



## Article

# Experimental Susceptibility of *Nyssomyia antunesi* and *Lutzomyia longipalpis* (Psychodidae: Phlebotominae) to *Leishmania (Viannia) lainsoni* and *L. (V.) lindenbergi* (Trypanosomatidae: Leishmaniinae)

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**Abstract:** The present work assessed the experimental susceptibility of *Nyssomyia antunesi* and *Lutzomyia longipalpis* to *Leishmania (Viannia) lainsoni* and *L. (V.) lindenbergi*. A *L. (Leishmania) chagasi*–*Lu. longipalpis* combination was used as a susceptible control. Wild-caught *Ny. antunesi* and laboratory-bred *Lu. longipalpis* were membrane-fed on blood with a  $5 \times 10^6$ /mL log-phase promastigote culture suspension and dissected on days 2 and 8 post-blood meal (pbm) for analysis focused on the assessment of parasitoses, as well as placement and promastigote morphotyping. Survival curves were constructed. In all combinations, promastigotes were observed on day 8 pbm. For both *Leishmania* species, in *Lu. longipalpis*, the presence of parasites was observed up to the stomodeal valve, while in *Ny. antunesi*, the presence of parasites was observed up to the cardia. There were no significant differences in parasitosis between *L. (V.) lainsoni* and *L. (V.) lindenbergi* in either *Ny. antunesi* or *Lu. longipalpis*. Six morphological promastigote forms were distinguished in Giemsa-stained gut smears. The survival curves of all combinations decreased and were affected differently by several *Lu. longipalpis*–parasite combinations, as well with *Lu. longipalpis*–uninfected blood. These findings stress *Lu. longipalpis* as experimentally susceptible to *Leishmania* spp. and suggest the putative susceptibility of *Ny. antunesi* to *L. (V.) lainsoni* and *L. (V.) lindenbergi*.

**Keywords:** *Nyssomyia antunesi*; *Lutzomyia longipalpis*; *Leishmania lainsoni*; *Leishmania lindenbergi*; experimental infection



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## 1. Introduction

Phlebotomines (Diptera: Psychodidae) are medically important insects implicated in the transmission of several pathogens, mainly *Leishmania* protozoa (Kinetoplastea: Trypanosomatidae) [1], which are the agents of leishmaniases, a group of neglected tropical diseases affecting millions of people worldwide [2].

*Leishmania* parasites have a digenetic life cycle and infect a wide range of vertebrate reservoir hosts and invertebrate vectors, mainly phlebotomines (Diptera: Psychodidae) [3–5]. The development of *Leishmania* within the phlebotomines is a complex process: after the ingestion of infected blood, amastigotes (nonflagellated forms) differ from promastigotes (flagellated forms) inside the insect's gut [6], overcoming adverse conditions such as physico-chemical barriers and excretion flow [7–10]. Several subtypes of promastigote forms are recognized according to their morphology, including procyclic, haptomone, nectomonad, paramastigote and metacyclic [11], the latter of which are infective to vertebrate hosts [12,13].

A fundamental aspect in determining whether phlebotomines are incriminated by the transmission of *Leishmania* is the differentiation of infectious metacyclic forms [14]. In this same sense, the parasitosis and placement of late-stage infection constitute important parameters for evaluating the vector competence of a particular phlebotomine species for the developmental success of a given *Leishmania* sp. [15].

In the Brazilian Amazon, a particular tegumentary leishmaniasis (TL) transmission scenario occurs mainly because of the etiology of *L. (Leishmania) amazonensis*, *L. (Viannia) lainsoni* and *L. (V.) lindenbergi* in the forest fragments of Belém city [16]. In these TL foci, with respect to *L. (L.) amazonensis*, *Bichromomyia flaviscutellata* has well-established vector evidence [17]; for *L. (V.) lainsoni*, species of *Trichophoromyia*, particularly *Th. Ubiquitalis* and *Th. brachipyga*, have been shown to be involved in transmission [18]; ultimately, for *L. (V.) lindenbergi*, *Nyssomyia antunesi* has received attention due to its abundance, dominance, spatiotemporal convergence with human disease, blood feeding on human and potential reservoirs of *Leishmania* [19,20]. Despite the lack of evidence of true and species-specific identifiable *Leishmania* infection, the vector role of *Ny. antunesi* remains undefined, not advancing on suspect status.

However, studies on the interactions between *Leishmania* and its vectors are required to advance the understanding of the processes involved in parasite development and transmission [6]. Some parasite–vector combinations have been studied under laboratory conditions; however, the majority of binomials inferred by field evidence still require laboratory investigation. Therefore, the present study aimed to fill the gap in vector knowledge on the development of *L. (V.) lainsoni* and *L. (V.) lindenbergi* in *Ny. antunesi* and *Lu. longipalpis*. A *L. (L.) chagasi*–*Lu. longipalpis* combination was used as a ‘positive control’.

