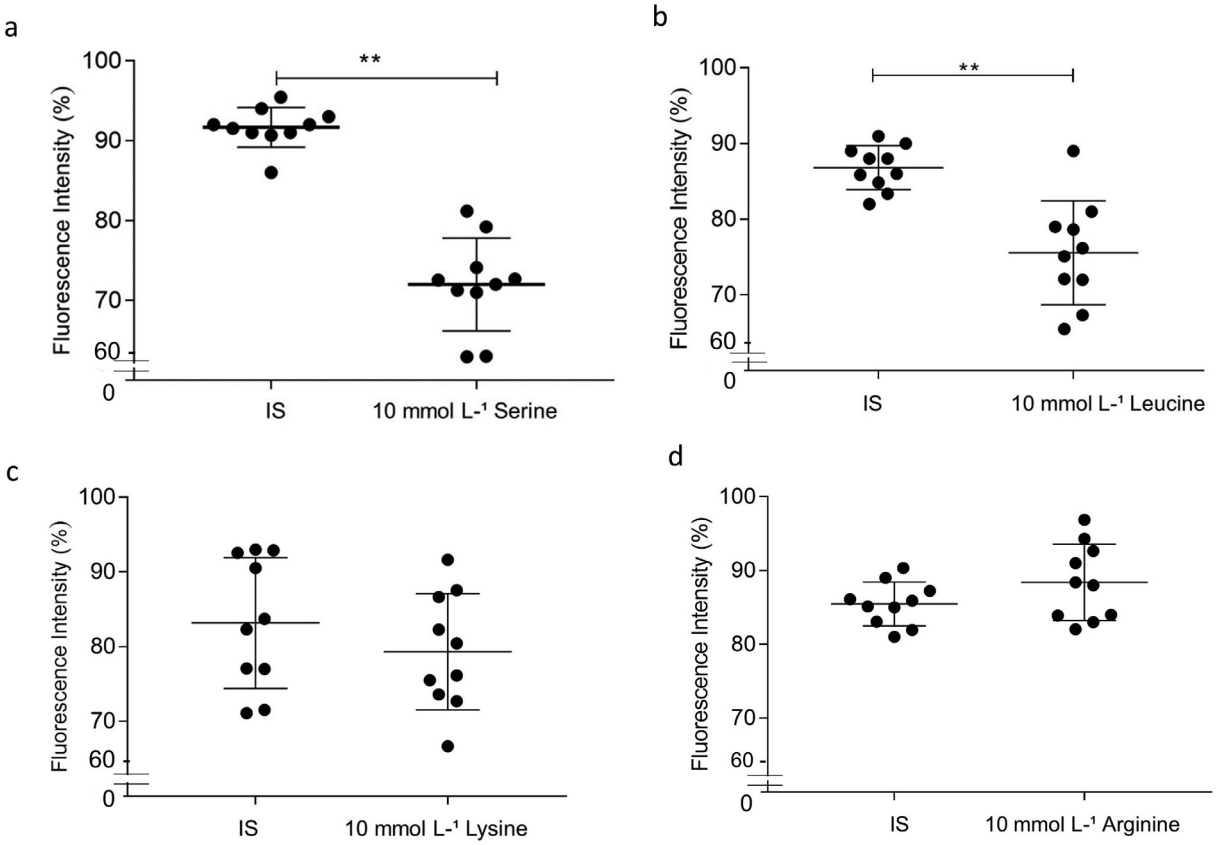

Fig. 8. Fluorescence intensity observed in opened *L. longipalpis* midguts after the addition of insect saline containing serine, leucine, lysine or arginine. Amino acids were individually dissolved in 10 mmol L^{-1} HEPES-buffered IS, pH 7, and added to opened midguts containing fluorescein. a: serine; b: leucine; c: lysine or d: arginine. The data correspond to the percentage of fluorescence emission two minutes after the treatments were performed (n = 10). The control group received insect saline without amino acids. The data were expressed as the mean ± standard deviation. *, $p < 0.05$; **, $p < 0.01$, unpaired Student's *t*-test.

they are orthologous (Fig. 2) and, thus, should present the same location and function. Similar to AaePAT1, LuloPAT1 must be located on the luminal surface. Assuming that this location is correct, the entry of amino acids into the enterocytes of unopened guts is expected to occur through different carriers present in the basolateral region of the gut epithelium. Accordingly, serine and leucine promote acidification only in opened midguts confirming the apical location of LuloPAT1 (or other PATs if present).

