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Impact of clinically acquired miltefosine resistance by *Leishmania infantum* on mouse and sand fly infection

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ABSTRACT

Objectives: This study evaluated the implications of clinically acquired miltefosine resistance (MIL-R) by assessing virulence in mice and sand flies to reveal the potential of MIL-R strains to circulate.
Methods: Experimental infections with the MIL-R clinical *Leishmania infantum* isolate MHOM/FR/2005/LEM5159, having a defect in the LiROS3 subunit of the MIL-transporter, and its syngeneic experimentally reconstituted MIL-S counterpart (LEM5159^{LiROS3}) were performed in BALB/c mice and *Lutzomyia longipalpis* and *Phlebotomus perniciosus* sand flies. In mice, the amastigote burdens in liver and spleen were compared microscopically using Giemsa smears and by bioluminescent imaging. During the sand fly infections, the percentage of infected flies, parasite load, colonization of the stomodeal valve and metacyclogenesis were evaluated. The stability of the MIL-R phenotype after sand fly and mouse passage was determined as well.
Results: The fitness of the MIL-R strain differed between the mouse and sand fly infection model. In mice, a clear fitness loss was observed compared to the *LiROS3*-reconstituted susceptible strain. This defect could be rescued by episomal reconstitution with a wildtype *LiROS3* copy. However, this fitness loss was not apparent in the sand fly vector, resulting in metacyclogenesis and efficient colonization of the stomodeal valve. Resistance was stable after passage in both sand fly and mouse.
Conclusion: The natural MIL-R strain is significantly hampered in its ability to multiply and cause a typical visceral infection pattern in BALB/c mice. However, this LiROS3-deficient strain efficiently produced mature infections and metacyclic promastigotes in the sand fly vector highlighting the transmission potential of this particular MIL-R clinical *Leishmania* strain.

1. Introduction

Leishmania spp. parasites are transmitted by phlebotomine sand flies in (sub)tropical regions and cause a complex of diseases called "leishmaniases" (Ponte-Sucre et al., 2017). Globally distributed, leishmaniases are poverty-related neglected tropical diseases with visceral leishmaniasis (VL) as the most severe clinical form affecting internal organs and with fatality rates up to 95% if left untreated (Alvar et al., 2012; Ibarra-Meneses et al., 2020). Miltefosine (MIL) is currently the only oral drug available for VL treatment (Palić et al., 2019). Due to the decreasing and disappointing efficacy of monotherapy with various antileishmanial drugs, including MIL, implementation of combination therapies is now warranted (Palić et al., 2019). Indeed, a decreased efficacy of MIL is found in the Indian sub-continent and the South American region with increasing treatment failure rates (Brambilla Carnielli Trindade et al., 2019; Rijal et al., 2013) mediated by parasite-, drug- and host-related factors (Barrett et al., 2019; Ponte-Sucre et al., 2017). Regarding the parasite-related factors, resistance in the Indian and Mediterranean region (Cojean et al., 2012; Hendrickx et al., 2014; Srivastava et al., 2017) is associated with mutations in the *MIL-transporter* (*MT*) gene (Cojean et al., 2012; Mondelaers et al., 2016; Perez-Victoria et al., 2006b), while the *L. infantum* strains isolated from Brazilian treatment failure cases often lack the MIL-sensitivity locus (MSL) (Brambilla Carnielli Trindade et al., 2019). The impact of drug resistance on parasite fitness is likely not only drug- and stage-dependent, but also species-dependent (Hendrickx et al., 2016, Hendrickx