## 2. Materials and Methods

### 2.1. Parasites

The World Health Organization reference strains of three different *Leishmania* species maintained in the cryobank of the ‘Ralph Lainson’ Leishmaniasis Laboratory, Instituto Evandro Chagas (IEC), Belém, Brazil, were used: *L. (V.) lainsoni* (MHOM/BR/1981/M6426), *L. (V.) lindenbergi* (MHOM/BR/1996/M15729) and *L. (L.) chagasi* (MHOM/BR/1981/M6445). Promastigotes were cultured in Schneider’s insect medium (SIM) supplemented with 100 U/mL penicillin, 100 g/mL streptomycin and 10% heat-inactivated fetal bovine serum. To carry out the subsequent experimental infection of phlebotomines, low-passage parasites were used. Before being mixed with the blood, the samples were washed by centrifugation ( $2400 \times g$  for 5 min) and resuspended in a sterile container with a saline solution [5].

### 2.2. Phlebotomines

Wild-caught *Ny. antunesi* were obtained from the Bosque Rodrigues Alves-Jardim Botânico da Amazônia ( $1^{\circ}25'48''$  S;  $48^{\circ}27'25''$  W), an urban park of Belém city in which the phlebotomine fauna has already been surveyed [21]. Captures were performed with CDC light traps set 1.5 m above ground level ( $n = 4$ ) and 20 m above ground level ( $n = 2$ ), operating from 6:00 p.m. to 6:00 a.m., from May to August 2023. The phlebotomines were visually screened, aspirated from the primary cage in the field, and transported to the laboratory under  $80 \pm 10\%$  relative humidity and 10% glucose solution offered ad libitum [22,23]. The phlebotomines were immediately transferred to a secondary nylon cage. Congested, gravid or semigravid females were excluded from the experiments [24].

Laboratory-bred *Lu. longipalpis* from an established Amazonian closed colony (Abaetetuba F236) were used. For the tests, adult female specimens 5–9 days old were used [5,25] and supplied with 10% glucose solution ad libitum [26] up to 24 h before the assays [27,28].

### 2.3. Parasite–Vector Systems

Experimental infections were carried out according to the artificial blood feeding protocol proposed by Sánchez Uzcátegui et al. [24]. Briefly, the groups of both wild-caught *Ny. antunesi* and laboratory-bred *Lu. longipalpis* were artificially fed in  $30 \text{ cm}^3$  nylon cages

for 3 h through a sausage membrane installed in a circulator device containing previously heat-inactivated serum (56 °C for 1 h) human blood and  $5 \times 10^6$  /mL promastigotes [5] from log-phase cultures [29]. For *Lu. longipalpis*, females that had fed on uninfected blood were also assessed. The engorged females were confined to 200 mL flasks, and the recipients were lined with moistened filter paper and given a 10% sucrose diet until dissection [5]. Females from each species/experiment were divided into two groups for dissection: one group was dissected before the females defecated (early stage of infection) on day 2 post-blood meal (pbm), and the other group was dissected after defecation (late stage of infection) on day 8 pbm [5,25].

#### 2.4. Parasite Detection and Development

Phlebotomines were monitored daily to account for dead females, and survival curves were constructed. Dead females were dissected, and only *Leishmania*-positive females were counted [30]. The proportion survived (lx) was calculated according to Rabinovich [31]. Survivorship curves were obtained for different parasite–vector combinations and were compared by the log-rank test using BioEstat 5.3 software [32]. On days 2 and 8 pbm, the females were removed from the oviposition glasses using a Castro aspirator, and placed at 4 °C for thermal immobilization by cooling. The females were washed once with a 0.9% NaCl solution plus 5% neutral detergent, and twice with 0.9% NaCl for subsequent dissection. Phlebotomines were placed in a drop of phosphate-buffered saline (PBS) on a microscope slide, and the head was separated from the thorax before the intestine was extracted through the apex of the abdomen. The intestines were individually observed under an optical microscope and examined to determine the development of flagellates in the guts [33] following the taxonomic statement of Lainson and Shaw [34], and classified with the semiquantitative parasitosis scale described by Myskova et al. [15], whereby parasite loads were graded as absent (0 parasites per gut), weak (less than 100 parasites per gut), moderate (100–1000 parasites per gut) or heavy (more than 1000 parasites per gut). Promastigotes from the gut were Giemsa-stained and microscopically assessed to infer distinguishable evolutive forms based on morphologic/morphometric criteria modified by Ticha et al. [5], focusing on identifying metacyclic-like forms on day 8 pbm. All experiments were repeated at least three times for each parasite–vector combination. Parasitoses were compared using the G test with BioEstat 5.3 software [32]. In all statistical analysis,  $p \leq 0.05$  was considered to indicate a 95% confidence interval.

