Functional Genomics in Sand Fly - derived *Leishmania* promastigotes

Leishmania Genomics within the Sand Fly

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Abstract

Background

Leishmania development in the sand fly gut leads to highly infective forms called metacyclic promastigotes. This process can be routinely mimicked in culture. Gene expression profiling studies by transcriptome analysis have been performed with the aim of studying promastigote forms in the sand fly gut, as well as differences between sand fly- and culture-derived promastigotes.

Principal findings

Transcriptome analysis has revealed the crucial role of the microenvironment in parasite development within the sand fly gut because substantial differences and moderate correlation between the transcriptomes of cultured and sand fly-derived promastigotes have been found. Sand fly-derived metacyclics are more infective than metacyclics in culture. Therefore, some caution should be exercised when using cultured promastigotes, depending on the experimental design. The most remarkable examples are HASP/SHERP, gp63, and autophagy gene up-regulation in sand fly-derived promastigotes compared to cultured promastigotes. Because HASP/SHERP genes are up-regulated in nectomonad and metacyclic promastigotes in the sand fly, the encoded proteins are not metacyclic-specific. Metacyclic promastigotes are distinguished by morphology and high infectivity. Isolating them from the sand fly gut is not exempt of technical difficulty because other promastigote forms remain in the gut even 15 days after infection. *L. major* procyclic promastigotes within the sand fly gut up-regulate genes involved in cell cycle regulation and glucose catabolism, whereas metacyclics increase transcript levels of fatty acid biosynthesis and ATP-coupled proton transport genes. Most signal transduction pathways remain uncharacterized. Future elucidation may improve understanding of parasite development, particularly signaling molecule-encoding genes in sand fly *vs*. culture, and between promastigote forms in the sand fly gut.

Conclusions

Transcriptome analysis has been demonstrated to be technically efficacious to study differential gene expression in sand fly gut promastigote forms. Transcript and protein levels are not well

correlated in these organisms (~25% quantitative coincidences), especially under stress situations, and particularly at differentiation processes. However, transcript and protein levels behave similarly in ~60% cases from a qualitative point of view (increase, decrease, or no variation) Changes in translational efficiency observed in other trypanosomatids strongly suggest that the differences are due to translational regulation, and regulation of the steady-state protein levels. The lack of low input sample strategies does not allow translatome and proteome analysis of sand fly-derived promastigotes so far.

Key learning points

1. Metacyclic promastigotes are highly infective forms, but no markers are available.

2. Accurate description of samples compared by means of high-throughput strategies and caution when comparing different studies is essential, and particularly important for samples obtained from the sand fly because different vector and parasite pairs are considered.

3. Transcriptome data and infection experiments support that sand fly-derived promastigotes are more infective than cultured ones.

4. Sand fly-derived promastigotes are more infective than cultured promastigotes to *in vitro* cultured human phagocytes, which combined with transcriptome profiles supports that metacyclogenesis is more successfully completed in the sand fly gut.

5. Transcriptome analysis in *L. infantum* and *L. major* promastigotes derived from the sand fly gut confirm that HASP, SHERP, and gp63 genes are involved in metacyclogenesis and already increased in nectomonad promastigotes, thus not being metacyclic promastigote markers.

6. Differential expression of several genes involved in gene expression regulation, signaling, and metabolic processes between sand fly-derived and cultured promastigotes supports an important influence of the microenvironment in differentiation.

7. Studying the translatome and the proteome is not feasible in sand fly-derived promastigotes so far. Transcriptomics is the only alternative, and interpretation of the results should be cautiously discussed because transcript levels do not always reflect protein levels.

Introduction: Why studying sand fly-derived promastigotes is important?

The *Leishmania* spp. (Kinetoplastida: Trypanosomatidae) life cycle is digenetic because two hosts are involved: a mammal and a sand fly (being the genera *Phlebotomus* and *Lutzomyia* proven vectors; Psychodidae: Phlebotominae). The promastigote is the motile stage which develops within the gut of the invertebrate host and is transmitted to the mammalian host during blood-sucking (reviewed in [1]). A small fraction of inoculated promastigotes is internalized by mononuclear phagocytic cells [2] and differentiate to the amastigote stage, which is the round non-motile dividing form (reviewed in [3, 4]). Eventually, a sand fly feeds from the blood of an infected mammal. Amastigotes are released and transform into promastigotes, which begin the complex developmental process within the sand fly gut becoming more infective for transmission to the mammalian host [5].