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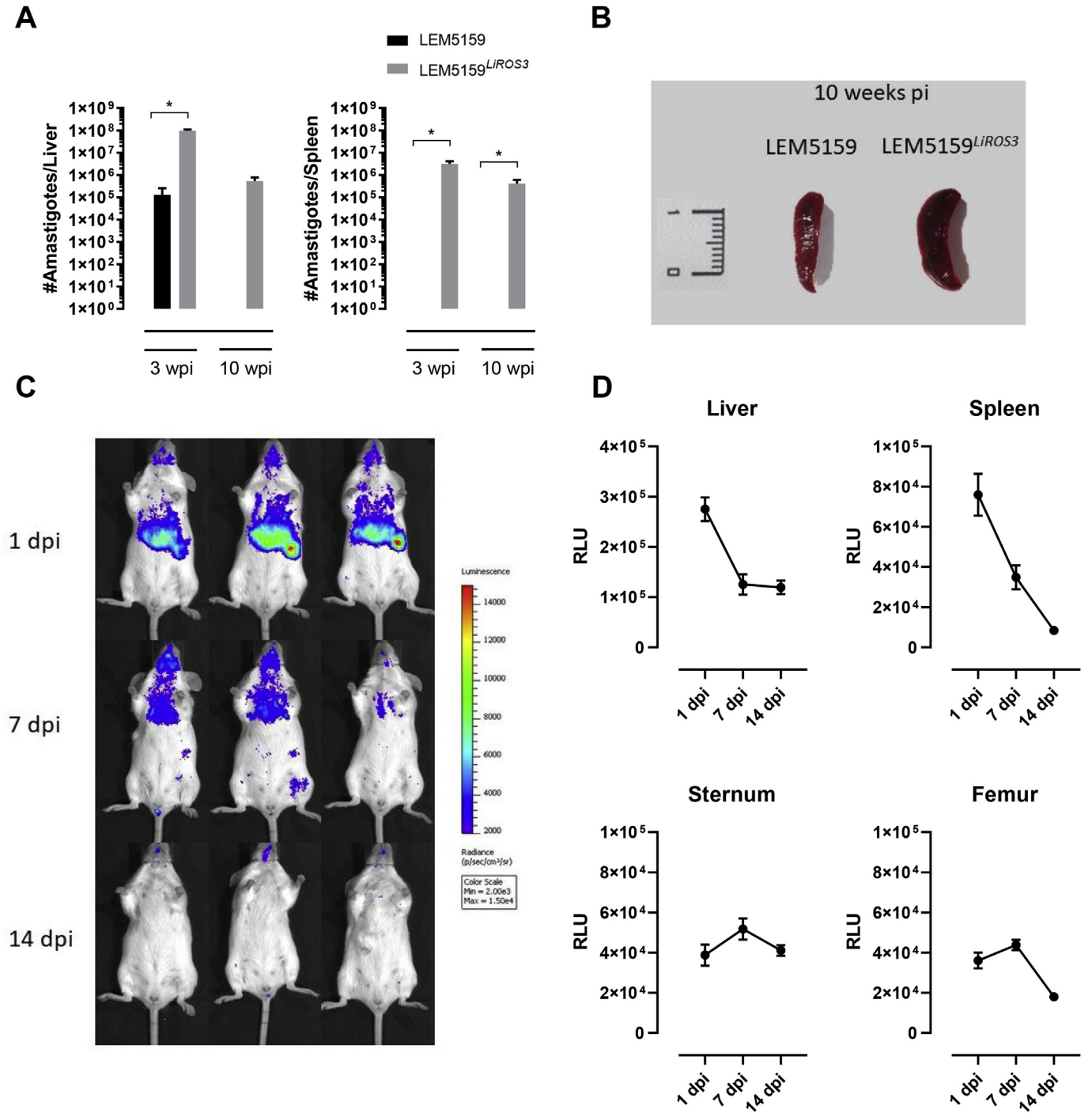

Fig. 1. ***In vivo*** **amastigote burdens of the MIL-R (LEM5159) and MIL-S (LEM5159^{LiROS3}) isolates in BALB/c mice after intravenous infection with 1 × 10^8 metacyclic promastigotes.** (**A**) Stauber index of liver and spleen; results are expressed as mean ± standard error of mean (SEM) (*$p < 0.05$). (**B**) Spleen isolated from BALB/c mice infected with LEM5159 (left) or LEM5159^{LiROS3} (right) at 10 WPI. (**C**) Bioluminescent imaging (BLI) of LEM5159^{PpYRE9} over time. (**D**) Parasite burdens obtained by BLI within regions of interest corresponding to the major target organs. Results are expressed as mean relative light units (RLU) ± SEM and are based on two independent repeats.

