Elucidating in vitro and in vivo phenotypic behaviour of L. infantum/L. major natural hybrids

S. Cortes1,*, A. Albuquerque-Wendt1,*,†, C. Maia1, M. Carvalho1,2, I. A. Lima3, L. A. R. de Freitas3, W. L. C. dos-Santos3 and L. Campino1

1Global Health and Tropical Medicine, GHTM, Instituto de Higiene e Medicina Tropical, IHMT, Universidade Nova de Lisboa, UNL, Rua Junqueira 100, 1349-008 Lisbon, Portugal. 2Grupo Galenus, Clinlab (Anatomia Patológica), Edifício Monumental Av. Fontes Pereira de Melo, 51 C, 1° Piso, 1000-120 Lisbon, Portugal and 3Fundação Oswaldo Cruz, Instituto Gonçalo Moniz, Salvador, BA, Brazil

Abstract

The clinical manifestation and course of Leishmania infections depend on factors such as species, virulence and host-immunity. Although trypanosomatids are considered to have clonal propagation, genetic hybridization has produced successful natural hybrid lineages. Hybrids displaying strong selective advantages may have an impact on pathogenesis and the epidemiology of leishmaniasis. Thus, characterization of phenotypic properties of Leishmania hybrids could bring significant insight into the biology, infectivity, pathogenicity and transmission dynamics of these atypical strains. The present study focuses on phenotypic features and survival capacity of Leishmania infantum/Leishmania major hybrid isolates as compared with representative putative parental species, L. infantum and L. major. In vitro assays (growth kinetics, susceptibility to different conditions) and in vivo infection (parasite detection and histopathological alterations) showed that hybrids present higher growth capacity and decreased susceptibility to reactive oxygen species. Furthermore, evaluation of infected spleen tissue suggests that hybrids induce a stronger immune reaction than their putative parents, leading to the development of white pulp hyperplasia in B-lymphocyte compartments. Overall, these hybrids have shown high plasticity in terms of their general behaviour within the different phenotypic parameters, suggesting that they might have acquired genetic features conferring different mechanisms to evade host cells.

Introduction

The family Trypanosomatidae comprises a large group of parasitic protozoa that cause important diseases in humans: Chagas disease, Human African Trypanosomiasis and Leishmaniasis. Leishmaniasis are endemic in 98 countries, representing a risk for 350 million people with an incidence of 1.3 million cases per year (WHO, 2015). Visceral leishmaniasis (VL) is the most severe clinical form, with 300,000 cases per year and 20,000–50,000 deaths per year. In the Old World, Leishmania donovani and L. infantum are the main aetiological agents of VL while L. aethiopica, L. infantum, L. major and L. tropica, are responsible for cutaneous leishmaniasis (CL). Clinical manifestations and course of infection depend on factors such as intrinsic parasites’ virulence, host genetic background and immune status. Although trypanosomatids are considered to be mainly clonal, genetic hybridization has produced successful hybrid lineages, which have, for example, influenced Trypanosoma cruzi evolution and the epidemiology of Chagas disease (Miles et al., 2009).

In the last two decades natural inter and intraspecific Leishmania hybrids have been found in the New World between L. braziliensis and L. peruviana (Dujardin et al., 1995; Kato et al., 2016), L. guyanensis and L. braziliensis (Delgado et al., 1997; Bahuls et al., 1999; Nolde et al., 2007) and, L. braziliensis and L. panamensis (Belli et al., 1994). In the Old World hybrids between L. infantum and L. major (Ravel et al., 2006), L. donovani and L. aethiopica (Odhuvor et al., 2011) and within L. donovani complex (Chargui et al., 2009; Gelanew et al., 2014; Rogers et al., 2014) have also been documented.

