

22 **Abstract**

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24 The leishmaniases are caused by *Leishmania* parasites and transmitted through the
25 bites of phlebotomine sand flies. During parasite development inside the vector's
26 midgut, promastigotes move towards the stomodeal valve, a mechanism that is crucial
27 for transmission. It has been reported that the sugar meal acquired by sand flies during
28 feeding between bloodmeals is essential for the development and migration of parasites.
29 We demonstrated that the distribution of *Leishmania mexicana* parasites was affected
30 by the sugar meals obtained by the sand flies. Promastigote migration towards the cardia
31 region seems to be only partially based on the stimuli provided by sugar molecules. In
32 the absence of sugars, significant amounts of parasites developed in the hindgut. In
33 addition, sugar meals were important for the survival of sand flies, especially during
34 blood digestion, presumably supporting their energy requirements.

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38 **Keywords:** *Leishmania mexicana*, *Lutzomyia longipalpis*, sugar

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46 *Leishmania* parasites develop as extracellular forms (promastigotes) in the gut of
47 their sand fly vectors and as obligate intracellular forms (amastigotes) inside the
48 phagolysosomes of infected macrophages in the vertebrate host. The development of
49 *Leishmania* parasites inside the vector is complex and dynamic. Depending on the
50 *Leishmania* subgenus a different pattern of development can be observed inside the gut
51 of the vector, for *Leishmania mexicana* (subgenus *Leishmania*) parasites develop

52 exclusively in the midgut and foregut of their vectors, which is known as suprapylarian
53 development. ⁽¹⁾

54 After ingestion of an infective blood meal by the sand fly, macrophages containing
55 parasites release their amastigotes forms into the blood meal, and the change in pH
56 conditions triggers the differentiation of amastigotes into promastigotes ⁽²⁾, a motile and
57 replicative form. These parasites have different developmental stages inside the gut of
58 the vector. For infection establishment, two cycles of multiplication occur during
59 parasites development. The first cycle occurs with the multiplication of procyclic
60 promastigotes inside the peritrophic matrix, in the blood meal phase ⁽³⁾. After the
61 digestion of blood, parasites escape from the peritrophic matrix, attach to the midgut
62 epithelium and migrate to the anterior midgut region ⁽⁴⁾. The second cycle of
63 multiplication takes place in the sugar meal phase with the leptomonad promastigotes,
64 which differentiate in the non-multiplicative infective metacyclic promastigote forms. It
65 is hypothesized that the presence of sugar ingested by the female sand fly between
66 bloodmeals triggers the multiplication of leptomonad promastigotes ⁽³⁾.

67 Between blood meal feeds, sand flies take sugar-rich meals that are stored in the
68 crop ⁽⁵⁾. The sugar meal is then released in small quantities into the midgut. After blood
69 meal digestion, the sugar meal rich that can contain sucrose, raffinose, melezitose,
70 starch, and cellulose (besides other types of sugars) is a potential source of nutrition for
71 parasites developing inside the vector gut. It is believed that the ingestion of sugar by
72 the vector impacts the developing promastigote parasite population ⁽⁶⁻⁸⁾. It was
73 described for different *Leishmania* species that they secrete glycosidases, enzymes
74 specialized in the digestion of sugars, like alpha-glucosidase, sucrases, invertases,
75 alpha-amylases, and others ⁽⁸⁻¹¹⁾. For *L. mexicana*, both invertase and sucrase activity

76 were identified as secreted by promastigotes ^(7,10). In this respect, *L. mexicana* might use
77 sugar meals as an exogenous source of energy for its development.

78 In addition, sugar ingestion by females sand flies creates a sugar gradient along the
79 midgut, and it was reported that this gradient provides the stimulus for parasite
80 migration towards the stomodeal valve region (critical for efficient transmission) by
81 mechanisms of chemo- and osmotaxis ⁽¹²⁻¹⁵⁾. However, studies investigating the effects
82 of the sugar meal on parasite migration and development using an in vivo model need to
83 be performed.