Studying sand fly-derived promastigotes is not exempt of difficulties for three reasons: first, few parasites can be isolated from the insect gut ($\sim 2 \times 10^5$ from the whole gut, $\sim 10^4$ promastigotes from the stomodeal valve –SV- area) [6, 7] compared to cultures ($2\text{-}4 \times 10^7$ promastigotes/mL) [8-10]; second, the promastigote populations are phenotypically heterogeneous and asynchronous in the sand fly gut [5, 11-14] and in culture [15]; and third, maintenance of sand fly laboratory colonies, experimental infection, and parasite isolation from the gut is not exempt of technical difficulties [16, 17], within reach of specialized laboratories. As a consequence, most research on the promastigote stage is performed in axenic culture, and the molecular, biochemical and physiological features of this stage have been scarcely described within its natural environment. As the genome sequences of these parasites are available [18,

19], high-throughput transcriptome analysis of sand fly-derived promastigotes has been performed in *L. infantum* and later on in *L. major*.

The main promastigote forms within the sand fly gut are procyclics, nectomonads, leptomonads, and metacyclics [14, 20]. These forms have also been found in culture [21]. The main metacyclic promastigote isolation method is based on the different agglutination ability in the presence of the peanut lectin (PNA), despite the structural differences in the lipophosphoglycan (LPG) [22]. Promastigote development in the sand fly gut was extensively reviewed [14, 20, 23].

In vitro infection of the human myeloid U937 cell line with *L. infantum* promastigotes showed that the peanut lectin non-agglutinating metacyclic subpopulation (LiPro-PNA$^-$) is more infective than the agglutinating subpopulation (LiPro-PNA$^+$) and the whole population in stationary phase of axenic culture (LiPro-Stat), from where both are isolated [24]. The same approach has revealed that LiPro-Stat and LiPro-PNA$^-$ are less infective (~50% and ~20-30%, respectively) than promastigotes isolated from the sand fly vector *P. perniciosus* (LiPro-Pper) SV [7, 25, 26]. Sand fly metacyclics are present in the SV vicinity, which is located in the thoracic midgut forefront and plays a crucial role in parasite injection into the mammalian host's dermis during blood meal intakes. In the case of the *P. perniciosus*-*L. infantum* vector-parasite pair, the metacyclic promastigote proportion in culture [24, 25] and within the sand fly gut [27] is approximately equal (~10%). The percentages are much higher, up to 90%, in other parasite and vector species [28, 29]. Culture passage also affects the yield in metacyclic promastigotes [28]. Therefore, higher infectivity levels of sand fly-derived promastigotes isolated from the SV are explained by a more advanced differentiation status (i.e., these promastigotes are more "metacyclic in character"), instead of a simple enrichment in metacyclics. Working with promastigotes from the gut is technically demanding, but transcriptome analysis and infection experiments indicate that using the culture model does not always lead to reliable results. Case-by-case decision making is required in the experimental design [7].

Promastigote development in the sand fly gut

According to Gossage et al.'s model [14], based on time course flow cytometry analysis, the *Leishmania* spp. life cycle is completed in three dividing phases, which are separated by non-dividing transmission stages. One of them is amastigote replication within mammalian phagocyte phagolysosomes. Then, the bloodmeal phase takes place in the sand fly abdominal midgut. This phase consists of procyclic promastigote replication followed by differentiation to nectomonad promastigotes. This is valid for suprapylarian species, which are grouped within the subgenus *Leishmania*. Peripylarian species (subgenus *Viannia*) begin development in the hindgut [30]. Nectomonads are non-dividing forms with an elongated flagellum which migrate towards the thoracic midgut. During the sugarmeal phase, they become leptomonads, which are able to divide. A few leptomonad promastigotes differentiate to metacyclic promastigotes, which are the highly infective stage (Figure 1A). Other forms like haptomonads and paramastigotes have been reported. This terminology is useful for understanding development. However, Gossage et al. [14] urge for finding molecular markers which may help in defining these stages more precisely. In *Leishmania* spp., the term metacyclic has been defined as the infective form or the end product of promastigote development within the sand fly vector [31], small rapid-swimming forms with an elongated flagellum differentiated from leptomonads [14]. Gossage et al. [14] highlighted the absence of parasite-sand fly interactions in axenic culture and warned about improper usage of the terms procyclics and metacyclics when identified with logarithmic and stationary phase promastigotes, respectively.