The existence of hybrids from two distant species, L. infantum and L. major, was firstly reported by Ravel et al. (2006), who utilized multilocus enzyme electrophoresis and multilocus sequence typing to show that probably these hybrids encompassed the complete genomes of both parental species. Volf et al. (2007) observed that these hybrids developed infections in Phlebotomus papatasi, which is a L. major-specific vector but refractory to L. infantum. Interestingly, several experimental studies have shown that genetic exchange in Leishmania occurs in the phlebotomine sand fly producing hybrid progenies, which inherited both parental alleles, and were capable of being transmitted to the mammalian vertebrate host (Akopyants et al., 2009; Sadlova et al., 2011; Inbar et al., 2013; Romano et al., 2014).

The existence of sexual recombination in Leishmania is also supported by multiple different population genetic studies (Galanew et al., 2014; Rougeron et al., 2015) although it may be of little consequence to a predominately clonal parasite with exponential growth in an ideal
environment. However, once conditions become stressful, the genetic exchange may be crucial to survival and expansion (Miles et al., 2009). Indeed, *L. braziliensis/L. peruviana* hybrid clones showed a higher plasticity and phenotypic diversity upon stressful *in vitro* conditions (Cortes et al., 2012). In addition, a high pathogenicity in mice infection was observed (Cortes et al., 2012). In an experimental infection, the existence of hybrids with strong selective advantage and increased plasticity may have an impact on pathogenesis and eco-epidemiology of leishmaniasis (Miles et al., 2009). *Leishmania* genomic plasticity (e.g. gene conversion, aneuploidy tolerance, extra-chromosomal elements and gene repair) is probably a key factor in its adaptation to different environmental conditions associated with the different phases of the *Leishmania* life cycle (Rogers et al., 2014). In fact, *Leishmania* parasites encounter some hostile conditions both inside phlebotomine sand fly’s midgut and inside macrophages of the vertebrate host, namely reactive oxygen and nitrogen species (ROS and RNS) (Vanaerschot et al., 2010). Therefore, characterization of phenotypic and genetic properties of *Leishmania* hybrids may bring additional relevant data relating to parasite infectivity, pathogenicity and the transmission dynamics of these atypical strains. In the present study, we focused on phenotypic features and compared the survival capacity of these atypical strains. In the present study, we focused on phenotypic features and compared the survival capacity of these atypical strains.

### Drug susceptibility assay

To assess the susceptibility of the parasites to amphotericin B (AmB, Sigma), promastigotes were plated in 96-well flat-bottomed microtiter plates, at a final parasite density of 10⁶ promastigotes mL⁻¹ in RPMI 1640 medium (Sigma) supplemented with 10% FBS plus 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (Sigma) (complete RPMI) in the presence of different drug concentrations (0–100 µg mL⁻¹) for 48 h. MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium] colorimetric assay was used to access parasite viability. Briefly, MTT (5 mg mL⁻¹) was added to each well, incubated for 4 h at 24 °C and centrifuged at 1800 g for 15 min. The supernatant was removed and the precipitated formazan was dissolved in dimethyl sulfoxide (DMSO). Optical density (OD) was measured spectrophotometrically at 595 nm. Relative viability was calculated from the ratio of optical density (OD) readings in parasites exposed to compounds vs those not exposed. The data were exported to GraphPad Prism 5 to calculate the average inhibitory concentrations that kill 50% of *Leishmania* promastigotes (IC₅₀) using a sigmoidal dose-response model with variable slope. Three independent experiments were performed with six replicates each.

### In vitro amastigote infection assay

*In vitro* intracellular amastigote infection rates were determined using monocytes derived from a Human histiocytic lymphoma U-937 cell line (ATCC® CRL-1593.2) maintained in complete RPMI at 37 °C and 5% CO₂. After 48 h differentiation of 5 × 10⁵ cells mL⁻¹ into macrophages in sterile 16-channel LabTek slides (Nunc) with 100 ng mL⁻¹ phorbol myristic acid (PMA) (Sigma), the cells were washed once with PBS, to remove non-differentiated and non-adherent cells and further infected with 2.5 × 10⁵ promastigotes mL⁻¹ in a 5:1 parasite-to-host cell ratio (Maia et al., 2007) for 48 h. Slides were gently washed once with PBS after this period, to remove non-internalized promastigotes, fixed with methanol (Sigma) and stained with Giemsa (Sigma). The Infection Index (II = % infected macrophages × no. of internalized amastigotes/infected macrophase) was estimated according to Vanaerschot et al. (2010). The results represent the average of two counts in two independent experiments.

