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ORIGINAL PAPER

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Development of *Leishmania chagasi* (Kinetoplastida: Trypanosomatidae) in the second blood-meal of its vector *Lutzomyia longipalpis* (Diptera: Psychodidae)

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Abstract Light and electron microscopy investigations were carried out to compare the development of *Leishmania chagasi* in *Lutzomyia longipalpis* females that took a second blood-meal and others that took a single blood-meal. The establishment of the parasite in the foregut and the thoracic midgut of the vector was not severely affected by the intake of the second blood-meal. At 3 days after the intake of the second blood-meal, a rapid increase in the proportion of metacyclic promastigotes detected in the midgut and proboscis of the vector was noticed. No similar increase in the proportion of metacyclic promastigotes observed in the females that took a single blood-meal was noticeable. The results indicate a higher probability of transmission of the parasite to the vertebrate host at the third bite of the vector than at the second bite.

Introduction

Studies on the life cycle of *Leishmania* parasites in the sandfly have been reviewed by Killick-Kendrick (1979, 1987, 1990), Molyneux et al. (1986), and Molyneux and Killick-Kendrick (1987). It has been confirmed that the parasites display a sequence of morphological forms related to the age of infection and the parts of the sandfly gut in which development takes place.Most of these studies have dealt with the development of the parasite in the first gonotrophic cycles of sandflies.

In the present paper we compare for the first time the development of *L chagasi* in *Lutzomyia longipalpis* females that took a second blood-meal and others that took a single blood-meal as observed by light and electron microscopy. Although the females that took a second blood-meal had not laid their eggs before re-feeding, the results of such investigations would be useful in indicating the probability and efficiency of transmission of *Leishmania* parasites at the third and subsequent bites of their sandfly vectors.

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Materials and methods

The parasite used in this study was a Brazilian strain of *Leishmania chagasi* (MHOM/BR/76/150406) isolated from a patient with visceral leishmaniasis and then maintained since 1976 by intraperitoneal passage in hamsters (*Cricetus auratus*) and cotton rats (*Sigmodon hispidus*). The *Lutzomyia longipalpis* sandflies used originated from females collected from L'Aguila (Tolima, Colombia) and were colonized in the laboratory using the methods described by Modi and Tesh (1983).

A group of 3- to −4 day-old females of *L. longipalpis* ($n = 300$) were infected with *L. chagasi* by feeding them on a suspension of amastigotes, which had been isolated from a hamster spleen and mixed with defibrinated, inactivated rabbit blood to a final concentration of 10^5 amastigotes/m (i.e. 10 amastigotes per average sandfly-meal). The engorged females were kept in a separate cage, maintained at 26°C and 95% relative humidity under a 12-/12-h photoperiod, offered fresh saturated sucrose solution continuously and examined for *Leishmania* infection by light and electron microscopy at 4, 5, 6, 7, and 11 days after the infective feed. At each of these times, Giemsa stained smears of 6 females and transmission electron microscope (TEM) sections of another 6 females were examined and the proportions of different morphological forms were estimated. Details of the methods used to carry out the infective feeds and of the light and electron microscopy investigations have been described in a previous paper (Elnaiem et al. 1992a). The parasite forms were identified following the morphological definitions and descriptions given by Killick-Kendrick (1979, 1990) and Molyneux and Killick-Kendrick (1987). The proportions of different morphological forms of *Leishmania* promastigotes in the midgut of the vector were estimated by counting the number of individuals of each form and all promastigotes of the parasite in 5 microscopic fields (magnification = × 1,125) of the Giemsa smear of each of the 6 separate flies and then calculating the respective mean percentages ±SD of each form per fly. The differences between the proportions of promastigote forms observed at different times of infection were statistically analyzed using Student's *t*-test. Probability levels (*P*) of less than 0.05 were considered to indicate significant differences.

A second batch of *L. longipalpis*, consisting of 300 females fed on the amastigote-blood suspension and maintained as described above, were offered a second blood-meal on 5 anaesthetized, uninfected 1-month old hamsters at 4 days after the initial infective feed. The hamsters were kept for 1 year, weighed monthly and

then sacrificed and examined microscopically for the presence of *Leishmania* amastigotes in their spleens and livers. The re-fed females were counted, isolated in a separate cage, offered fresh saturated sucrose solution continuously and examined for *Leishmania* development at 12 h, 24 h, 3 days and 5 days after the second blood-meal as described above. In addition to the Giemsa-stained smears and the TEM sections, parasagittal (5 μm) histology sections of six 12 h and six 24 h re-fed females were prepared, using the methods described by Walters et al. (1987) and then examined for the location of the parasites and the second blood-meal.