Bates [20], Dostálová and Volf [23] reviewed promastigote-sand fly interactions during development and the hypotheses about the metacyclic promastigotes transmission mechanisms. During the bloodmeal phase, blood is digested within the chitinous peritrophic matrix (PM) whereas embedded procyclic promastigotes proliferate [32]. Then, nectomonads accumulate at the anterior part of the matrix and are able to escape [33, 34] thanks to the chitinase secreted by the gut epithelium [35, 36]. Nectomonads are able to migrate forward and firmly attach to the gut epithelium microvilli. These facts contribute to explain why the sand fly is a true vector because promastigotes are not expelled during defecation and continue their developmental process. One of the attachment mechanisms in *L. major* within *P. papatasi* is the

lipophosphoglycan (LPG) interaction with gut epithelium galectins. However, the presence of LPG-receptors in other sand fly species remains unclear, and LPG-independent development has been reported. In fact, LPG composition is variable across species. The LPG together with certain proteophosphoglycans (PPG) may also have a major role in resistance to proteolysis within the gut (reviewed in [23]). Once nectomonads reach the SV, they become leptomonads and divide [14]. Leptomonads produce the promastigote secretory gel (PSG) [37], mainly composed of filamentous PPG [38], which also let them bind to the epithelium to some extent. A small fraction of leptomonads become haptomonad promastigotes [39], which tightly attach to the epithelium through hemidesmosome-like structures [40, 41], probably priming PSG plug formation [20] and/or favouring blockage [42, 43], while some others differentiate to metacyclic promastigotes [37]. This process is called metacyclogenesis and is defined as the transformation of poorly infective to highly infective promastigotes [28, 44]. In the sand fly gut, metacyclic promastigotes de-differentiate back into leptomonad-like promastigotes, which have been called retroleptomonad promastigotes, when a second blood meal is ingested by an infected sand fly. Interestingly, retroleptomonad promastigotes rapidly differentiate to metacyclic promastigotes, which causes an important increase in promastigote numbers and infectiousness [29]. Culture passage also causes promastigote de-differentiation (see below).

According to the blocked fly hypothesis, the PSG plug obstructs the SV until it is removed by regurgitation during blood meal intakes [45], being leptomonads embedded, and most metacyclics located in the plug poles [20]. A different hypothesis is passive inoculation of promastigotes found in the proboscis only [46-48]. Both hypotheses are not mutually exclusive because both mechanisms may participate in transmission [20]. In fact, low-dose and high-dose bite patterns have been observed and may correlate to the respective aforementioned transmission mechanisms [49]. In addition, chitinase-mediated damage was observed in the stomodeal valve [33], supporting the regurgitation hypothesis. The pharyngeal and cibarial pumps would contribute to the process [42, 43]. PSG high solubility explains why a few metacyclic promastigotes are released from the PSG plug pole when it contacts blood being ingested (reviewed in [50]). PSG and sand fly saliva egestion accompanying metacyclic

promastigotes probably play a role in the initial infection steps [51], including immune response modulation [52-54].

The phenotypical features of the different promastigote forms found in the sand fly gut differ between species. Separately studying each form is challenging. For example, the binding ability is strictly stage-dependent, as nectomonads and leptomonads are considerably bound to the epithelium according to the different mechanisms mentioned above and further explained in the next section, whereas procyclics and metacyclics are non-binding forms. Nonetheless, the relative binding ability is variable between different species, and in certain cases, a mild binding tendency has been observed in procyclics and metacyclics. For example, nectomonads bind tighter than leptomonads in *L. infantum*, whereas no substantial differences have been observed in the case of *L. mexicana*, and, unlike in *L. infantum*, *L. mexicana* metacyclics bind slightly tighter than procyclics[55].