### In vivo infection

Female BALB/c mice, with 4–5 weeks’ age were purchased from Harlam Interfauna Ibérica SL (Barcelona, Spain) and housed at the animal facilities of IHMT under stable climatic and dietary conditions.

The virulence of *Leishmania* parasites was maintained by the animal passage. Promastigotes were used at days 6–7 in culture, corresponding to the highest parasite density and a high.
percentage of infective metacyclic promastigote forms (Almeida et al., 1993). Four groups (corresponding to the different *Leishmania* strains) of 20 randomly selected animals were inoculated both intraperitoneal and sub-cutaneous in the left footpad with $10^7$ promastigotes mL$^{-1}$ (100 and 25 mL$^{-1}$, respectively). The control groups consisted of 20 animals inoculated with the same volume of 0.9% saline solution. Prior to infection, mice were anaesthetized with 100 mL solution of 150 mg mL$^{-1}$ of ketamine (Imalgene® 1000, Rhône Mérieux) and 15 mg mL$^{-1}$ of xylazin (Rompun®, Bayer). Within each treatment group (inoculated species and control), animals were randomly separated and caged together in groups of five. Animals were weighed weekly and examined for the presence of clinical signs. At each time-point (days 14, 28, 42 and 56 post-infection) five collectively housed mice per treatment group were sacrificed. The spleen (SP), liver (LV), draining lymph nodes (LN) and skin from the inoculation site (SKI, left footpad) and from skin distant from the inoculation site (SKD, right footpad) were aseptically harvested for parasite detection and SP and SKI were also subjected to histopathological analysis. Each five mice tissue samples were treated as replicates.

**Parasite detection (cultures and polymerase chain reaction)**

Tissues of each animal were homogenized in complete Schneider medium (WHO, 2010) and for DNA extraction. Cultures were incubated at 24 °C and observed weekly for the presence of promastigotes up to 4 weeks, including those with samples from control tissues infected with the studied strains. When the null hypothesis was rejected, multiple comparisons were implemented to determine which strains differ from each other, using a significance level of 5% ($P < 0.05$). Statistical significance ($P < 0.05$) of amphotericin B $IC_{50}$ and infection index was assessed by one-way ANOVA followed by Bonferroni’s $t$ test using GraphPad Prism 5 (GraphPad Software, Inc.).

**Histopathological analysis**

Spleen and skin tissue slices with a thickness of 4 mm were collected from each animal, fixed in formalin and embedded in paraffin. Sections 3–5 µm thick were cut and stained with haematoxylin and eosin (H&E) and analysed by optical microscopy. Leucocytes were characterized morphologically according to previously described criteria (Silva-O’Hare et al., 2016).

The densities of different leukocyte populations were scored as follows: 0 = no cells, 1 = the less frequent leukocyte population observed in most of the examined × 400-magnified microscopic fields, 2 = the second more frequent leukocyte population observed in most of the examined × 400-magnified microscopic fields, and 3 = the most frequent leukocyte populations observed in most of the examined × 400-magnified microscopic fields. Furthermore, the relative size of the white pulp lymphoid follicles and the number of lymphoid follicle per mm$^2$ were estimated by morphometry using the Image ProPlus (Media Cybernetics).

**Results**

**In vitro assays: growth kinetics, oxidative stress, drug susceptibility and intracellular infection**

Growth kinetics and promastigote densities of *L. infantum/L. major* hybrids and of two putative parental *L. major* and *L. infantum* strains were compared during 12 days in the culture medium. Hybrid IMT 208 reached the highest parasite density between the 5th and 6th days (2.38 × 10$^7$ parasites mL$^{-1}$) (Fig. 1). Hybrid IMT 211 presented lower densities than hybrid IMT 208 until peak at days 5–6. Between days 10 and 11 it presented a second growth peak.