et al., 2020; Turner et al., 2015; Vanaerschot et al., 2011). Although experimentally selected MIL-resistant (MIL-R) strains demonstrated a clear fitness cost (Eberhardt et al., 2019; Hendrickx et al., 2016; Turner et al., 2015; Van Bockstal et al., 2019), increased infectivity has been suggested for MIL treatment relapse isolates (Rai et al., 2013), making the impact of MIL-R on parasite fitness still debatable (Hendrickx et al., 2020), hence calling for additional studies on a larger panel of VL isolates.

The present study evaluated the impact of naturally acquired MIL-R on *L. infantum* parasite fitness by assessing its capacity (i) to survive, expand and produce infectious metacyclic stages in the sand fly vector and (ii) to infect the vertebrate host (Vanaerschot et al., 2013). The MIL-R clinical *L. infantum* isolate MHOM/FR/2005/LEM5159 was compared with the experimentally reconstituted syngeneic MIL-susceptible (MIL-S) strain MHOM/FR/2005/LEM5159^{LiROS3} in BALB/c mice and in the sand fly vectors *Lutzomyia longipalpis* and *Phlebotomus perniciosus*.

Table 1
Promastigote and amastigote susceptibility of the MIL-R and MIL-S strains before and after passage in mice (BALB/c) or sand fly (*L. longipalpis*). Results are based on two independent repeats in triplicate and are expressed as mean IC_{50} value (μM) ± standard error of the mean (SEM).

Strains	Promastigote IC_{50} ± SEM (μM)	Post-mice promastigote (spleen) IC_{50} ± SEM (μM)	Post-fly promastigote IC_{50} ± SEM (μM)
MIL-R	> 40.0	N.D.	> 40.0
MIL-S	4.2 ± 3.0	3.5 ± 2.0	2.1 ± 0.1
	Intracellular amastigote IC_{50} ± SEM (μM)	**Post-mice amastigote (spleen)** IC_{50} ± SEM (μM)	**Post-fly amastigote** IC_{50} ± SEM (μM)
MIL-R	> 20.0	N.D.	> 20.0
MIL-S	0.7 ± 0.3	5.2 ± 0.6	2.2 ± 0.7

2. Materials and methods

2.1. Ethics statement

Experiments using laboratory rodents were carried out in strict accordance with all mandatory guidelines (European Union directives, including the Revised Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes that came into force on 01/01/2013, and the Declaration of Helsinki in its latest version) and were approved by the ethical committee of the University of Antwerp, Belgium (UA-ECD 2017–78 and UA-ECD 2015–90).

2.2. Mice and sand flies

Female Swiss and BALB/c mice (20–25 g) were purchased from Janvier (Le Genest-Saint-Isle, France). Food for laboratory rodents (Carfil, Arendonk, Belgium) and drinking water were available *ad libitum*. Sand flies (*L. longipalpis* and *P. perniciosus*) were maintained as described elsewhere (Volf and Volfova, 2011) at the Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp, Belgium and at the Department of Parasitology, Charles University, Czech Republic, respectively.