#### 2.5. Ethical Approval

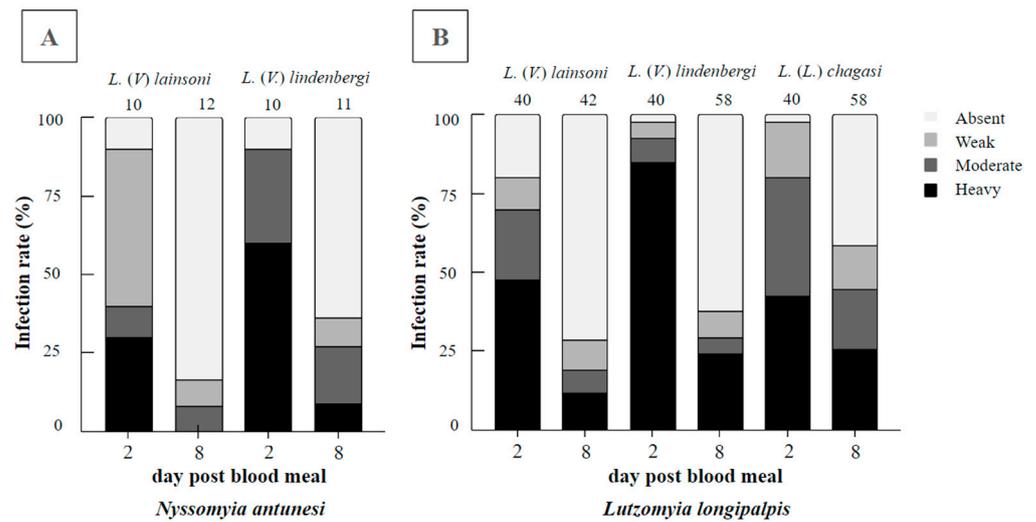
The capture and processing of invertebrate fauna (phlebotomines) were authorized by the ‘Sistema de Autorização e Informação em Biodiversidade’ under protocol no. 70142-2. Animals used for the blood feeding of phlebotomine colonies were maintained and handled at the Instituto Evandro Chagas animal facility, in accordance with institutional guidelines and Brazilian legislation (Federal Law no. 11.794, 8 October 2008). In vivo blood feeding standard operational procedures were approved by the Ethics Committee on Animal Use (CEUA/IEC), under certificate no. 30/2021.

### 3. Results

#### 3.1. Susceptibility of *Ny. antunesi* to *L. (V.) lainsoni* and *L. (V.) lindenbergi*

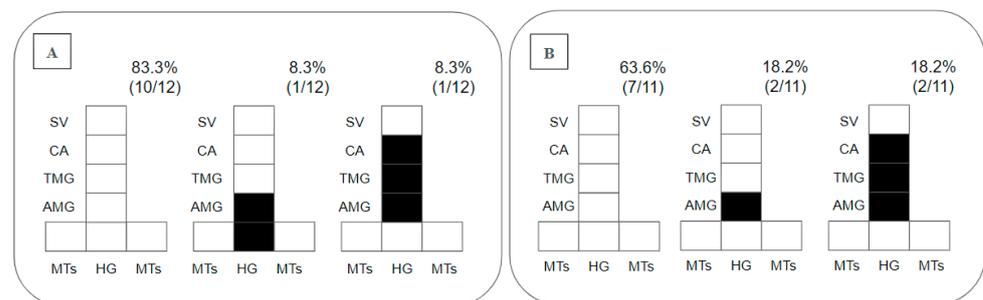
In total, 43 blood-fed *Ny. antunesi* females were dissected, 22 of which were exposed to *L. (V.) lainsoni* and 21 to *L. (V.) lindenbergi*. On day 2 pbm, the infection rates were 90% for both parasite–vector combinations, recording weak, moderate and heavy parasitoses, respectively; 50%, 10% and 30%, respectively, for *L. (V.) lainsoni*; and 0%, 30% and 60%, respectively, for *L. (V.) lindenbergi*. Promastigotes were limited to the endoperitrophic space within the ingested blood meal. On day 8 pbm, the infection rates were 16% and 36% for *L. (V.) lainsoni* and *L. (V.) lindenbergi*, respectively. For *L. (V.) lainsoni*, parasitoses were assessed as 8% weak and 8% moderate, whereas for *L. (V.) lindenbergi*, parasitoses were assessed as 9% weak, 18% moderate and 9% heavy (Figure 1A). The results from the assess-

ment of parasitoses between these *Leishmania* species were not significant (G test = 2.2148, df = 3, p = 0.5290).



**Figure 1.** Infection rates and parasitoses of *Leishmania* (*Viannia*) *lindenbergi* (MHOM/BR/1996/M15729) and *L. (V.) lainsoni* (MHOM/BR/1981/M6426) in (A) *Nyssomyia antunesi* and (B) *Lutzomyia longipalpis*. The well-known susceptible *L. (L.) chagasi*–*Lu. longipalpis* combination was also performed as the control. Intestines were dissected on days 2 and 8 post-blood meal (pbm). Parasitoses were classified into four categories: weak (less than 100 parasites per gut), moderate (100–1000 parasites per gut) and heavy (more than 1000 parasites per gut). The number of females evaluated can be found above the columns.