The entry of amino acids such as lysine or arginine activates alkalinization via a LuloPAT1-independent mechanism (Fig. 9a, b). Other noninvestigated amino acids may also be involved in this process. Interestingly, lysine and arginine did not enter the enterocytes via PAT carriers because acidification of enterocytes was not observed in opened guts (Fig. 8c, d). The cytoplasmic perception of lysine or arginine is most likely performed by the TOR system if TOR is involved in this process.

The relative contributions of the three mechanisms to alkalinization of the abdominal midgut are unknown. Most likely, their contributions vary during the digestive process. As reported in a previous study (Santos, 2008), blood exposed to the air undergoes rapid alkalinization as a consequence of CO_2 release. Given the nature of this mechanism, the release of CO_2 is likely effective during a bite. The juxtaposition of phlebotomine mouthpieces is not perfect and could allow the blood being ingested to contact the atmosphere, facilitating CO_2 release.

In a study involving AaePAT1, Evans and colleagues (2009) obtained indirect evidence of cytoplasmic acidification by showing that the entry of amino acids into *Xenopus* oocytes was electrogenic. The fact that AaePAT1 is electrogenic and belongs to the SLC36 family indicates that the ion responsible for this characteristic is the H^+ ion. In the present study, we provided direct evidence of the acidification of *L. longipalpis'* enterocytes by transporting amino acids to the cytoplasm through the apical region in opened midguts. In another study with *Musca domestica* larvae, Barroso and colleagues (2019) obtained experimental evidence of the involvement of PATs in the alkalinization of a midgut portion. It seems that PAT carriers probably play a similar hole in different insects.

Other amino acid transport systems are most likely present and assist in the transport of amino acids from the lumen to the enterocytes. Since blood is rich in Na^+ and K^+ (sodium exists at a high concentration in plasma and potassium inside blood cells), it is likely that a Na^+ or K^+ / amino acid symporter functions during the digestion of blood. The presence of these other transporters is probably necessary because when the luminal pH is sufficiently alkaline (approximately pH 8.15; Santos, 2008), the low availability of H^+ in the lumen could decrease the efficiency of PAT carriers. Additionally, Na^+ and K^+ ions must be transported to the hemolymph to be excreted by the Malpighian tubules. Sodium or K^+/amino acid symporters are potential candidates for this role.

The presence of efficient amino acid transporters is fundamental. It is known in *L. longipalpis* and other hematophagous insects from the suborder Nematocera that blood ingestion is important for vital events such as vitellogenesis. In mosquitoes, blood meal-derived amino acids are the main stimulus that activates nutrient signaling pathways that stimulate the fat body to initiate vitellogenesis (Hansen et al., 2004; Hansen et al., 2005; Hansen et al., 2011; Carpenter et al., 2012). In fact, amino acid transporters can be considered to represent the initial link between a cell (in a multicellular organism) and its environment, sensing and monitoring changes in extracellular and intracellular amino acid availability, which allows cells to efficiently adapt to changes in nutritional status (Hundal and Taylor, 2009)