Results

Parasite development in sandflies that took a single blood-meal

Figure 1 shows the proportions of the different parasite forms observed in the midgut, at 4–11 days after the infective feed. At 4 and 5 days after the infective feed, the most dominant parasite form detected in the midgut was the haptomonad, which represented mean (±SD) proportions of 58.15% ± 12.27% of the 173 promastigotes counted /6 flies at day 4 and 63.92% ± 10.32% of the 164 promastigotes counted at day 5. It was followed by the nectomonad which accounted for 39.60% ± 11.30% of the 173 promastigotes counted at day 4 and 27.62% ± 10.04% of the 164 promastigotes counted at day 5. At both days, the proportions of the haptomonad were significantly higher than those of the nectomonad ($P = 0.024$ at day 4 and 0.0001 at day 5). During this period, the metacyclic promastigote represented much lower proportions (2.25% ± 2.70% of the173 promastigotes counted at day 4 and 8.46% ± 2.24% of the 164 promastigotes counted at day 5, which were significantly lower than those of the nectomonad form ($P = 0.0005$ at day 4 and 0.006 at day 5). A similar order of dominance was observed at 7–11 days, with the haptomonad showing significantly higher proportions than the nectomonad ($P = 0.0009$ at day 7 and 0.0002 at day 11). During this period, however, the proportions of the metacyclic form were similar to those of the nectomonad ($P = 0.36$ at day 7 and 0.28 at day 11).

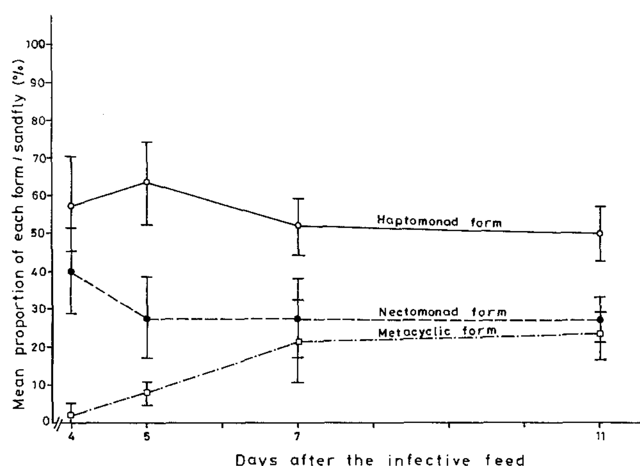

Fig. 1 Proportions of different morphological forms of *Leishmania chagasi* detected in the midgut of *Lutzomyia longipalpis* at 3–11 days after an infective feed; total number of parasites = total number/5 microscopic fields for each fly/6 flies = 173 parasites at 4 days, 164 parasites at 5 days, 187 parasites at 7 days and 91 parasites at 11 days