Regarding the placement of parasites in the gut, as promastigotes were limited to the endoperitrophic space on day 2 pbm, this parameter was only considered on day 8 pbm, when 83% of the *Ny. antunesi*–*L. (V.) lainsoni* combinations did not sustain infection; 8.3% presented peripylarian development with colonization in the hindgut (HG) and abdominal midgut (AMG); and 8.3% presented suprapylarian development, with colonization in the AMG, thoracic midgut (TMG) and cardia (CA) (Figure 2A). In *Ny. antunesi*–*L. (V.) lindenbergi* combinations, 63.6% of the strains did not sustain infection, and 36% presented suprapylarian development with colonization in the AMG, TMG and CA (Figure 2B).



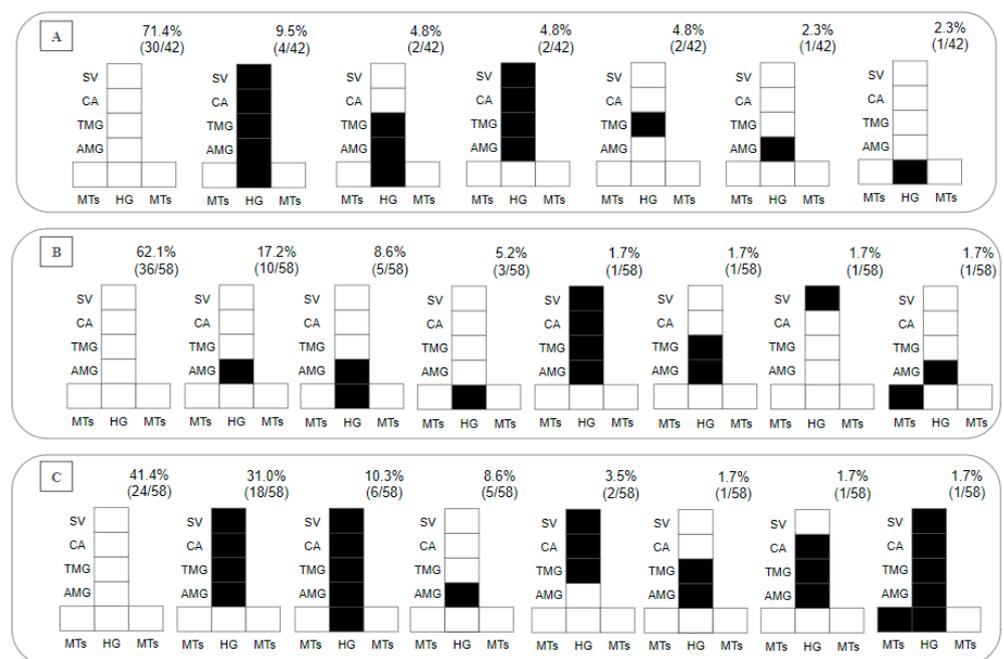
**Figure 2.** Diagrammatic placement of *Leishmania* spp. in the gut of *Nyssomyia antunesi* on day 8 post-blood meal (pbm). (A): *Leishmania* (*Viannia*) *lainsoni* (MHOM/BR/1981/M6426); (B): *L. (V.) lindenbergi* (MHOM/BR/1996/M15729). HG, hindgut; MTs, Malpighian tubules; AMG, abdominal midgut; TMG, thoracic midgut; CA, cardia; SV, stomodeal valve. Percent distribution of localization patterns among the infected females is shown in the top right of each diagram.

### 3.2. Susceptibility of *Lu. longipalpis* to *L. (V.) lainsoni* and *L. (V.) lindenbergi*

In total, 180 blood-fed *Lu. longipalpis* females were dissected, 82 were exposed to *L. (V.) lainsoni* and 98 were exposed to *L. (V.) lindenbergi*. On day 2 pbm, the infection rates

were 81% for *L. (V.) lainsoni* and 98% for *L. (V.) lindenbergi*, with promastigotes found in the endoperitrophic space only within the ingested blood meal. Parasitoses were assessed as 10% weak, 23% moderate and 48% heavy for *L. (V.) lainsoni*, whereas parasitosis were assessed as 5% weak, 8% moderate and 85% heavy for *L. (V.) lindenbergi*. On day 8 pbm, the infection rates were 29% for *L. (V.) lainsoni* and 38% for *L. (V.) lindenbergi*, with parasitoses assessed as 10% weak, 7% moderate and 12% heavy for *L. (V.) lainsoni*, whereas parasitosis were assessed as 9% weak, 5% moderate and 24% heavy for *L. (V.) lindenbergi* (Figure 1B). The results from the assessment of parasitoses between these *Leishmania* species were not significant (G test = 1.7129, df = 3,  $p = 0.6341$ ).