84 In this work, we demonstrated that the distribution of *Leishmania mexicana* along
85 the gut of *Lutzomyia longipalpis* is reliant on sugar feeding by phlebotomines. In the
86 absence of sugar meals, although the parasites are capable of reaching the stomodeal
87 valve region, a significant population of parasites instead develop in the hindgut of the
88 insect. Also, although sugar feeding was not necessary for the complete development of
89 parasites, the survival of *Lu. longipalpis* was drastically affected by the absence of sugar
90 feeding, especially after blood-feeding. In this respect, we emphasize the importance of
91 sugar meals during the life cycle of both sand fly vectors and *Leishmania* parasites.

92 For this investigation insectary-reared *Lu. longipalpis* (Jacobina, Bahia, Brazil),
93 maintained at Lancaster University (United Kingdom), were used for experiments.
94 Insects were kept under standard laboratory as described in Moraes et al. ⁽¹⁶⁾. For
95 experiments, groups of recently emerged females (0 - 3 hours) were separated into small
96 cages, kept for three days with access to water only, followed by blood feeding or
97 infected blood feeding using a Hemotek apparatus (Discovery Workshops), with
98 chicken skin membranes held at 37 °C for 1 hour, and then maintained under different
99 conditions with access to water or 1.2 M sucrose.

100 In this study, *L. mexicana* (World Health Organization strain
101 MNYC/BZ/1962/M379) from an axenic culture of amastigote-like forms was used for
102 infections. Amastigote-like culture and sand fly infections were performed as described
103 by Moraes et al. ⁽¹⁶⁾. For infections, a concentration of 2×10^6 parasites/mL, estimated
104 with Neubauer chambers, was used. Briefly, after centrifugation at 2000 x g for 5 min,
105 the supernatant was removed, and parasites were mixed with sheep blood and offered to
106 3-day old females maintained with water (unfed). After blood feeding, unfed females
107 were discarded, and the fed ones were kept with water only or 1.2 M sucrose.

108 For estimation of *L. mexicana* infections, the whole gut of infected females was
109 dissected and analyzed under light microscopy at 3, 6 and 10 days after blood feeding to
110 check for establishment. Dissections were conducted in PBS on microscope slides using
111 needles. Dissected guts were transferred to polypropylene tubes containing 20 μ L PBS
112 and 2 % paraformaldehyde, used to immobilise parasites. After homogenization and
113 dilution, a 10 μ L sample was transferred to Neubauer chambers, and the total number of
114 parasites was determined. We also analyzed the number of metacyclic promastigotes on
115 in day six samples. The identification of metacyclic promastigotes followed the
116 characteristics described for the identification of *Leishmania* different developmental
117 stages ⁽¹⁷⁾. On the third and sixth days after the blood feeding, the number of parasites
118 was also estimated in the hindgut and midgut, separately, using the same procedure
119 described above.

120 We also analyzed the longevity of *Lu. longipalpis* under different conditions.
121 Mortality was evaluated, and dead insects were removed from cages daily. For each
122 biological replicate and condition tested 100 females were used. As specified above,
123 emerged females (0-3 hours) were separated, and six different feeding conditions were

124 monitored. The following groups were analyzed: unfed maintained with water, fed on
125 1.2 M sucrose (SF), blood-fed (infective meal or not) maintained with 1.2 M sucrose or
126 blood-fed (infective meal or not) maintained with water.

127 All statistical analysis on parasite infections was performed with GraphPad Prism
128 6.0 for Windows (San Diego, California, USA), and the D'Agostino-Pearson Omnibus
129 K2 normality test was used. The outliers were identified with the ROUT method, and Q
130 was established as 1 %. One-way ANOVA (multiple comparisons) followed by
131 Tukey's multiple comparison tests and significance was considered when $p < 0.05$. For
132 survival, results were analyzed using the Kaplan-Meier survival curve obtained with
133 GraphPad Prism 6.0 for Windows (San Diego, California, USA) and thus the average
134 survival time was determined in each condition. The log-rank Mantel-Cox test was used
135 to compare survival curves. Significance was considered when $p < 0.05$.