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**Table 1. Characterization of the four *Leishmania* strains used in the study**

<table>
<thead>
<tr>
<th>Species, zymodeme</th>
<th>Lab code</th>
<th>WHO strain code</th>
<th>Clinical form</th>
<th>Human Host</th>
<th>Geographic origin</th>
<th>Clinical history</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. infantum</em>, MON1</td>
<td>IMT151</td>
<td>MHOM/PT/88/ IMT151</td>
<td>VL, bone marrow</td>
<td>Child, Immunocompetent</td>
<td>PT</td>
<td>No treatment</td>
<td>Maia et al. (2013)</td>
</tr>
<tr>
<td><em>L. infantum/L. major</em> hybrid</td>
<td>IMT208</td>
<td>MHOM/PT/94/ IMT208</td>
<td>VL, peripheral blood</td>
<td>Adult, HIV co-infection (IDU)</td>
<td>PT</td>
<td>After treatment with Glucantime*</td>
<td>Ravel et al. (2006)</td>
</tr>
<tr>
<td><em>L. infantum/L. major</em> hybrid</td>
<td>IMT211</td>
<td>MHOM/PT/94/ IMT211</td>
<td>VL, pleural fluid</td>
<td>Adult, HIV co-infection (IDU)</td>
<td>PT</td>
<td>After treatment with Glucantime* and Fungizone*</td>
<td>Ravel et al. (2006)</td>
</tr>
</tbody>
</table>

HIV, human immunodeficiency virus; IDU, intravenous drug user; VL, Visceral leishmaniasis; CL, Cutaneous leishmaniasis; PT, Portugal; IS, Israel.
DNA were detected in LN of animals from the infected groups, persisting until the end of the experiment. However, in this organ (LN) mice infected with hybrid IMT 208 significantly persisted at high levels in comparison with their putative parents (P < 0.05). The increased LFD was accompanied by an increase of relative lymphoid follicle size, reaching a maximum at day 42 and returning to a lower size at day 56, with exception of L. major LV561 (Fig. 4B). In addition, germinal centre size showed to be relatively constant throughout the course of infection (Fig. 4C). Infection with hybrids and L. infantum IMT 151 resulted in a general increase in white pulp size (Fig. 4D), with hybrid IMT 208 presenting 2-fold increase in comparison with its putative parent L. infantum.

Furthermore, infection with hybrid strains was associated with lymphoid follicle hyperplasia and increased B cells (follicular compartment) of the spleen (Fig. 5). At day 56 monocytic and macrophage infiltrations containing amastigote forms were observed in the skin of mice infected with hybrid strains, as well as in L. major infected mice, with high inflammatory infiltrates with heavily amastigote-infected macrophages (Fig. 6). In contrast, no infiltrates were found in L. infantum infected mice (data not shown).

Discussion

Genetic exchange between species and strains has been documented for the trypanosomatids that cause human disease. Namely, in T. cruzi two of the six major circulating genetic lineages are hybrids that are frequently isolated from humans in regions where chronic Chagas disease is particularly severe (Lewis et al., 2011). Although genetic exchange in Leishmania is predicted to be a rare event, with an estimated frequency of \(\approx 2.5 \times 10^{-6}\) or
less per cell (Akopyants et al., 2009), it is nowadays considered that *Leishmania* presents a mix-mating model of reproduction with clonality occurring in the vertebrate and invertebrate hosts, and genetic recombination within the insect vector (Rougeron et al., 2017). Many questions have been raised concerning hybrids, such as the occurrence and frequency of genetic exchanges as well as their plasticity and maintenance in adverse environments.