Regarding the presence of parasites in the gut on day 8 pbm, within 28.7% of the *Lu. longipalpis*–*L. (V.) lainsoni* positive combinations, 2.4% presented with hypopylarian, 14.3% with peripylarian and 12% with suprapylarian development. The peripylarian patterns included colonization in the HG, AMG, TMG, CA and stomodeal valve (SV) (9.5%), and in the HG, AMG and TMG (4.8%). The suprapylarian patterns included colonization in the AMG, TMG, CA and SV (4.8%), and in the TMG (4.8%) and AMG (2.3%) (Figure 3A). Within 37.8% of the *Lu. longipalpis*–*L. (V.) lindenbergi* positive combinations, hypopylarian (5.2%), peripylarian (10.3%) and suprapylarian (22.3%) development was observed. The hypopylarian pattern included colonization in the HG (5.2%); peripylarian comprised colonization in the HG and AMG (8.6%), and MTs and AMG (1.7%); and more frequent suprapylarian colonization was in the AMG (17.2%), while other infection patterns did not exceed 2% (Figure 3B).



**Figure 3.** Diagrammatic placement of (A) *Leishmania (Viannia) lainsoni* (MHOM/BR/1981/M6426), (B) *L. (V.) lindenbergi* (MHOM/BR/1996/M15729) and (C) *L. (L.) chagasi* (MHOM/BR/1981/M6445) in the gut of *Lu. longipalpis* on the 8th day post-blood meal (pbm). HG, hindgut; MTs, Malpighian tubules; AMG, abdominal midgut; TMG, thoracic midgut; CA, cardia; SV, stomodeal valve. In the upper right part of each diagram is the percentage distribution obtained for each *Leishmania* species within the intestine of *Lu. longipalpis*.

### 3.3. Susceptibility of *Lu. longipalpis* to *L. (L.) chagasi* (Control Experiment)

In total, 98 *Lu. longipalpis* exposed to *L. (L.) chagasi* were dissected. On day 2 pbm, the infection rate was 99%, recording 18% weak, 38% moderate and 43% heavy parasitoses. On day 8 pbm, the infection rate was 59%, recording 14% weak, 19% moderate and 26% heavy parasitoses. Peripylarian (12.1%) and suprapylarian (44.8%) development was observed.

Peripylarian pattern comprised colonization in the HG, AMG, TMG, CA and SV (10.3%); suprapylarian comprised colonization in the AMG, TMG, CA and SV (31%), exclusively in the AMG (8.6%), and TMG, CA and SV (3.5%); and other infection patterns did not exceed 2% (Figure 3C).

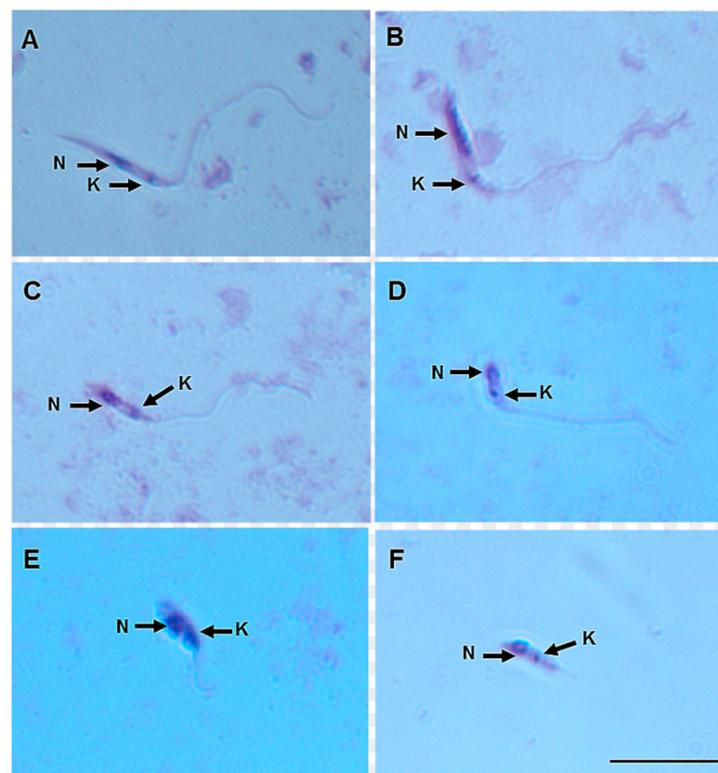
On the other hand, the results of the evaluation of parasites among the *Leishmania* species were significant on day 8 pbm (Table 1).

**Table 1.** Summary statistics for the comparison of parasitosis on day 8 post-blood meal in different parasite–vector combinations. Significant differences are highlighted in bold.