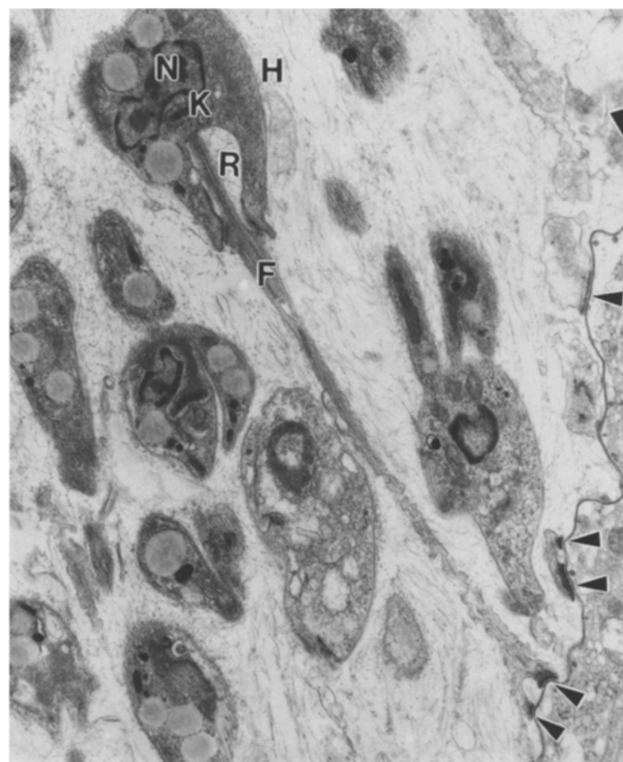

Fig. 2 Electron micrograph of the pharyngeal lumen of *L. longipalpis* obtained 7 days after a blood meal infected with *L. chagasi* amastigotes, showing a haptomonad promastigote (*H*) attaching its flagellum (*F*) to the cuticular intima of the gut wall. (*K* Kinetoplast. *N* nucleus; *R* flagellar reservoir, *arrowheads* hemidesmosomal points of attachment) × 8,800)

The TEM sections showed that the various parasite forms exhibited different distributions in different parts of the midgut. In the abdominal midgut, the most common form detected was the nectomonad, of which many individuals oriented themselves towards and inserted their flagella between the microvilli of the gut wall. In the thoracic midgut, close to the stomodaeal valve, the most dominant form observed was the haptomonad.

The parasites were seen in the foregut of most sandflies examined between 4 and 11 days after the infective feed. In the oesophageal region, the commonest form was the haptomonad, of which some individuals attached their flagella to the cuticular lining of the gut wall through hemidesmosomes. In the pharynx, paramastigotes and haptomonads occupied the gut lumen and attached their flagella to the cuticular lining of the gut wall (Fig. 2).

With the exception of flies sampled at 11 days after the infective feed, no parasite was seen in the cibarium. The forms seen in the cibarium of 11 day infected flies

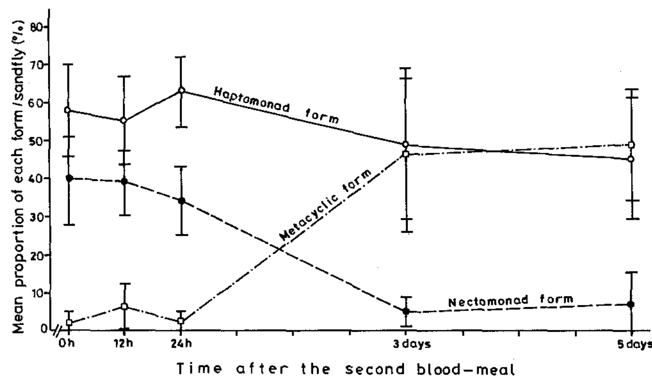

Fig. 3 Proportions of different morphological forms of *L. chagasi* detected in the midgut of *L. longipalpis* during the period of 0 h to 5 days after the second blood-meal; total number of parasites = total number/5 microscopic fields for each fly/6 flies = 173 parasites at 0 h, 124 parasites at 12 h, 324 parasites at 24 h, 532 parasites at 3 days and 484 parasites at 5 days

were the paramastigote and the metacyclic promastigote. In all samples, including the 11 day infected flies, no parasite was seen in the proboscis.

Transmission attempt with 4-day-infected sandflies

Of the 4-day-infected flies offered the second blood-meal ($n = 300$), 147 females engorged or took partial blood-meals on hamsters (49% re-feeding rate). At 11–12 months after the feed, the dissected spleens and livers of all five hamsters were found to be uninfected.

Development of *Leishmania* in the second blood-meal in the vector at 12–24 h after the second feed

The proportions of the different promastigote forms observed in the midgut during the period of 12 h to 5 days after the second blood-meal are shown in Fig. 3. At between 12 and 24 h after the second meal, the order of dominance of the different forms was similar to that observed between 4 and 5 days after the infective feed in the group of females that took a single blood-meal (Fig. 1).

At 12 h after the second blood-meal, TEM sections showed many haptomonads and paramastigotes in the pharynges and the oesophagi of the flies. At this time, the parasagittal sections showed that while the abdominal midguts were distended with blood, the oesophagi, stomodaeal valves and the thoracic midguts were packed with promastigotes.