136 Our results demonstrated that the number of parasites present in the whole gut was
137 not affected by the presence of sucrose. Comparisons were performed three and six days
138 after infection (Fig 1). Due to high mortality, it was not possible to evaluate infections
139 at ten days in water-fed females. Furthermore, the number of parasites did not increase
140 following the days after infection for either water or sucrose fed females. After six days,
141 we also analyzed the number of *Leishmania* metacyclic forms in the midgut (including
142 stomodeal valve), and no significant difference was detected in the numbers when
143 comparing water fed to 1.2 M sucrose fed females, with 1200 ± 200 and 1400 ± 200
144 metacyclic promastigotes per midgut, respectively. So, we demonstrate that *L. mexicana*
145 can develop inside *Lu. longipalpis*, even in the absence of sugar feeding by the
146 phlebotomine host. There was no significant difference comparing the total number of
147 parasites inside the gut, or the number of metacyclic forms, in water or sugar-fed

148 females. Previous works discuss the importance of sugar feeding by phlebotomine sand
149 flies for parasite development ^(6-8,10). According to them, *Leishmania* promastigotes
150 depend on the diet of their phlebotomine host to sustain their growth. Sugars may also
151 prevent the egestion of *Leishmania* during defecation of blood meal remnants ⁽¹⁸⁾.

152 In our work, we demonstrated the multiplication and development of promastigote
153 and metacyclic forms in the guts of water-fed flies, although the infectivity of these
154 parasites in a second blood meal remains to be addressed. In addition, we do not know if
155 the absence of sugar can affect the appearance of different promastigotes forms during
156 development. The development of *L. mexicana* parasites into different promastigotes
157 forms was described as sugar dependent ⁽³⁾. Protocols for studying the effect of a second
158 blood meal in *Lu. longipalpis* under laboratory conditions have been recently developed
159 ⁽¹⁶⁾ and it has been recently demonstrated that the ingestion of a second non-infecting
160 blood meal by infected sand flies leads to enhanced disease transmission by amplifying
161 the number of parasites acquired in the infected blood meal. The process occurs by
162 dedifferentiation of the metacyclic promastigotes into replicative “retroleptomonad”
163 promastigote forms, which leads to increased infection ⁽¹⁹⁾. Although components
164 present in plasma were reported to trigger dedifferentiation, we cannot rule out the
165 hypothesis that sugar absence might also have an effect in the appearance of
166 promastigote retroleptomonad forms or affect the numbers of parasites in the case of a
167 second blood meal. So, we expect to extend these observations in the future.

168 Our data suggest that nutrients obtained from sugar meals are not strictly necessary
169 for parasite growth and differentiation, and the parasites are presumably obtaining
170 nutrients released from blood hydrolysis in the absence of a sugar meal. The nutrients
171 obtained from blood are likely to be especially necessary for the early phases of

172 development when the parasite is trapped inside the peritrophic matrix. Differently from
173 the results demonstrated in our work, in infections of *Lu. longipalpis* with *Leishmania*
174 *donovani*, a regular sugar meal was shown to enhance the number of parasites inside the
175 gut of the vector ⁽²⁰⁾, and also in *Lu. youngi* the efficiency of infection with *L.*
176 *amazonensis* was affected by the type of sugar used to feed the sand flies ⁽⁶⁾. It is
177 possible that, under our conditions, the presence or absence of sugar meals impacts the
178 development and survival of parasites only after more extended periods after the blood
179 feed, and further studies must confirm or reject this hypothesis.