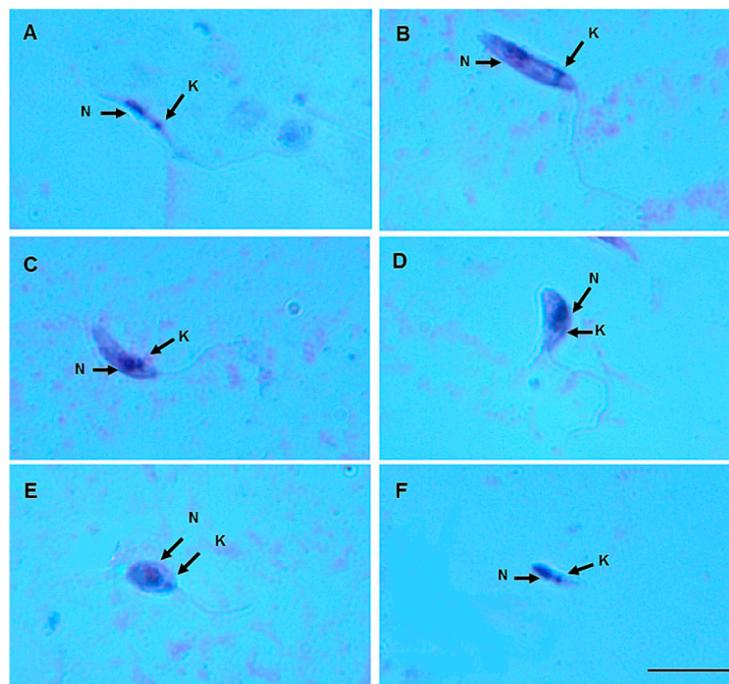
Vector	Parasite	n	Statistics
<i>Ny. antunesi</i>	<i>L. (V.) lainsoni</i>	12	$G \text{ test} = 2.2148, df = 3, p = 0.5290$
	<i>L. (V.) lindenbergi</i>	11	
<i>Lu. longipalpis</i>	<i>L. (V.) lainsoni</i>	42	$G \text{ test} = 1.7129, df = 3, p = 0.6341$
	<i>L. (V.) lindenbergi</i>	58	
<i>Lu. longipalpis</i>	<i>L. (V.) lindenbergi</i>	58	$G \text{ test} = 8.636, df = 3, p = \mathbf{0.0345}$
	<i>L. (L.) chagasi</i>	58	
<i>Lu. longipalpis</i>	<i>L. (V.) lainsoni</i>	42	$G \text{ test} = 8.0092, df = 3, p = \mathbf{0.0458}$
	<i>L. (L.) chagasi</i>	58	

#### 3.4. Morphology of *L. (V.) lainsoni* and *L. (V.) lindenbergi* Promastigotes in *Ny. antunesi*

On day 8 pbm, six promastigote morphotypes of *L. (V.) lindenbergi* (Figure 4) and *L. (V.) lainsoni* (Figure 5) were observed in the gut of *Ny. antunesi*: elongated nectomonad, short nectomonad, metacyclic promastigote, rounded metacyclic promastigote, rounded paramastigote and haptomonad.



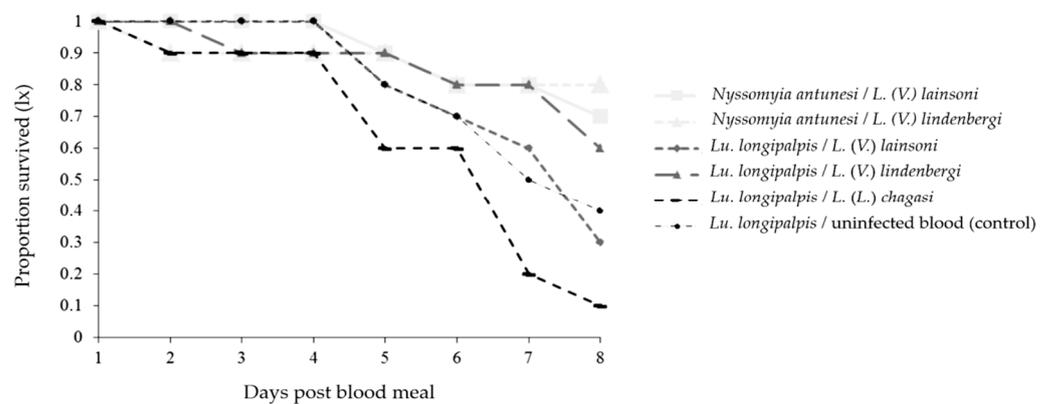
**Figure 4.** *Leishmania (Viannia) lindenbergi* (MHOM/BR/96/M15729) morphological form development in the gut of *Nyssomyia antunesi*: (A) elongated nectomonad; (B) short nectomonad; (C) metacyclic promastigote; (D) rounded metacyclic promastigote; (E) rounded paramastigote; (F) haptomonad. N, nucleus; K, kinetoplast (stained by Giemsa). Bar = 10  $\mu\text{m}$ .



**Figure 5.** *Leishmania (Viannia) lainsoni* (MHOM/BR/1981/M6426) morphological form development in the gut of *Nyssomyia antunesi*: (A) elongated nectomonad; (B) short nectomonad; (C) metacyclic promastigote; (D) rounded metacyclic promastigote; (E) rounded paramastigote; (F) haptomonad. N, nucleus; K, kinetoplast (stained by Giemsa). Bar = 10  $\mu$ m.