Sand fly – *Leishmania* interactions

Few molecular interactions between *Leishmania* spp. and the sand fly gut have been revealed [23]. The innate immune response to pathogens has been profusely studied in insects, including receptors, signaling pathways, and effectors (antimicrobial peptides, reactive oxygen species (ROS), autophagy, etc.) [56-60]. Defensins, a caspar-like protein, and ROS were associated to innate immunity of the sand fly against *Leishmania* spp. [23, 61-65]. Midgut transcriptomic analysis in *Lutzomyia longipalpis*, *P. papatasi*, and *P. perniciosus* [66-69] revealed important data about molecules which potentially interact with *Leishmania* spp. molecules.

The bloodmeal induces digestive enzymes, fundamentally trypsins and chymotrypsins. These are serine-proteases [66-72] like other enzymes induced at the transcript level in the midgut, such as an alanyl aminopeptidase, a novel serine protease, astacin-like metalloproteases, and metallocarboxypeptidases [73]. Resistance to proteases is variable depending on the *Leishmania* species. This feature is crucial for vector competence, defining compatible and non-compatible vectors with a given *Leishmania* species [74-76]. At least half of the amastigote population transforming into immature promastigotes during the first hours of gut colonization

248 are killed, even in compatible species [37]. At the early development stages, the parasite is able
249 to control protease activity levels and timing [66, 72, 77-80] through gene expression
250 modulation and production of serine protease inhibitors (ISP) in the sand fly midgut when the
251 vector is compatible. The *L. major* genome encodes for serine protease inhibitors which do not
252 have targets in the parasite's proteome [18] but have been shown to be active against
253 mammalian host phagocyte's proteases [81] and trypsin activity from sand fly guts [82].
254 Amastigotes and metacyclic promastigotes are resistant to sand fly gut's proteases, but not
255 procyclic promastigotes, namely in the first 2-8 h of amastigote-to-promastigote transition [83].
256 Phosphoglycans (PG) and the secreted acid phosphatase (SAP) are essential for resistance [31].
257 For example, LPG acts as a shield against proteolytic activities.

258 The PM is composed of peritrophins, which contain one or more chitin-binding domains
259 (CBD), which has been predicted in most cases [66, 67, 69]. Multiple CBD peritrophins
260 probably cross-link PM chitin fibrils. PM formation is an extrinsic protection mechanism for
261 promastigotes during bloodmeal digestion [83, 84]. The sand fly midgut transcriptionally
262 regulates peritrophins in the presence of promastigotes [66, 67]. The PM starts to disintegrate
263 about 2 days after ingestion. A necessary but not sufficient condition for successful
264 promastigote development within the sand fly gut is PM breakage allowing nectomonad
265 promastigote release. This is not always possible depending on parasite and vector species, and
266 parasite's chitinase implication is controversial [33, 66, 67, 85-88]. Hemoglobin inhibits
267 *Leishmania* spp. chitinase. For this reason, the parasite is not able to escape the PM until blood
268 has been digested [89]. However, chitinases from a given *Leishmania* species are not able to
269 break the PM of all sand fly vector species, and not escaping from the PM leads to parasite
270 elimination through defecation. Therefore, this mechanism contributes to parasite-vector
271 competence [86].

272 Once nectomonads escape the PM, attachment to the gut epithelium is required to avoid
273 clearance and then progressively ascend throughout the gut. It has been shown that nectomonad
274 and leptomonad promastigotes specifically attach to the gut microvilli, and the mechanism
275 depends on the parasite-vector pair [55, 90]. A molecule involved in attachment is the