In the present work, we compared the phenotypic behaviour of two *L. infantum/L. major* hybrid strains, and the two putative parental species. Phenotypic characterization can be relevant for associating differences in parasites’ growth, virulence and plasticity. Promastigotes from hybrid strains presented higher capacity to grow in culture than the two putative parental species, with special emphasis to hybrid IMT 208 which presented the earliest and highest parasite density. In addition, the hybrid IMT 211 was able to survive in culture with high density and for a longer period, suggesting that these hybrids may present differences in nutrient requirements, and/or possibly altered tolerance to medium acidification, as observed in other *Leishmania* species such as *L. donovani*, *L. major* and *L. mexicana* (Zilberstein and Shapira, 1994; Vanaerschot et al., 2010). Concerning natural *Leishmania* hybrids, just a limited number of *in vitro* phenotypic studies have been reported (Torrico et al., 1999; Cortes et al., 2012).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tissues/Days post-infection</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
<th>14</th>
<th>28</th>
<th>42</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. infantum</em> (IMT 151)</td>
<td>SP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>LV</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td></td>
<td>LN</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td></td>
<td>SKI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td></td>
<td>SKD</td>
<td>–</td>
<td>–</td>
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</tr>
</tbody>
</table>
|                               |                             | SP, spleen; LV, liver; LN, lymph nodes; SKI, skin from the inoculation site; SKD, skin distant from the inoculation site; +, positive culture or DNA amplification in at least 1 out of 5 animals; –, negative cultures or no DNA amplification.

**Table 2.** Detection of parasites through NNN (Novy, MacNeal and Nicolle medium) cultures and PCR from BALB/c mice inoculated with different *Leishmania* strains and necropsied at 14, 28, 42 and 56 days post-infection.
that a combination of strains’ subculturing passages as well as AmB formulation, could explain these differences. Nevertheless, it is clear that hybrid IMT 208 and IMT 211 promastigotes present a susceptibility to AmB that is similar to L. major.

Footpad inoculation of stationary phase promastigotes of the L. major parental strain led to rapid lesion development, similar to that observed elsewhere (Romano et al., 2014). The dermotropic of L. major parental species is evidenced by the significant (P < 0.05) dissemination of parasites to the right footpad in early time-points (Table 2). Interestingly, although L. major parasites have been detected in mice’ spleens, they were not found in the livers. In other studies, L. major DNA has also been detected in internal organs of North African hedgehogs from Algeria and Tunisia (Tomás-Pérez et al., 2014; Chemkhı et al., 2015) and in the liver and spleen of Baluchistan gerbils and brown rats (Motazedian et al., 2010). In contrast, even though parasites were detected and recovered from L. infantum and hybrids infected tissues, no skin lesions were detected up to 8 weeks post-infection (data not shown). Similar results were observed following experimental cross-species mating efforts used to generate L. infantum/L. major hybrids, where upon infection of BALB/c mice no lesions were observed for either L. infantum parental strain or hybrid infected tissues (Romano et al., 2014). No parasites were detected in the skin from the inoculated footpad of animals infected with L. infantum IMT 151 and hybrid IMT 211 after day 42, whereas parasites were detected throughout the whole course of infection in hybrid IMT 208 and L. major infected animals. Together, these data suggest that hybrids display differences in their ability to grow in the skin or viscera of mice, with hybrid IMT 208 being significantly (P < 0.05) more dermotropic than hybrid IMT 211. IMT 211 presented a viscerotropic behaviour, similar to that of the L. infantum IMT 151, whereas hybrid IMT 208 presented a dermotropic behaviour like that more closely mimics L. major LV561. The phenotypic diversity noted between the hybrids analysed here is consistent with previous observations made within the Viannia complex (Cortes et al., 2012).