2.3. Leishmania strains

The MIL-R clinical *L. infantum* isolate MHOM/FR/2005/LEM5159 was isolated from an HIV-patient who received multiple rounds of MIL-treatment. Whole-genome sequencing of LEM5159 revealed a SNP in the aminophospholipid translocase *LiMT* gene resulting in an E to Q substitution and a frameshift mutation in the *LiROS3* gene causing an early stop codon (Mondelaers et al., 2016). Episomal transfection with the wild-type *LiROS3* gene ($LEM5159^{LiROS3}$) restored full MIL-susceptibility (Mondelaers et al., 2016). The LEM5159 strain was transfected with the red-shifted firefly luciferase gene variant *PPyRE9* as described previously (Eberhardt et al., 2019). Promastigotes were grown in HOMEM (Gibco®, Life Technologies, Ghent, Belgium) supplemented with 200 mM L-glutamine, 16.5 mM $NAHCO_3$, 10% heat-inactivated FCS, 40 mg/L adenine, 3 mg/L folic acid, 2 mg/L D-biotin and 2.5 mg/L hemin at 25 °C and were sub-cultured twice weekly. The $LEM5159^{LiROS}$ strain was grown under hygromycin pressure.

2.4. Animal infection

For each strain, five BALB/c mice were infected in the tail vein with 1×10^8 metacyclic promastigotes in 100 μL RPMI. Bioluminescent imaging (BLI) was performed at 1, 7 and 14 days post-infection (DPI) using the IVIS® Spectrum In Vivo Imaging System (Pelkin Elmer) upon intraperitoneal injection of 150 mg/kg D-Luciferin (Beetle Luciferin Potassium Salt; Promega) and under 2% isoflurane anesthesia. Images were analyzed using Living Image v4.3.1 within organ-specific regions of interest (Eberhardt et al., 2019). At pre-defined time points, organ parasite burdens were determined by microscopic evaluation of Giemsa-stained tissue imprints using the Stauber index (Stauber, 1958). Ten weeks post infection (WPI), a promastigote back-transformation was performed on liver and spleen in HOMEM medium at 25 °C (Hendrickx et al., 2015). When sufficiently dense log-phase post-mice cultures were obtained, a standard MIL-susceptibility assay was performed.

2.5. Sand fly infections

Sand fly females were fed with heat-inactivated heparinized mouse blood (*L. longipalpis*) or heat-inactivated rabbit blood (*P. perniciosus*) containing 5×10^6/mL (*L. longipalpis*) or 1×10^6/mL (*P. perniciosus*) promastigotes from log-phase cultures through a chicken skin membrane. Blood-fed females were separated 24 h after feeding, kept in the same conditions as the colony and dissected on 2, 5, 7, 9, 12 and 15 days post blood meal (DPBM) to microscopically check the presence and localization of parasites and infective metacyclic promastigotes. By crushing the total gut content in 50 μL PBS, the parasite load was microscopically quantified using a KOVA counting chamber. At 9 DPBM, colonization of the stomodeal valve and flagellum/cell body length ratios were determined as described previously (Van Bockstal et al., 2019). Parasites isolated from *L. longipalpis* guts on 9 DPBM were cultured in HOMEM promastigote medium supplemented with 5% penicillin-streptomycin. When sufficiently dense, log-phase cultures were obtained, a standard MIL-susceptibility assay was performed.

2.6. MIL susceptibility assay

Standard promastigote and intracellular amastigote susceptibility assays were performed to assess MIL susceptibility (Vermeersch et al., 2009). In brief, 50% inhibitory concentrations (IC_{50}) were determined by exposing log-phase promastigotes to two-fold serial dilutions of MIL starting from 40 μM. After 72 h incubation, promastigote susceptibility was determined by viability testing upon addition of resazurin and fluorescence reading (Tecan®, GENios). For amastigote susceptibility determination, primary peritoneal macrophages were harvested from starch-stimulated female Swiss mice. About 24 h after seeding, cells were infected with stationary-phase promastigotes at a 10:1 multiplicity of infection. Residual extracellular promastigotes were removed 24 h later by washing and two-fold MIL dilutions were added. After 96 h of drug exposure at 37 °C and 5% CO_2, plates were stained with Giemsa and IC_{50} values were determined microscopically by comparing the intracellular amastigote burdens between treated and untreated cells.