180 In our work, although no differences were found in the total number of parasites
181 after different feeding regimes, we observed a difference in the pattern of parasite
182 distribution. In females fed with water after three and six days of infection, we found a
183 large number of parasites in the hindgut. In Fig 2, we present images obtained by light
184 microscopy demonstrating the presence of parasites in the hindgut of water-fed females
185 (Figs 2A and 2B), with many parasites in this region. The hindgut of these insects is
186 filled with parasites, but these do not seem to be attached to the cuticle (supplementary
187 video 1). In water-fed females, parasites were also present in the midgut and cardia
188 region (Fig 2C), while for sugar-fed females parasites are not distributed along the
189 hindgut (Fig 2D). Considering the migration of parasites to the hindgut, we evaluated
190 the number of parasites in this compartment in water and sucrose-fed females. The
191 percentage of infected females presenting parasites in the hindgut was larger for water-
192 maintained flies. After three days post-blood feeding 70 % of analyzed insects had
193 parasites in the hindgut compared with 20 % in sugar-maintained insects (Fig 3A). Six
194 days after blood-feeding almost 90 % of water-maintained females had parasites in the
195 hindgut (Fig 3A). For the midgut, the number of parasites (Fig 3B) was consistent with

196 the same pattern demonstrated in Fig 1, in both water and sugar-maintained insects a
197 massive number of parasites concentrated in this region. In contrast, although the
198 absolute numbers recorded are lower, the number of parasites in the hindgut of water-
199 fed females was significantly higher compared to sucrose-fed females (Fig 3C), even
200 though, the parasites number in the hindgut did not increase from 3 to 6 days. However,
201 the data obtained in these assays suggest that the number of parasites quantified in the
202 hindgut of water-fed flies, compared to what we can observe in the images (Fig 2B),
203 was underestimated, possibly due to the limitation of the technique of rupturing the gut
204 for separation of midgut and hindgut.

205 During the development of parasites inside the gut of the vector, the movement of
206 promastigotes to the anterior region of the sand fly midgut, with the accumulation of
207 metacyclic promastigotes in the stomodeal valve, is critical, causing a distension of the
208 valve and transmission to a mammal when a next blood-feeding occurs ^(2,17). Taxis is a
209 phenomenon where an organism responds to specific stimuli by movement. It was
210 proposed that during development the promastigotes could be attracted by the sugar
211 meals ingested by sand flies, then migrating to the anterior region of the midgut ⁽²¹⁾.
212 Some works described that *Leishmania* promastigotes undergo chemotaxis in a gradient
213 constituted of different sugars ^(13,15) and likewise by serum albumin, hemoglobin,
214 besides others ⁽¹⁵⁾. The movement is also due to the osmotic gradient generated by the
215 presence of sugars ⁽¹²⁾. For *L. amazonensis* it was demonstrated that the parasite was
216 able to respond both to chemotactic and osmotactic stimuli ⁽¹⁴⁾. In this respect, both
217 mechanisms of chemotaxis and osmotaxis play a role in the direction of parasites to the
218 stomodeal valve region. We demonstrated that parasites are more frequently found in
219 the hindgut (not attached) of water-fed compared to sugar-fed females. However, in

220 both conditions, a high number of parasites were also able to reach the stomodeal valve.
221 In this respect, the presence of sugar, creating an osmotic and chemical gradient, seems
222 to be important but not obligatory to direct the migration of parasites toward the cardia
223 region. In normal conditions where the sugar concentration is much higher than the
224 other components, it might function as the central stimulator for parasite migration.
225 However, in a situation where a large quantity of sugars is not present, the movement
226 towards the stomodeal valve might be explained by water flow or by the presence of
227 other components inside the vector gut that might also create an orientation stimulus for
228 parasite migration. The midgut of the vector is divided into specialized regions with a
229 variety of chemical and structural features that *Leishmania* parasites might exploit for
230 orientation. Some studies have demonstrated that chemotaxis in *Leishmania* could be
231 elicited by a wide range of compounds ^(13,15), and saliva components might also work as
232 toxic agents. It was proposed that the receptors involved in chemotaxis possess low
233 specificity and a wide range of affinity, the same receptor might be able to bind
234 structurally related molecules ⁽¹⁴⁾.