Visceral leishmaniasis is associated with spleen white pulp hyperplasia in susceptible hosts (Veress et al., 1983; Keenan et al., 1984). Surprisingly, livers of animals infected with L. infantum seem to lack established infections or have perhaps controlled the infections in an early phase, as we were not able to isolate parasites nor detect parasite DNA in this tissue. This observation could also be related to other factors such as inter-, intra-specific variations or even due to different strain tissue preferences (Garin et al., 2001; Méndez et al., 2001). Overall, our data suggest that the hybrid strains induce a strong white pulp hyperplasia affecting predominantly B-lymphocyte compartments in splenic tissue. In
a recent study, it was observed that livers of \textit{L. donovani} infected BALB/c mice presented early granulomatous lesions with T- and B-cells being predominant in more advanced granuloma stages (Salguero et al., 2018). Moreover, evidence exists that lymphoid disorganization and atrophy may follow the hyperplasia in severe forms of visceral leishmaniasis (Veres et al., 1983; Santana et al., 2008). Apoptosis of T-lymphocytes and follicular dendritic cells may be involved in this process (Smelt et al., 1997; De Lima et al., 2012). Further studies are needed to investigate if the more intense hyperplasia induced by these hybrid strains contributes to a faster or deeper white pulp disruption.

Calvo-Álvarez et al. (2014) experimentally obtained a \textit{L. infantum} hybrid lineage which presented a lower virulence and parasite load in the spleen and liver, in comparison with its parental strain. Moreover, Romano et al. (2014) showed that \textit{L. major} and \textit{L. infantum} hybrid progeny seem to have differentially inherited genes controlling the respective tissue tropisms of their parents, indicating that at least one of the parental strains is heterozygous for these genes. Interestingly, Volf et al. (2007) experimentally infected \textit{P. papatasi} with one of the hybrid strains used in this study (IMT 208) and found out that hybrids express \textit{L. major} lipophosphoglycan (LPG) which present a crucial role in the attachment to midgut and survival within the vector. LPG is a known virulence factor with a role in skin inflammation and pathology (Villacorch–Cardoso et al., 2008; Zamora-Chimal et al., 2017). It should be emphasized that these hybrids have shown plasticity in terms of their general behaviour within the different phenotypic parameters, suggesting that they might have acquired additional genetic features conferring environmental adaptation mechanisms to evade/resist to the immune response of their host cells.

Our study has shown that the genetic differences are revealed by the diversity of the phenotypic characteristics, highlighting the relevance in characterizing the fitness and genetic background of natural hybrids. To a broader extent, it may impact on the prevention and case management in terms of treatment approaches and disease progression. Although one strain of each parental visceral and cutaneous species was evaluated, other strains from the same species might differ on their genetic identity and phenotypic diversity with implications beyond what was perceived in this study. Nevertheless, this exploratory study was done in order to find trends of phenotypes preferences and profiles, thus similar experiments using different strains as well as clonal lineages are advised. Moreover, we believe that other different characteristics are to be expected which may lead to adaptation to new ecological niches, vectors and even hosts, including humans and domestic animals. With further insight into the complexity, prevalence and significance of \textit{Leishmania} hybridization, we might expect to observe novel epidemiological trends, clinical outcomes and therapeutic responses. To further investigate the full consequences of the genetic background of \textit{Leishmania} natural hybrids, and their eco-epidemiological implications, more studies should be carried out which should shed some light on the traits of these strains.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0031182018001993.

**Author ORCIDs.** S. Cortes 0000-0001-5850-6950 and A. Albuquerque-Wendt 0000-0001-5794-5417.

**Acknowledgements.** The authors would like to thank José Manuel Cristovão for excellent technical assistance, Prof. Philipp Sibbeth (Leibniz Universität Hannover) for statistical advice and Dr Ciara McCoy for the English review of this manuscript.

**Financial support.** This work was supported by funds from Fundação para a Ciência e a Tecnologia (FCT), Ministério da Ciência, Tecnologia e Ensino Superior to the project PTDC/CVT/112371/2009 and GHTM (UID/Multi/04413/2013) and Rede CYTED (RIMLEV Propuesta P210RT0565). S. Cortes and C. Maia have the support of FCT, through the Investigator Starting Grants IF/0773/2015 and IF/01302/2015, respectively.

**Conflict of interest.** None.

**Ethical standards.** All procedures with animals were carried out according to the Ethics Committee of the IHMT and Portuguese Veterinary Official Authorities (‘Direcção Geral de Veterinária’, approval ID 1443/DSSPA) and followed the guidelines of the Portuguese legislation (Lei no 113/2013, 7.8).

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