At 24 h after the second feed, the midgut was still distended with the blood-meal, which appeared to be surrounded by a peritrophic membrane. The parasites were lying in the blood-meal near the peritrophic membrane. However, in TEM sections of two specimens, part of the blood-meal and many promastigotes were seen adjacent to the microvilli. In most flies the parasite density observed in the abdominal midgut was much lower than that seen in the thoracic region, which appeared to be free of the blood-meal. The only change observed in the morphology of the promastigotes was that their cytoplasm contained numerous lipid vacuoles.

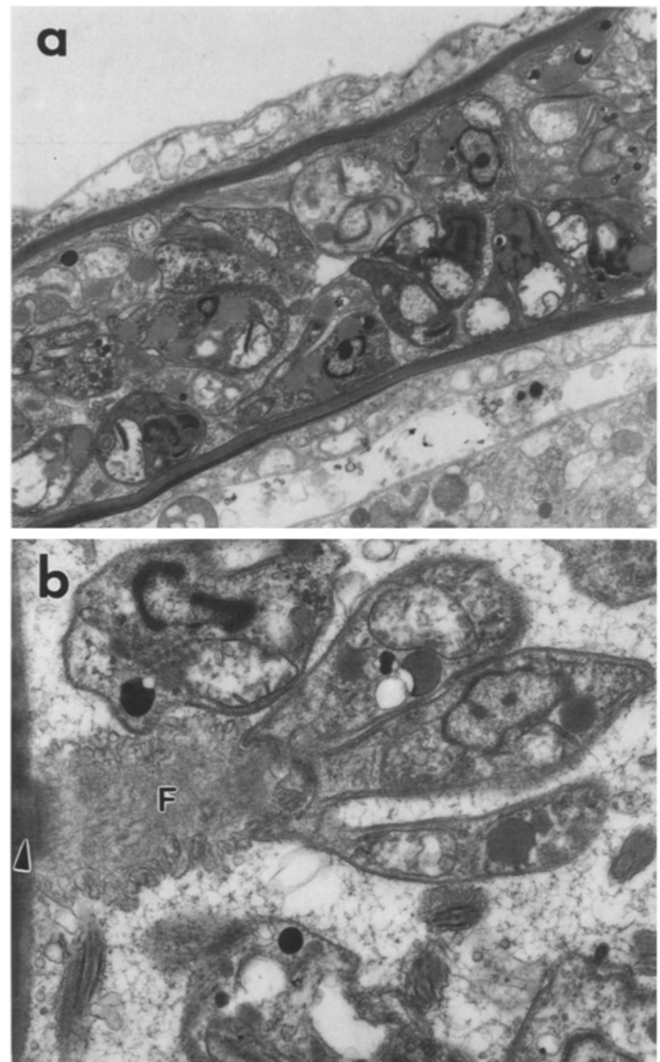

Fig. 4 a, b Electron micrographs of the pharynx of a *L. chagasi*-infected *L. longipalpis*, obtained 5 days after a second blood-meal, **a** showing a high degree of infection and **b** a flagellum (*F*) attached to the cuticular intima of the gut through a hemidesmosome (*arrowhead* Hemidesmosomal attachment) **a** × 6,700, **b** × 13,100)

Parasite development during the period of 72 h to 5 days after the second blood-meal

During this period, no peritrophic membrane was observed and the parasites noticed in the midgut were lying within traces of the digested meal. The metacyclic form showed a remarkable increase in its proportions, accounting for 46.57% ± 20.71% of the 532 parasites counted at 72 h and 48.13% ± 14.55% of the 484 para-