2.7. Statistics

Graphs were made with GraphPad Prism. Mann-Whitney tests were performed for the Giemsa imprints, the total parasite load, metacyclic promastigotes and colonized stomodeal valve in the sand fly. An unpaired *t*-test was performed for the flagellum/cell body length ratio in GraphPad Prism. A chi square test was performed in SPSS for the percentage infected flies.

3. Results

3.1. Comparative fitness of MIL-R and MIL-S parasites in BALB/c mice

The survival and growth of the resistant (LEM5159, MIL-R) and the syngeneic sensitive ($LEM5159^{LiROS3}$, MIL-S) strains were evaluated in BALB/c mice. Higher spleen burdens were detected for MIL-S compared to MIL-R at 3 WPI ($p = 0.0286$) and 10 WPI ($p = 0.0159$). Liver

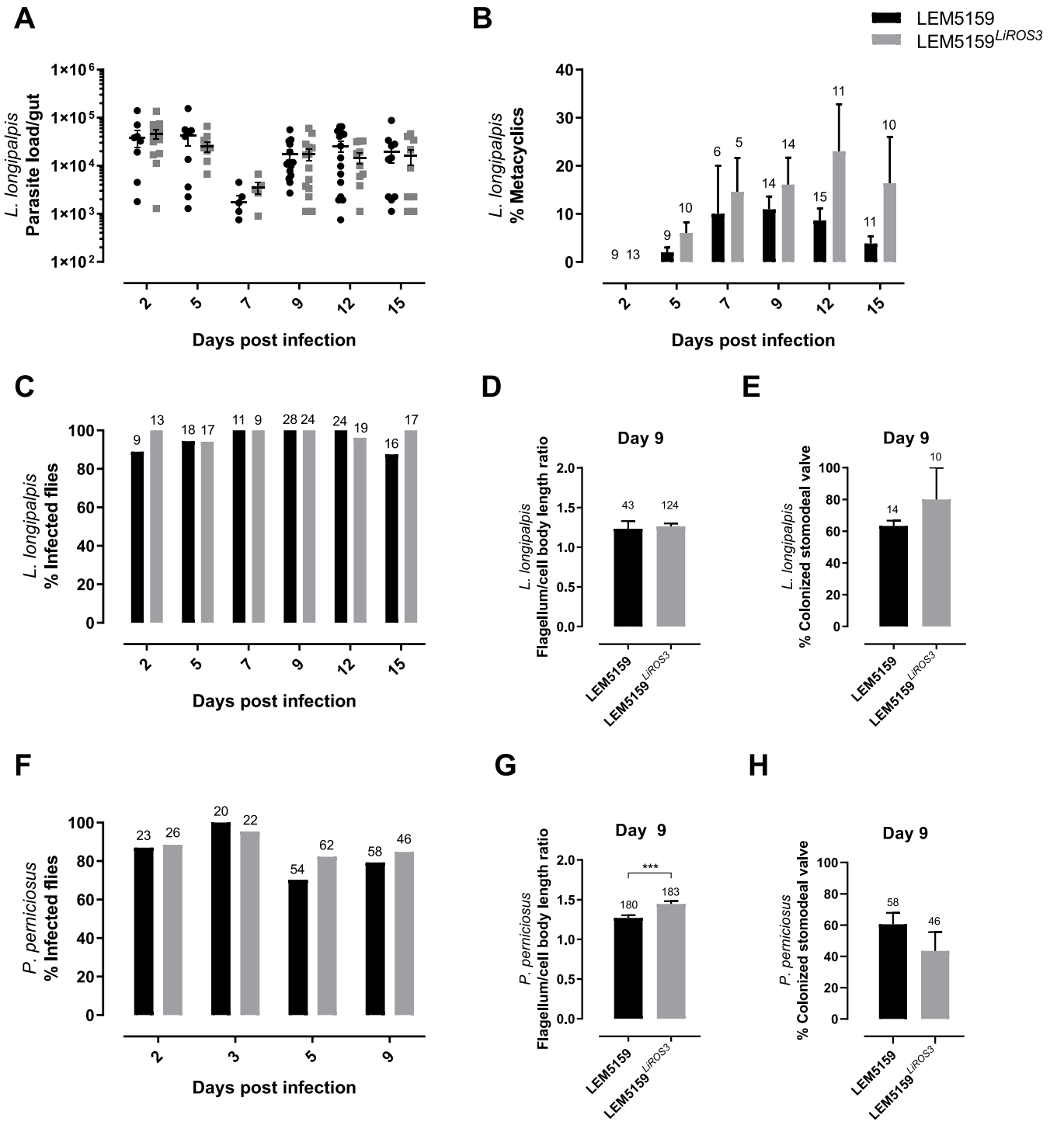

Fig. 2. Development of MIL-R (LEM5159) and MIL-S (LEM5159^{LirOS}) parasites in *L. longipalpis* (A–E) and *P. perniciosus* (F–H). (**A**) Parasite load at different time points post-infection. (**B-D-G**) Metacyclogenesis determined by (**B**) light microscopy in a KOVA chamber, numbers of dissected females are shown above bars, and by (**D-G**) measuring flagellum/cell body length ratio on Giemsa smears, number of parasites are shown above bars. (**C–F**) Infection rates (% infected females) at different time points post-infection. Numbers of dissected females are shown above bars. (**E-H**) Colonization of stomodeal valve. Numbers of dissected females are shown above bars. Results are expressed as mean ± SEM and are based on two independent repeats. Differences between groups were evaluated using Mann-Whitney (**A-B-E-H**), Chi-square test (**C–F**) or an unpaired *t*-test (**D-G**).

burdens were higher for MIL-S at 3 WPI ($p = 0.0286$) but not at 10 WPI ($p = 0.1667$) (Fig. 1A). Autopsy at 10 WPI revealed a clear splenomegaly in animals infected with MIL-S, but not with MIL-R (Fig. 1B).