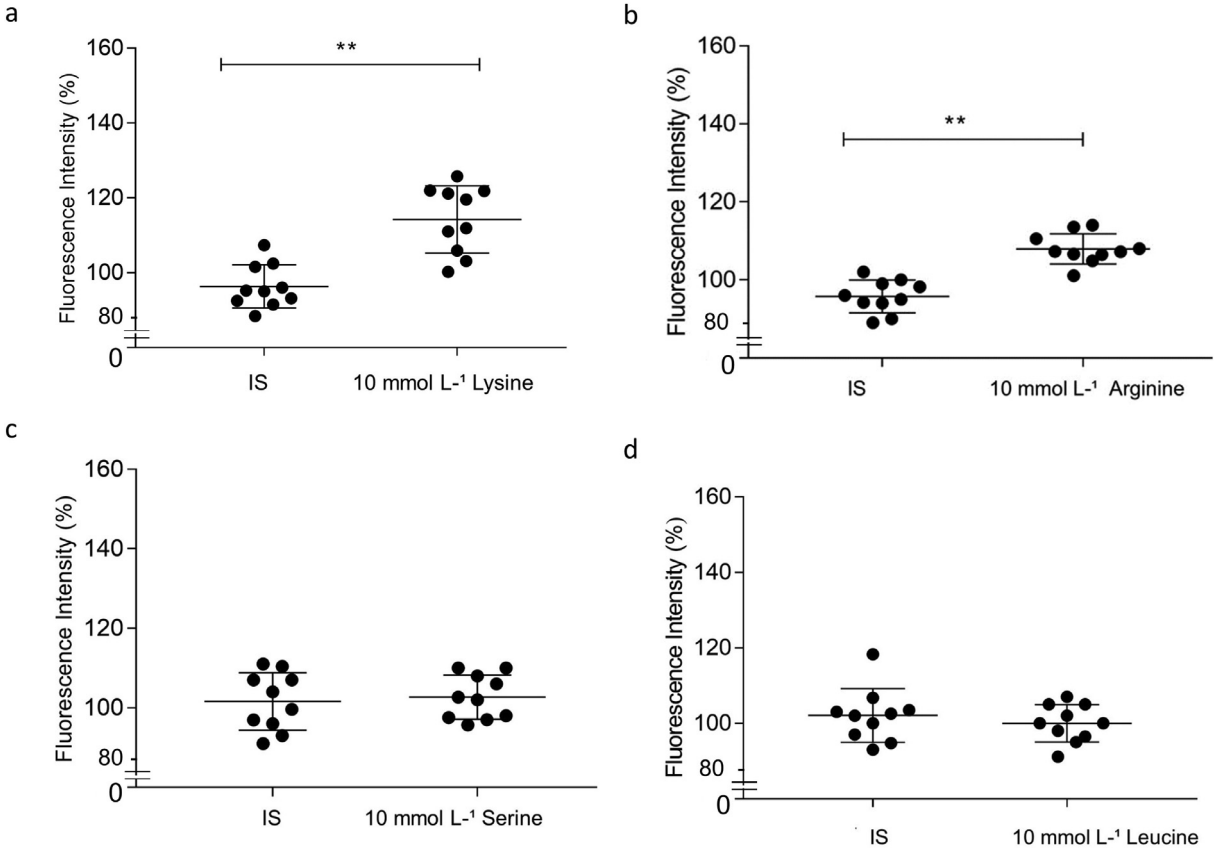

Fig. 9. Fluorescence intensity observed in unopened *L. longipalpis* midguts after the addition of insect saline containing serine, leucine, lysine or arginine. Ten mmol L^{-1} amino acids were dissolved individually in 10 mmol L^{-1} HEPES-buffered IS, pH 7, and added to unopened midguts containing fluorescein. a: lysine; b: arginine; c: serine or d: leucine. The data correspond to the percentage of fluorescence emission two minutes after the treatments were performed (n = 10). The control group received insect saline without amino acids. The data were expressed as the mean ± standard deviation. *, $p < 0.05$; **, $p < 0.01$, unpaired Student's *t*-test.

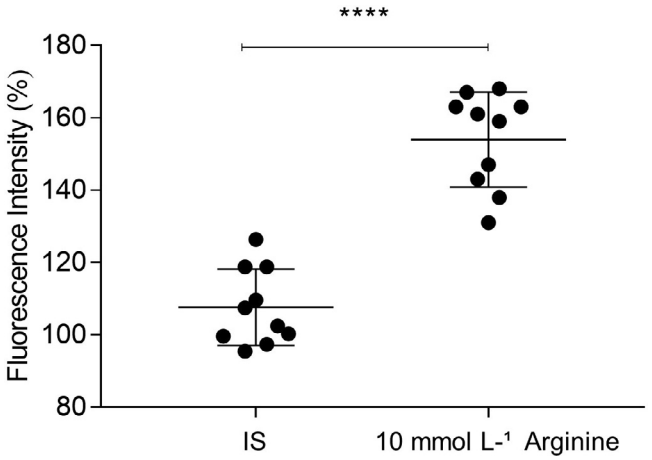

Fig. 10. Fluorescence intensity observed in unopened *L. longipalpis* midguts after the addition of insect saline at pH 6 containing arginine. Arginine was dissolved at 10 mmol L^{-1} in 10 mmol L^{-1} MES-buffered IS, pH 6, and added to unopened midguts containing fluorescein. The data correspond to the percentage of fluorescence emission two minutes after the treatment was performed (n = 10). The control group received 10 mmol L^{-1} MES-buffered IS, pH 6 without amino acids. The data were expressed as the mean ± standard deviation. *, $p < 0.05$; **, $p < 0.01$, unpaired Student's *t*-test.

The use of fluorescein in the study of H^+/amino acid symport was quite simple, informative and inexpensive. It is possible that this methodology can be adapted for the study of other systems in which acidification or alkalinization occurs near pH 7.

The participation of amino acid/H^+ transport in the process of midgut alkalinization is likely a common strategy between Diptera and even other insect species.

Funding

Funding for this study was partially provided by the following Brazilian agencies: Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), INCT – Entomologia Molecular and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2019.103973.