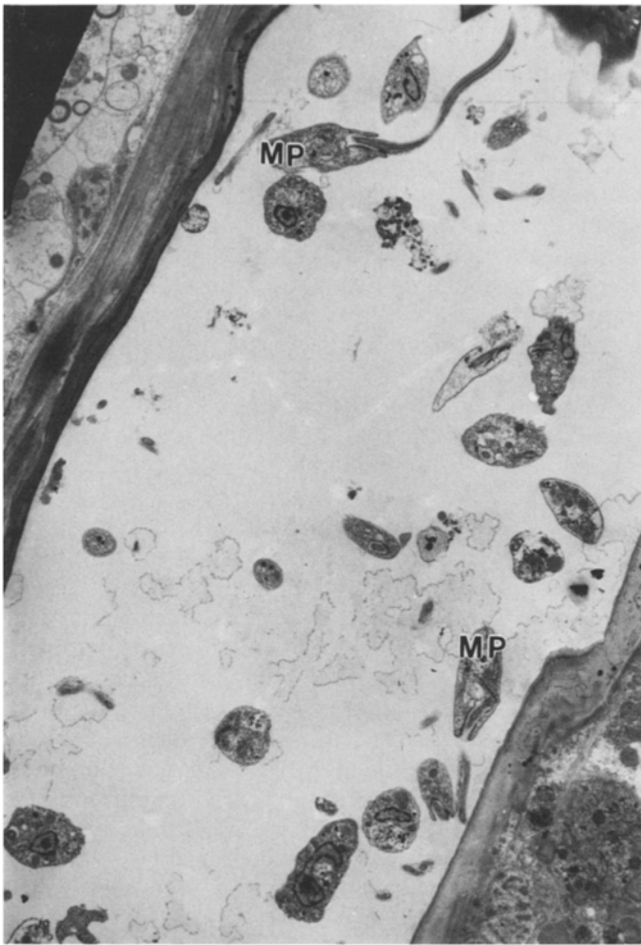

Fig. 5 Electron micrograph of the cibarium proboscis junction of *L. chagasi* -infected *L. longipalpis*, obtained 3 days after a second blood-meal showing sections through metacyclic promastigotes (*MP*) lying freely in the lumen, × 4,000

sites counted at day 5. These proportions were statistically similar ($P = 0.86$ at 72 h and 0.75 at 5 days) to those observed for the haptomonad, which represented $48.67\% \pm 19.31\%$ of the 532 parasites counted at 72 h and $45.5\% \pm 15.59\%$ of the 484 parasites counted at day 5. The nectomonad showed significantly lower proportions ($P = 0.0046$ at 72 h and 0.0005 at 5 days as compared with the metacyclic form), representing $4.76\% \pm 3.76\%$ of the 532 parasites counted at 72 h and $6.65\% \pm 8.24\%$ of the 484 parasites counted at 5 days.

In the midgut, stomodaeal valve and oesophagus, the parasites maintained distributions and orientations similar to those described for the 4- to −11-day-infected flies in the group of females that took a single blood-meal. More anteriorly, the pharyngeal lumen was full of haptomonads and paramastigotes (Fig. 4a), of which many individuals were attaching to the gut wall. The mode of attachment of the flagellum to the cuticular intima of the pharynx was similar to that observed before the second blood-meal (Fig. 4b). The metacyclic form was encountered in the cibarium-proboscis junction and proboscis of all flies dissected at 72 h after the second blood-meal (Fig. 5). These parasites were unattached measuring approximately 2.0 μm in diameter in exact cross section.

Discussion

Development of *Leishmania* in sandflies taking a single blood-meal

The results of the present investigations on infected females taking a single blood-meal generally confirm the previous findings on the life cycle of *Leishmania* in the sandfly (reviewed by Killick-Kendrick 1979, 1990; Molyneux and Killick-Kendrick 1987). The observations that the parasite invaded the foregut of the fly as early as 4 days after the infective feed support the findings of Lainson et al. (1977) and Walters et al. (1989), who considered such an early migration as a basic adaptation that favours the transmission by bite.

It has been suggested that the orientation of the nectomonad promastigotes to the midgut wall and the insertion of their flagella between the microvilli is advantageous for the parasite in anchoring itself against the peristaltic movements of the gut and in avoiding being evacuated with the remains of the blood-meal to the hindgut (Killick-Kendrick 1979). Similarly, it has been suggested that the attachment of the flagella of the haptomonads and paramastigotes to the foregut wall may be important in maintaining the parasites' position in a floating aqueous environment (Jefferies et al. 1986).

The absence of the parasite from the proboscis after 11 days following the infective feed confirms many previous observations (e.g. Lainson et al. 1977; Walters et al. 1989).

Intake of the second blood-meal by 4-day-infected flies and the lack of parasite transmission at this time

The reluctance of 51% of the females to engorge on the hamster may have been due to feeding difficulties caused by parasites blocking the pharynges, oesophagi and stomodaeal valves of the flies (Beach et al. 1985; Molyneux and Jefferies 1986), or to egg retention caused by the females not being offered suitable oviposition conditions. If egg retention is the reason, then the proportion of females that fully engorged on the hamster may represent the individuals that opt to take a second blood-meal before oviposition (Elnaiem et al. 1992b).