235 *Leishmania* parasites have been classified as suprapylarian, peripylarian or
236 hypopylarian, based on the region of their development along the gut of the sand fly
237 vector ⁽¹⁾. *Leishmania* species that exclusively develop in the gut regions anterior to the
238 pylorus are considered suprapylarian and belong to the subgenus *Leishmania*.
239 *Leishmania* species that also colonize the abdominal gut regions, around the pylorus, are
240 named peripylarian, and belong to the New World *Viannia* subgenus. *Leishmania*
241 species that develop mainly in the hindgut are named hypopylarian, and belong to the
242 subgenus *Sauroleishmania* and infect reptiles. Interestingly, our data suggest that the
243 distribution of *Leishmania* parasites along the gut of sand flies also depends on the

244 sugar meal of the vector, as in our conditions, *L. mexicana*, a suprapylarian parasite
245 from the subgenus *Leishmania*, shows considerable development in the hindgut in the
246 absence of sugars in the phlebotomine diet.

247 Finally, we examined the longevity of *Lu. longipalpis* under different feeding
248 conditions, and this demonstrated that the median survival was drastically reduced from
249 25 days to 5 days, for sucrose fed females compared to water fed (starving) insects (Figs
250 4A and 4B). The results also demonstrate that blood-feeding detrimentally affects the
251 survival of the sand flies, but not the presence of *L. mexicana* parasite, at least under
252 these conditions. In sugar-fed females, the mean survival was reduced from 25 to 7
253 days, almost a 70 % reduction, in blood-fed females (infected or not infected) compared
254 to the non-blood fed ones. For water-maintained females, the median survival was
255 reduced from 5 to 3 days, in blood-fed females (infected or not infected), compared to
256 the non-blood fed females.

257 Although the parasite does not seem to have an absolute requirement for sugar to
258 undergo development in our conditions, sugar is essential for phlebotomine survival.
259 Without sugar meals, the mortality of sand flies was drastically enhanced, especially
260 when females were also blood-fed. Sugar feeding appears to be vital to the metabolic
261 demands of phlebotomine sand flies. The glucose, for example, obtained from sugar
262 hydrolysis could be taken up by enterocytes, and converted to trehalose or stored as
263 glycogen to supply the energetic demands of insects, like flight. In a starving condition,
264 the reserves of glycogen and triglycerides are mobilized ^(22,23). During blood digestion,
265 nutrients as heme and amino acids are present in excess, and these molecules need to be
266 detoxified by disposal or converted to advantageous derivatives. The release of heme is
267 toxic because it potentiates oxygen-reactive species and can permeate membranes ⁽²⁴⁾.

268 Moreover, there is enhanced microbial growth after blood feeding that needs to be
269 controlled ⁽²⁵⁾. Briefly, we suggest that during the blood digestion, there is an energetic
270 demand to maintain the homeostasis in the organism. In a starving phlebotomine sand
271 fly, weakened by the lack of energy, the hazardous effects of molecules or pathogens
272 increased during blood digestion would be enhanced, and the pathways used for
273 detoxification of these compounds or control of pathogens might be restricted.

274 In this respect, according to the results reported here, the development and migration
275 of *L. mexicana* towards the stomodeal valve region, a mechanism essential for
276 transmission, is not strictly dependent on sugar feeding by the phlebotomine host, but
277 the sugar meals are necessary to supply the energy requirements for the survival of sand
278 flies, especially during blood digestion. The survival of sand flies for an extended time
279 is crucial for *Leishmania* transmission to the mammalian host since a minimum of two
280 blood feeds are necessary for this. Thus, even with the viable development of the
281 parasites in the absence of sugar, the transmission cycle might not occur, because the
282 sand flies do not survive long enough to perform two blood feeds.