No promastigotes were observed in the back-transformations from any of the spleen and liver samples of LEM5159-infected animals. Therefore, no IC_{50} values for the post-mouse cultures were obtained for this strain (Table 1). After 10 WPI in BALB/c mice, the MIL-S parasites isolated from the spleen were still drug-susceptible at the promastigote and amastigote level (Table 1).

The *in vivo* fitness of MIL-R was also evaluated with bioluminescent imaging (Fig. 1C). LEM5159^{PpYRE9} parasites failed to establish in all target organs with a rapid decline to undetectable bioluminescence in liver, spleen and bone marrow by 2 WPI (Fig. 1D) confirming the microscopic data (Fig. 1A).

3.2. Fitness of MIL-R parasites in L. longipalpis and P. perniciosus

Comparatively for MIL-R and MIL-S, parasite load, percentage of infected sand flies and percentage of metacyclic promastigotes over time were determined in two sand flies. In L. *longipalpis* (Fig. 2A–C), no differences could be identified and the stomodeal valve colonization and flagellum/cell body length ratio at 9 DPBM did not significantly differ either (Fig. 2D–E). After L. *longipalpis* passage, MIL-S parasites were still susceptible and MIL-R remained resistant both at the level of the promastigote and amastigote (Table 1). In *P. perniciosus,* no differences in percentage of infected sand flies, nor in the stomodeal valve colonization were identified between the two strains (Fig. 2F–H). However, when using the flagellum/cell body length ratio as indicator for metacyclogenesis at 9 DPBM, MIL-R displayed a significantly lower ($p = 0.0004$) ratio (1.27 ± 0.03 μm) compared to MIL-S (1.45 ± 0.04 μm) (Fig. 2G).