References

Abbot, E.L., Grenade, D.S., Kennedy, D.J., Gatfield, K.M., Thwaites, D.T., 2006. Vigabatrin transport across the human intestinal epithelial (Caco-2) brush-border membrane is via the H(+)-coupled amino-acid transporter hPAT1. Br. J. Pharmacol. 147, 298–306. https://doi.org/10.1038/sj.bjp.0706557.

Bates, P.A., Tetley, L., 1993. *Leishmania mexicana*: Induction of metacyclogenesis by cultivation of promastigotes at acidic pH. Exp. Parasitol. 76, 412–423. https://doi.org/10.1006/expr.1993.1050.

Barroso, I.G., Santos, C.S., Bertotti, M., Ferreira, C., Terra, W.R., 2019. Molecular mechanisms associated with acidification and alkalization along the larval midgut of Musca domestica. Comp. Biochem. Physiol., Part A, Mol. Integrative Physiol. 237, 110535. https://doi.org/10.1016/j.cbpa.2019.110535.

Boll, M., Daniel, H., Gasnier, B., 2004. The SLC36 family: proton-coupled transporters for the absorption of selected amino acids from extracellular and intracellular proteolysis. Pflugers Arch. 447, 776–779. https://doi.org/10.1007/s00424-003-1073-4.

Boudko, D.Y., Tsujimoto, H., Rodriguez, S.D., Meleshkevitch, E.A., Price, D.P., Drake, L.L., Hansen, I.A., 2015. Substrate specificity and transport mechanism of amino-acid transceptor Slimfast from *Aedes aegypti*. Nat Commun. 9 (6), 8546. https://doi.org/10.1038/ncomms954.

Carpenter, V.K., Drake, L.L., Aguirre, S.E., Price, D.P., Rodriguez, S.D., Hansen, I.A., 2012. SLC7 amino acid transporters of the yellow fever mosquito *Aedes aegypti* and their role in fat body TOR signaling and reproduction. J. Insect Physiol. 58, 513–522. https://doi.org/10.1016/j.jinsphys.2012.01.005.

Clements, A.N., 1992. The Biology of Mosquitoes. London: Chapman and Hall Press.

Dillon, R.J., Lane, R.P., 1993. Blood meal digestion in the midgut of *Phlebotomus papatasi* and *Phlebotomus langeroni*. Med. Vet. Entomol. 7, 225–232. https://doi.org/10.1111/j.1365-2915.1993.tb00681.x.

Dillon, R.J., El-Kordy, E., 1997. Carbohydrate digestion in sandflies: alfa-glucosidase activity in the midgut of *Phlebotomus langeroni*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 116B, 35–40. https://doi.org/10.1016/S0305-0491(96)00195-2.

Evans, A.M., Aimanova, K.G., Gill, S.S., 2009. Characterization of a blood-meal-responsive proton-dependent amino acid transporter in the disease vector, *Aedes aegypti*. J. Exp. Biol. 212, 3263–3271. https://doi.org/10.1242/jeb.029553.

Fan, S.-J., Goberdhan, D.C.I., 2018. PATs and SNATs: amino acid sensors in disguise. Front Pharmacol. 9, 640. https://doi.org/10.3389/fphar.2018.00640.

Foltz, M., Boll, M., Raschka, L., Kottra, G., Daniel, H., 2004. A novel bifunctionality: PAT1 and PAT2 mediate electrogenic proton/amino acid and electroneutral proton/fatty acid symport. FASEB J. 18, 1758–1760. https://doi.org/10.1096/fj.03-1387fje.

Fredriksson, R., Nordström, K.J.V., Stephansson, O., Hägglund, M.G.A., Schiöth, H.B., 2008. The solute carrier (SLC) complement of the human genome: phylogenetic classification reveals four major families. FEBS Lett. 582, 3811–3816. https://doi.org/10.1016/j.febslet.2008.10.016.

Gontijo, N.F., Almeida-Silva, S., Williams, P., Mares-Guia, M.L., Melo, M.N., 1998. *Lutzomyia longipalpis*: pH in the gut, digestive glycosidases, and their relationship with *Leishmania* development. Exp. Parasitol. 90, 212–219. https://doi.org/10.1006/expr.1998.4336.

Hansen, I.A., Attardo, G.M., Park, J.-H., Peng, Q., Raikhel, A.S., 2004. Target of rapamycin-mediated amino acid signaling in mosquito anautogeny. PNAS 101, 10626–10631. https://doi.org/10.1073/pnas.0403460101.