283

284 **Author's Contributions**

285

286 Conception and design of the work: SGC, CSM, PB, RJD, and FG. Obtaining
287 experimental data: SGC and CSM. Data analysis: SGC and FG. Writing and revision of
288 the manuscript: SGC, CSM, PB, RJD, and FG. All authors read and approved the final
289 version.

290

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294

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363 **Legend of Figures**

364 **Fig 1:** *L. mexicana* parasite quantification in the gut of *Lu. longipalpis* females at
365 different days following the blood feeding. Recently emerged (0-3 h) females were
366 collected and maintained with water for 3 days before feeding with an infected blood
367 meal. After feeding, insects were maintained with sucrose 1.2 M (grey circles) or water
368 (black circles). Circles represent the number of parasites per individual gut. The results
369 are the mean \pm SEM of two independent experiments. One-way ANOVA was
370 performed followed by Tukey multiple comparison test. **ns:** non-significant difference.

371

372 **Fig 2:** Light microscopy images of infected *Lu. longipalpis* females 6 days after blood
373 feeding. Recently emerged (0-3 h) females were collected and maintained with water
374 for 3 days before feeding with an infected blood meal. After feeding, insects were
375 maintained with water (**A, B, C**) or sucrose 1.2 M (**D**). **A:** hindgut 20 X magnification
376 of infected water-maintained females. **B:** 40 X magnification of delineated section from
377 figure 2A. **C:** 40 X magnification of the cardia of infected water-maintained females. **D:**
378 hindgut 40 X magnification of infected sugar-maintained females. Note gut epithelium
379 (black arrowhead), *Leishmania* parasites (black arrows), Malpighian tubules (red
380 arrows) and cardia (red arrowhead).

381

382 **Fig 3:** *L. mexicana* parasite quantification in the midgut and hindgut of *Lu. longipalpis*
383 after 3 and 6 days following blood feeding. Recently emerged (0-3 h) females were
384 collected and maintained with water for 3 days before feeding with an infected blood
385 meal. After feeding, insects were maintained with sucrose 1.2 M or water. **A:** Infection
386 rate of hindgut of water or sugar-maintained females. The black background indicates
387 the percentage of positive samples containing parasites in the hindgut and the grey
388 background represents the percentage of negative samples **B:** Quantification of parasites
389 in the midgut of water or sugar-maintained females after 3 days (**black bars**) and 6 days
390 (**grey bars**). **C:** Quantification of parasites in the hindgut of water or sugar-maintained
391 females after 3 days (**black bars**) and 6 days (**grey bars**). One-way ANOVA was
392 performed, followed by Tukey multiple comparison tests. Different letters indicate
393 statistically significant differences in quantification, $p < 0.001$.

394

395 **Fig 4:** Survival curves of *Lu. longipalpis* females in different feeding conditions,
396 maintained under controlled humidity and temperature conditions. Recently emerged (0-
397 3 h) females were collected and maintained with water for 3 days before feeding with an
398 uninfected or infected blood meal. After blood feeding (infected or not), insects were
399 maintained with 1.2 M sucrose or water. Control groups were only fed with 1.2 M
400 sucrose or water (no blood meal). **A:** non-fed females maintained with water (**grey**
401 **line**), blood-fed females maintained with water post blood feeding (**green line**),
402 infective blood-fed females maintained with water after infection (**black line**). **B:** 1.2 M
403 sucrose fed females (**grey line**), blood-fed females maintained with 1.2 M sucrose post
404 blood feeding (**green line**), infective blood-fed females maintained with 1.2 M sucrose
405 after infection (**black line**). The results are representative of three independent
406 experiments. For each replicate, at least 100 females were used. The Log-rank Mantel-
407 Cox test was performed, and the survival curves were significantly different at
408 $p < 0.0001$.

409

410

Additional files

411 **S1 Video:** Video demonstrating the hindgut of water-fed *Lu. longipalpis* infected with
412 *L. mexicana* 6 days post infection.