Survival Curves

The survival of both phlebotomine species decreased up to day 8 pbm for all combinations (Figure 6). The survival of *Lu. longipalpis* was affected differently in some *Lu. longipalpis* combinations, and the survival was higher when the phlebotomine species were infected with *L. (V.) lindenbergi* than when they were infected with *L. (V.) lainsoni* ( $p < 0.0001$ ) or *L. (L.) chagasi* ( $p < 0.0001$ ). Moreover, the survival was higher when the phlebotomine species were infected with *L. (V.) lainsoni* compared with *L. (L.) chagasi* ( $p < 0.0001$ ); and when they were infected with uninfected blood compared with those infected with *L. (V.) lindenbergi* ( $p = 0.0246$ ), or *L. (L.) chagasi* ( $p < 0.0001$ ) (Table 2).



**Figure 6.** Survival curves of *Nyssomyia antunesi* and *Lutzomyia longipalpis* blood-fed on a *Leishmania* spp. suspension (or not) up to day 8 post-blood meal.

**Table 2.** Log-rank test significance on the comparison of survival curves obtained for the different parasite–vector combinations up to day 8 post-blood meal. Significant differences are highlighted in bold.

Vector	Parasite	<i>n</i>	Log-Rank Test Significance
<i>Ny. antunesi</i>	<i>L. (V.) lainsoni</i>	16	$p = 0.6014$
	<i>L. (V.) lindenbergi</i>	10	
<i>Lu. longipalpis</i>	<i>L. (V.) lainsoni</i>	159	<b><math>p &lt; 0.0001</math></b>
	<i>L. (V.) lindenbergi</i>	142	
<i>Lu. longipalpis</i>	<i>L. (V.) lindenbergi</i>	142	<b><math>p &lt; 0.0001</math></b>
	<i>L. (L.) chagasi</i>	94	
<i>Lu. longipalpis</i>	<i>L. (V.) lainsoni</i>	159	<b><math>p &lt; 0.0001</math></b>
	<i>L. (L.) chagasi</i>	94	
<i>Lu. longipalpis</i>	Uninfected blood	78	<b><math>p = 0.0246</math></b>
	<i>L. (V.) lindenbergi</i>	142	
<i>Lu. longipalpis</i>	Uninfected blood	78	$p = 0.0954$
	<i>L. (V.) lainsoni</i>	159	
<i>Lu. longipalpis</i>	Uninfected blood	78	<b><math>p &lt; 0.0001</math></b>
	<i>L. (L.) chagasi</i>	94	

#### 4. Discussion

Several parasite–vector combinations were studied under laboratory conditions to evaluate interaction patterns, including the ability of vectors to support the late-stage development of parasites, suggesting the well-recognized classification of restrictive and permissive vectors. In the former category, phlebotomines present a remarkable specificity for a single (or some closely related) *Leishmania* species; while in the latter, phlebotomines allow for the development of a broad range of apart-related *Leishmania* species [35,36]. In this sense, the present study assessed *Ny. antunesi* and *Lu. longipalpis* in the development of medically important parasites in the Amazon biome, *L. (V.) lainsoni* and *L. (V.) lindenbergi*, until day 8 pbm, when late-stage promastigote forms were supposed to colonize the foregut, thus providing advanced inferences on their susceptibility.

After exhaustive attempts, the unsuccessful colonization of *Ny. antunesi* led the researchers to challenge wild-caught specimens. Although the unknown life status of these specimens and an apparently low number of assessments may compromise experimental reproducibility, it is believed that the field background brought about by nature adds pivotal elements for genuine parasite–vector interactions. *Nyssomyia antunesi* has been recognized as a suspected vector of *L. (V.) lindenbergi* based on some eco-epidemiological evidence [19–21], although no natural infection has been ascribed to this parasite. The present findings demonstrate that *Ny. antunesi* can develop at least a small population of late-stage promastigotes of both *L. (V.) lainsoni* and *L. (V.) lindenbergi* (a taxonomically distinct species) with no difference in parasitosis on day 8 pbm, suggesting that this species could be further investigated as a possible permissive vector, although it has never been found to be naturally infected by *L. (V.) lainsoni* in wild-caught specimens that have been examined. Interestingly, the present findings support early microscopic and current molecular-based evidence, which suggests that *Ny. antunesi* can harbor *Trypanosoma* sp. [37,38], *Porcisia* sp. [39], and a wide range of *Leishmania* spp. [40–49]. Most of these detections do not provide evidence of late-stage promastigote forms, which are insufficient to characterize *Ny. antunesi* as a true vector. Other supporting information for the present results is related to O-glycosylated proteins with N-acetylgalactosamine (GalNAc) epitopes, which are likely reported exclusively for permissive species [35], as has been suggested to be present in the midgut epithelial cells of *Ny. antunesi* [50].