4. Discussion

Even though MIL-monotherapy has been replaced by a single dose of AmBisome® as first-line treatment in the Indian subcontinent, its application in combination with paromomycin still remains one of the second-line options in the kala-azar elimination program (Goyal et al., 2019; Sundar et al., 2008). Although the risk of drug resistance is significantly reduced in drug combinations (Hendrickx et al., 2017), the emergence of resistance needs close monitoring (Vanaerschot et al., 2014). Whether or not drug-resistant parasites will propagate mainly relies on the fitness cost/gain associated with the acquired resistance (Vanaerschot et al., 2014). Full MIL-resistance is related to mutations in the *miltefosine transporter* (MT) or the *ROS3* gene (Cojean et al., 2012; Mondelaers et al., 2016; Srivastava et al., 2017) which jointly encode for the transporter complex responsible for MIL uptake (Dorlo et al., 2012; Perez-Victoria et al., 2006a). A more subtle MIL-R phenotype is described for *L. donovani* strains characterized by a lower copy number of chromosome 13 harboring the *MT* gene (Hendrickx et al., 2020; Shaw et al., 2016). This 'intermediate' MIL-R phenotype was also found in strains with an overexpression of ABC transporters, responsible for enhanced efflux of MIL (Castanys-Muñoz et al., 2008).

To determine the potential epidemiological impact of MIL-resistance, this study in BALB/c mice and *L. longipalpis* and *P. perniciosus* sand flies compared the fitness of the MIL-R clinical *L. infantum* isolate MHOM/FR/2005/LEM5159 with a defective *LiROS3* gene (frameshift mutation resulting in an early stopcodon) and its experimentally reconstituted MIL-S counterpart. In BALB/c mice, MIL-resistance arising from ROS3-deficiency resulted in reduced parasite fitness with lower parasite burdens in liver and spleen. However, this fitness loss was not observed in the sand fly vectors showing formation of infective metacyclics that are necessary for transmission to the mammalian host (Bates, 2007). Importantly, the MIL-R phenotype was preserved upon sand fly passage, indicating a potential risk of resistance to spread, as recently also observed for paromomycin (Hendrickx et al., 2020) and pentavalent antimonial (Seblova et al., 2014). In a previous study performed at our lab (Van Bockstal et al., 2019), the impact of experimental MIL-resistance (caused by a *MT* mutation) on development in the sand fly revealed a clear fitness loss in both sand fly species (*L. longipalpis* and *P. perniciosus)* characterized by a reduced capacity to infect and form infective metacyclic stages. Intermediate resistance in *L. donovani* following repeated *in vitro* or *in vivo* MIL-exposure, resulting in a reduced ploidy of chromosome 13, was well tolerated in sand flies. Here, we report that MIL-resistance due to a LiROS3 deficiency is also able to develop and form metacyclic stages in *L. longipalpis*, although some impact on metacyclogenesis in *P. perniciosus* could be observed. However, these changes are less outspoken compared to the previously reported *MT*-mutation (Van Bockstal et al., 2019), indicating that the particular *LiROS3* mutation (Mondelaers et al., 2016) is better tolerated for development in the sand fly vector.

This study has made a detailed characterization of one natural fully MIL-R isolate for all stages of the life cycle. Extending this type of research to additional circulating resistant strains would provide more detailed information about the propagation of resistant traits. The present work not only corroborates earlier findings of a lower infectivity of fully MIL-R parasites in the BALB/c model, but also shows that the fitness profiles in the vertebrate host and insect vector are not necessarily congruent, since successful development in the sand fly was achieved by the MIL-R LEM5159 strain. The decreased virulence in mammalian hosts for MIL-R *L. infantum* strains resulting from either ROS3- or MT-deficiency in the studied strains (Eberhardt et al., 2019; Hendrickx et al., 2016) likely explains why no widespread MIL-resistance is yet observed in the field. It is therefore not surprising that the LEM5159 strain has been isolated from a severely immunocompromised HIV-patient (Hendrickx et al., 2014; Mondelaers et al., 2018) compatible with uncontrolled parasite dissemination. In conclusion, results of this study indicate that beside the potential spread of intermediate MIL-resistance, the ROS3-deficient parasite strain may still be transmitted by sand flies. The severe fitness cost of this mutation in the mammalian host is most likely the reason why it mainly poses a threat to immunocompromised patients.

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