Hansen, I.A., Attardo, G.M., Roy, S.G., Raikhel, A.S., 2005. Target of rapamycin-dependent activation of S6 kinase is a central step in the transduction of nutritional signals during egg development in a mosquito. J. Biol. Chem. 280, 20565–20572. https://doi.org/10.1074/jbc.M500712200.

Hansen, I.A., Boudko, D.Y., Shiao, S.H., Voronov, D.A., Meleshkevitch, E.A., Drake, L.L., Aguirre, S.E., Fox, J.M., Attardo, G.M., Raikhel, A.S., 2011. AaCAT1 of the yellow fever mosquito, *Aedes aegypti*: a novel histidine-specific amino acid transporter from the SLC7 family. J. Biol. Chem. 286, 10803–10813. https://doi.org/10.1074/jbc.M110.179739.

Hundal, H.S., Taylor, P.M., 2009. Amino acid transceptors: gate keepers of nutrient exchange and regulators of nutrient signaling. Am. J. Physiol. Endocrinol. Metab. 296, E603–E613. https://doi.org/10.1152/ajpendo.91002.2008.

Killick-Kendrick, R., Leaney, A.J., Ready, P.D., 1977. The establishment, maintenance and productivity of a laboratory colony of *Lutzomyia longipalpis* (Diptera: Psychodidae). J. Med. Entomol. 13, 429–440. https://doi.org/10.1093/jmedent/13.4-5.429.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Martin, M.M., Lindqvist, L., 1975. The pH dependence of fluorescein fluorescence. J. Lumin. 10, 381–390. https://doi.org/10.1016/0022-2313(75)90003-4.

Meleshkevitch, E.A., Assis-Nascimento, P., Popova, L.B., Miller, M.M., Kohn, A.B., Phung, E.N., Mandal, A., Harvey, W.R., Boudko, D.Y., 2006. Molecular characterization of the first aromatic nutrient transporter from the sodium neurotransmitter symporter family. J. Exp. Biol. 209, 3183–3198. https://doi.org/10.1242/jeb.02374.

Modi, G.B., 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. https://doi.org/10.1093/jmedent/20.5.568.

Molyneux, D.H., Killick-Kendrick, R., Ashford, R.W., 1975. Leishmania in phlebotomid sandflies. III. The ultrastructure of Leishmania mexicana amazonensis in the midgut and pharynx of Lutzomyia longipalpis. Proc. R Soc. Lond. B Biol. Sci. 190, 341–357. https://doi.org/10.1098/rspb.1975.0098.

Nepomuceno, D.B., Santos, V.C., Pereira, M.H., Araujo, R.N., Sant'anna, M.R.V., Gontijo, N.F., 2017. pH control in the midgut of Aedes aegypti under different nutritional conditions. J. Exp. Biol. 220, 3355–3362. https://doi.org/10.1242/jeb.158956.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.

Santos, V.C., Araujo, R.N., Machado, L.A.D., Pereira, M,H., 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. 21, 2792-2798. https://doi.org/10.1242/jeb.019836.

Santos, V.C., Nunes, C.A., Pereira, M.H., Gontijo, N.F., 2011. Mechanisms of pH control in the midgut of Lutzomyia longipalpis: roles for ingested molecules and hormones. J. Exp. Biol. 214, 1411–1418. https://doi.org/10.1242/jeb.051490.

Santos, V.C., Vale, V.F., Silva, S.M., Nascimento, A.A.S., Saab, N.A.A., Soares, R.P.P., Michalick, M.S.M., Araújo, R.N., Pereira, M.H., Fujiwara, R.T., Gontijo, N.F., 2014. Host modulation by a parasite: how Leishmania infantum modifies the intestinal environment of Lutzomyia longipalpis to favor its development. PLoS ONE 9 (11), e111241. https://doi.org/10.1371/journal.pone.0111241.

Soares, R.P., Turco, S.J., 2003. Lutzomyia longipalpis (Diptera: Psychodidae: Phlebotominae): a review. An Acad. Bras Cienc. 75 (4), 441. https://doi.org/10.1590/S0001-37652003000300005.

Taylor, P.M., 2014. Role of amino acid transporters in amino acid sensing. Am. J. Clin. Nutr. 99, 223S–230S. https://doi.org/10.3945/ajcn.113.070086.

Zuckerkandl, E., Pauling, L., 1965. Molecules as documents of evolutionary history. J. Theor. Biol. 8, 357–366. https://doi.org/10.1016/0022-5193(65)90083-4.

Wolfersberger, M.G., 2000. Amino acid transport in insects. Annu. Rev. Entomol. 45, 111–120. https://doi.org/10.1146/annurev.ento.45.1.111.