On day 8 pbm, *L. (V.) lainsoni* and *L. (V.) lindenbergi* were observed up to the cardia of *Ny. antunesi*, probably because these *Leishmania* species need more time to advance to the stomodeal valve under laboratory conditions, as has been suggested for the binomial *Phlebotomus arabicus*–*L. (L.) infantum* [15]. On several occasions, it was noted that *Ny. antunesi* only partially fed under experimental conditions (Sánchez-Uzcátegui, personal

observation), which could possibly be important information from an epidemiological point of view. In this sense, multiple bloodmeals during a single gonadotrophic cycle have been reported for *Lu. longipalpis* [51], which has a potential impact on survival and *Leishmania* transmission, as suggested by Killick-Kendrick [52], for *Ph. papatasi* and *L. major*. Moreover, this characteristic would improve vector competence since the development of a successful infection in wild phlebotomines is a gradual process that depends on the parasite's action, which is amplified and enhanced by the ingestion of multiple blood meals [53]. Thus, all these facts add weight to the hypothesis that *Ny. antunesi* is an important vector from a medical point of view, but other criteria still need to be evaluated.

When evaluating the experimental infection of *Lu. longipalpis* with *L. (V.) lainsoni* and *L. (V.) lindenbergi*, it has been demonstrated that both parasite species can develop up to day 8 pbm with no difference in parasitosis between these combinations. Preliminary experimental infections have already been performed and the descriptions of these experiments with the two *Leishmania* species have focused on determining the developmental pattern for taxonomic purpose [19,38], thus not extending to the observation of late-stage promastigote forms. *Lutzomyia longipalpis* is well known as the major natural vector of *L. (L.) chagasi* [54], which is laboratory-supported as a permissive vector and competent for experimentally transmitting *L. (V.) braziliensis* [55], *L. (L.) chagasi* [56], *L. (L.) mexicana* [57], *L. (L.) major* [58,59] and *L. (L.) amazonensis* [60].

On day 8 pbm, parasitosis in *Lu. longipalpis* was higher in *L. (L.) chagasi* than in *L. (V.) lainsoni* and *L. (V.) lindenbergi*, reinforcing the status of the ancient and well-established *L. (L.) chagasi*–*Lu. longipalpis* natural binomial [54,61], herein regarded as the control experiment. Naturally, this combination [6,56,62–64] is overexploited due to its medical importance, as well as the manageable laboratory adaptation of *Lu. longipalpis* and consequent successful establishment of colonies [65], with effective rates of artificial blood feeding [22,24,66]. In addition, the results from developmental studies of *L. (L.) chagasi* in other phlebotomine species were also verified [67–69].

As expected, the predominant gut development reported for the studied *Leishmania* species (i.e., peripylarian for *L. (V.) lainsoni* and *L. (V.) lindenbergi*, and suprapylarian for *L. (L.) chagasi*) is in agreement with the taxonomic positions of these species originally described by Lainson and Shaw [34]. Few specimens with hindgut development were recorded for all combinations, which was exclusively attributed to heavy parasite loads throughout the phlebotomine gut. On that gut site, only adhered promastigotes were considered, avoiding artifactual observation due to back-wash [70]. In contrast, in the midgut, free-living promastigotes were considered. Killick-Kendrick [71] has shown striking features separating metacyclic forms from others in the phlebotomine gut, including a lack of attachment to the epithelium, high motility, a small body size and the presence of a long free flagellum.

The survival curves significantly decreased for all combinations, but were differentially affected in *Lu. longipalpis*–parasite combinations and uninfected blood. The reduced longevity of experimentally *Leishmania*-infected phlebotomines was documented [30,72,73], without evidence of a strain-specific impact [30]. Moreover, the results reported herein support the classical hypothesis that successful transmission in nature also depends on equilibrate parasitosis, which is sufficient for the inoculum but within the limits of vector tolerance, preserving its longevity [74].

## 5. Conclusions

In summary, wild-caught *Ny. antunesi* and laboratory-bred *Lu. longipalpis* have been suggested to be experimentally susceptible to *L. (V.) lainsoni* and *L. (V.) lindenbergi*, which results in the development of at least a small population of late-stage parasites up to day 8 pbm in the cardia or stomodeal valve. The putative permissiveness of *Ny. antunesi* has not been discarded, however, still requiring further assessment. The unsuccessful establishment of life cycles with these parasite–vector combinations in nature may result from non-negligible ecological field-driven elements. Indeed, the putative susceptibility

of phlebotomines suggested herein is worthy of important epidemiological consequences because it enables a successful adaptation of *Leishmania*. A successful colonization of *Ny. antunesi* could provide a considerable number of specimens that allow for an in vivo and in vitro assessment of *Leishmania*–phlebotomine interactions, and thus definitively determine its permissiveness and vector competence status.

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**Data Availability Statement:** All data supporting the conclusions are included within the article. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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