Leishmania spp. FLAG1/SMP1 flagellar protein [91]. According to these interactions, sand fly vectors are classified in restrictive, which are compatible with one or very few *Leishmania* species, and permissive, which support development of multiple *Leishmania* species [92-94]. The most studied parasite-sand fly interaction is the species and strain-specific *Leishmania* LPG – sand fly midgut galectin attachment mechanism [95, 96]. This interaction has been only demonstrated in the *L. major* Friedlin V1 strain – *P. papatasi* or *P. duboscqi* pairs, but other *L. major* strains are not able to bind. The LPG is composed of a glycosylphosphatidyl inositol (GPI) anchor and a glycan backbone composed of phosphoglycan (PG) units and attached to the anchor through a hexasaccharide core [97]. Side chain composition varies depending on the species and strain [98]. Monogalactosylation is the optimal pattern for galectin recognition, which has been shown through engineered *L. donovani* for galactosylation pattern optimization [99]. Also, LPG side chain composition is stage-dependent. Arabinose residues are cap side chain galactose residues in *L. major*, thus allowing promastigote release from galectins [98]. Alternative interaction mechanisms remain undiscovered. Galectins are absent in the midgut of permissive species such as *Lu. longipalpis* and *P. perniciosus* [66], which allow survival of LPG-deficient *L. major* and *L. mexicana* promastigotes in their guts in an LPG-independent manner [23, 45]. However, this is controversial because other authors reported that LPG composition mediates *Leishmania* spp. competence in different vectors [100]. This statement was hypothesized to be valid only in specific vectors [101]. While LPG-based attachment - release mechanisms in different *Leishmania* spp. - sand fly pairs have been reported, the receptors have not been identified yet (see next section). In summary, it is known that different mechanisms mediate attachment of nectomonad promastigotes to the sand fly gut microvilli, but most remain uncharacterized, and there is controversy about LPG roles in different parasite species.

Finally, the sand fly gut conditions may contribute to promastigote differentiation. An acidic environment, nutrient depletion, and probably scarce tetrahydrobiopterin levels induce metacyclogenesis. In this process, endosome-sorting and autophagy are essential [102], as well

as several *L. major* proteins of unknown function encoded in the HASP/SHERP gene cluster (hydrophilic surface proteins and small hydrophilic ER proteins) [103].

The axenic culture model: strengths and limitations

The first axenic culture of *Leishmania* parasites was performed by Nicolle in the Nicolle-Novy-McNeal medium [104]. Since then, an increasing number of culture media has been developed, leading to easy, fast and highly productive promastigote cultures. Regarding cell cycle and differentiation, promastigote populations in axenic culture, like in the sand fly gut, are complex and asynchronous. It is assumed that development within the sand fly gut is mimicked in axenic culture at 26-27ºC in undefined media containing heat-inactivated mammalian serum [105-110]. Stationary phase promastigotes are infective despite the absence of parasite-sand fly interactions. However, cultured promastigotes are less infective than metacyclic promastigotes obtained from the sand fly gut, at least in *L. infantum* and *L. major* [7, 25, 111]. In fact, infectivity is attenuated as the number of culture passages increases. For this reason, passages through laboratory animals are required (reviewed in [109]). These observations highlight the importance of the promastigote-sand fly interactions and suggest that adaptation to the culture conditions results in a progressive loss of the infective properties. Like in the sand fly gut, promastigote populations are heterogeneous in culture, and only a small fraction is metacyclic. The most widespread and successful method to isolate subpopulations of metacyclic promastigotes from cultures is based on LPG agglutination in the presence of the PNA. During metacyclogenesis, the LPG is modified, which leads to the loss of agglutination capability in the presence of PNA [22]. The modifications consist of adding α-D-arabinopyranose residues to the β1,3-D-galactose residue (βGal) side chains [112, 113]. Therefore, the PNA metacyclic selection method is negative. The agglutinating (PNA^+) subpopulation is less infective than the non-agglutinating (PNA^-) subpopulation in *L. major*, *L. infantum* [22, 24]. However, the LPG structure in *L. infantum* [114], including *L. infantum chagasi* [115], is different and varies depending on the strain, including side chains composed of glucose monomers or oligomers [114]. The LPG of a sudanese *L. donovani* strain agglutinates at early differentiation stages when in contact with PNA [113, 116, 117] but