The unsuccessful transmission observed in the present study may indicate that the timing of the second blood-meal was too early for the deposition of infective forms or that the flies were denied some essential factors that may be found in their natural sugar-meals (Shortt 1945; Killick-Kendrick 1979; Young et al. 1980; Molyneux et al. 1986; Warburg and Schlein 1986; Moore et al. 1987; MacVicker et al. 1991).

Development of the parasite in the second blood-meal of the vector

The consequences of the intake of a second blood-meal by *Leishmania* infected sandflies on the development of the parasite have been investigated, on only one previous occasion. In *Phlebotomus argentipes* infected with *L. donovani*, Shortt (1928) found that the second blood-meal had no deleterious effect on the development of the parasite. On the basis of this finding, Killick-Kendrick (1979) predicted that the *Leishmania* parasites transmitted in nature by the gonotrophic discordant *P. papatasi* would have evolved in accord with multiple blood-meal behaviour. Similarly, the present observations suggest that the establishment of *L. chagasi* in the foregut and thoracic midgut of its vector is not severely affected by the intake of the second blood meal. It seems that the presence of the parasites in the periphery of the gut lumen, which would experience a lower speed of blood flow than the centre, coupled with their attachment to the gut wall, somehow enable them to escape being washed backwards by the second blood-meal. The possibility that the parasites may have actually been carried back to the midgut and then re-migrated to the foregut during the following 12 h seems remote as the second peritrophic membrane would reduce such forward movement. Furthermore, with the presence of the second blood-meal in the midgut, it is unlikely that quick forward re-migration for nutritional reasons occurred, since most of the nutritional requirements of the parasites were present posteriorly.

Midguts distended with the second blood-meals were seen in flies showing high degrees of infection in their thoracic midguts, stomodaeal valves, oesophagi, and pharynges before the feed. These observations, which contradict the views of Jefferies et al. (1986), support the hypothesis of Adler and Theodor (1935) that in sandflies infected with *Leishmania*, the strong dilator muscles activating the cibarium and pharynx of the sandfly would widen the lumen sufficiently for the blood to pass without difficulty.

In some flies, many promastigotes and part of the second blood-meal were found outside the second peritrophic membrane, close to the microvilli, indicating that the presence of the parasites may disturb the formation of this membrane. Since one of the functions attributed to the peritrophic membrane is the protection of the gut epithelium from fresh blood-meals (Gemetchu 1974; Blackburn et al., 1988), it is suggested that this phenomenon may have some effects on the physiology of the vector and the sandfly-*Leishmania* host-parasite relationship.

It is interesting that during the period of 24–72 h after the second blood-meal, a remarkable increase in the proportion of the metacyclic form occurred. This result contradicts the common observation on laboratory-cultured parasites that the addition of fresh media usually favours the production of the logarithmic phase form (corresponding to the long nectomonads), whereas depleted media are dominated by the stationary form (corresponding to the metacyclic promastigote); (Chang and Hendricks 1985).

The observed increase in the proportion of the metacyclic promastigote and its presence in the proboscis at 3 days after the second blood-meal indicates that the transmission of the parasites by the bite of the vector may become more efficient after this meal. This form, which is characterized by special adaptations such as resistance to the complement-fixation reactions and to the respiratory burst of macrophages, is considered to be the promastigote form infective to the vertebrate host (Sacks and Perkins 1984; reviewed by Sacks 1989; Killick-Kendrick 1990). It is therefore recommended that future studies should attempt to compare the efficiency of transmission before and after the intake of the second blood-meal.

It is unfortunate that adequate egg-laying conditions were not provided for the infected flies in this study and that the intake of the second blood-meal did not follow oviposition by the vector. Nevertheless, the results of the present investigation provide invaluable information on the fate of the parasite in females that take multiple blood-meals, a case that has been proven for some sandflies (e.g. *P. papatasi*) and may be quite natural for *Lutzonyia longipalpis* (Elnaiem et al. 1992b). Since the physiology of the blood digestion and its assimilation by the fly is associated with egg production, it is recommended that future investigations be carried out on the effect of post oviposition intake of the second blood-meal on the development of the parasites.

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