metacyclic forms fail to agglutinate [24, 113, 117-119]. *L. infantum* PNA^- promastigotes are more infective than PNA^+ promastigotes [24] and the whole stationary phase population [25], which suggests that the LPG participates in alternative attachment mechanisms. Soares et al. [115] reported an *L. infantum* LPG - *Lu. longipalpis* midgut epithelium interaction based on PG receptors. The interaction is based in β1,3-glucosylation, and release is caused by glucose residue removal. The same mechanism was previously described for an indian *L. donovani* strain and the vector *P. argentipes* [117]. To add more complexity, the mechanism is opposite in *L. braziliensis* because glucose residue addition leads to *ex vivo* detachment from *Lu. longipalpis* gut explants [120]. In summary, the LPG - gut interaction and release mechanisms differ between species and are not related to PNA-based separation of procyclics and metacyclics. The minimum agglutinating amount of PNA is variable between *L. infantum* strains starting at 50µg/mL [24, 118]. The different LPG composition in the aforementioned species explains these observations. Interestingly, PNA^- and PNA^+ forms can be isolated in the monogenetic trypanosomatid *Crithidia fasciculata* [121], but the implications for life cycle understanding are unknown.

In vitro infection experiments of the human myeloid U937 cell line with *L. infantum* promastigotes have shown that the LiPro-PNA^- metacyclic subpopulation is more infective than the agglutinating LiPro-PNA^+ and the whole population in stationary phase of axenic culture (LiPro-Stat) from where both are isolated [24]. The same approach has revealed that LiPro-Stat and LiPro-PNA^- are less infective (~50% and ~20-30%, respectively) than promastigotes isolated from the stomodeal valve of the sand fly vector *P. perniciosus* (LiPro-Pper) [7, 25, 26]. Sand fly metacyclics are found in the SV vicinity. In the case of the *P. perniciosus-L. infantum* vector-parasite pair, the proportion of metacyclic promastigotes in culture [24, 25] and within the sand fly gut [27] is approximately equal (~10%). The percentages are much higher, up to 90%, in other parasite and vector species [28, 29]. Culture passage also affects the yield in metacyclic promastigotes [28]. Therefore, higher infectivity levels of sand fly-derived promastigotes isolated from the SV are explained by a more advanced differentiation status (i.e.,

these promastigotes are more "metacyclic in character") rather than a simple enrichment in metacyclics.

Considering how challenging working with promastigotes from the gut is, the cost-benefit balance presumably tilts to axenic culture in principle, but this is not as clear when considering results obtained by means of transcriptome analysis. Alternative methods for isolation of metacyclic promastigotes like centrifugation in Percoll gradient have been described, which are out of the scope of this review.

Transcriptome analysis of sand fly-derived promastigotes: technical considerations and current datasets

Microarrays are dense molecular probe matrixes on a solid surface. DNA microarrays contain thousands of genes, gene fragments and/or non-coding sequences which are hybridized with one or more labelled nucleic acid samples (DNA, cDNA, or RNA) for different purposes such as gene expression profiling. In this case, total RNA or mRNA samples are directly labelled, amplified and labelled, or reversely transcribed in order to obtain directly or indirectly labelled cDNA. The fluorescent labels enable measuring the relative levels of each target sequence once emission signals have been acquired with a specialized scanner (Figure 2). Bioinformatics analysis is relatively simple because probes are usually identified beforehand, and just two basic steps are required: normalization and statistical analysis of differential gene expression. More technical details on microarray analysis can be found in Mantione et al. [62] and Lowe et al. [63] reviews. A review of the DNA microarray technology impact in *Leishmania* research is also available [65].

RNA sequencing (RNA-seq) is a high-throughput approach based in Next Generation Sequencing (NGS) which consists of genome-scale amplification and NGS of short cDNA fragments generated from RNA samples. For this purpose, double-stranded cDNA is generated and PCR amplified, incorporating appropriate linkers for NGS. The primers used in all steps and the PCR conditions are designed according to the desired fragment size range, which is typically comprised between 0.1 and 1Kbp. The products are fragmented and subjected to NGS

in any of the platforms commercially available (464-pyrosequencing, Illumina, Ion Torrent, etc.) (Figure 2). Alternatively, fragmented RNA can be used as the input in the library preparation protocol. Bioinformatic analysis is complex because up to 300bp reads [122] must be mapped on the genome sequence, which requires demanding skills. Further information on technical details has been reviewed by Mantione et al. [62] and Lowe et al. [63].

Nowadays, transcriptome analysis is a routine technical approach thanks to the development of the DNA microarray technology during the mid-1990s, which has been extensively used during the last two decades and is being replaced by RNA-seq. At this point, it is important to remark that the condition of a technical approach to be valid is reliability rather than novelty. Both DNA microarray hybridization analysis and RNA-seq are reliable for gene expression profiling or differential gene expression analysis (DGE), although RNA-seq is a more powerful and robust approach [123, 124]. Microarrays and RNA-seq are technically reproducible (>99%) and accurate (~90%) high-throughput approaches. Both can detect splice variants. However, RNA-seq requires much less input RNA sample amount to reach the same genome coverage, is ~1,000 times more sensitive, and is characterized by lower background levels and a dynamic range ~100-1,000 times higher. In addition, RNA-seq is appropriate for SNP detection and UTR analysis, and does not necessarily require a reference genome sequence [67, 123, 124].

Before execution of a DGE analysis, biological samples must be examined to determine whether they are appropriate to address the proposed hypothesis. For example, the main features of metacyclic promastigotes are high infectivity and morphology (fusiform, small size, showing an elongated flagellum). Therefore, metacyclic promastigotes can be identified for downstream DGE by infection experiments (Figure 1B and C) [7, 25, 26] or morphological features [125]. Promastigotes de-differentiate once isolated because they are non-dividing forms [14]. In fact, *Leishmania* spp. is adapted to respond very quickly to different environments [126]. Considering the replacement principle, experimentation with animals can be substituted by *in vitro* infection of established myeloid cell lines. Given the scarce number of promastigotes obtained from each sand fly, each sample should be composed of a mixture of promastigotes

from different sand flies. A fraction of the sample should be immediately processed for RNA isolation upon extraction from the gut (e.g. lysed in Trizol reagent), and the remaining fraction used as soon as possible for the infection experiment [7, 25, 26]. As a difference with RNA-seq, which always includes a PCR amplification step, DGE based on the DNA microarray technology is not suitable for very low input samples unless RNA is amplified. Thanks to RNA amplification, as low as 20ng of LiPro-Pper total RNA per replicate sample were sufficient to conduct transcriptome comparisons with intracellular amastigotes, stationary phase promastigotes, and PNA^- promastigotes using microarray analysis [7, 25, 26]. Reliability of microarray results is not compromised by RNA amplification as suggested otherwise [125]. In fact, reliability is improved regardless of whether it is required for sample expansion [127-129]. The amplification procedure consists of double-stranded cDNA synthesis starting from a poly(T) oligonucleotide incorporating the T7 promoter sequence upstream, followed by linear amplification by means of *in vitro* transcription (IVT) with the T7 RNA polymerase, obtaining reverse complement RNA molecules ready for synthesis of labelled cDNA and subsequent hybridization with shotgun or oligonucleotide DNA microarrays. Preparation of RNA-seq libraries also requires synthesis of double-stranded cDNA and amplification, and the *L. major* RNA input was 5-20ng [125]. The basic conceptual difference relies on PCR instead of IVT for required amplification for subsequent processing through high-throughput sequencing or labelled-cDNA synthesis and hybridization, respectively (Figure 2). Primer design is performed according to each high-throughput sequencing platform (e.g. Illumina adaptors and sequencing primers). Moreover, index sequences can be added for multiplexed sequencing. RNA-seq data analysis demands considerably more bioinformatics skills and computer resources than microarray analysis does [123].

The presence of tissue from the sand fly host should be minimized when isolating the biological sample. Microarray cross-hybridization controls were performed to select specific hybridization conditions and remove the few cross-hybridizing spots found from analysis [7, 25, 26]. Specific sequence alignment against the parasite's genome sequence would presumably remove most noise from sand fly sequences, but it may interfere in quantification of conserved

sequences. Spliced-leader RNA-seq (slRNA-seq) is a fast, simple and selective method that overcomes this inconvenience without biasing the results which would be obtained otherwise with a regular RNA-seq procedure [130, 131]. slRNA-seq allows for low input amount of *L. donovani* RNA (1ng) samples embedded in a human RNA amount 1,000 times larger, although these samples should be sequenced more deeply to reach the same coverage as for pure *Leishmania* spp. RNA [130]. Once analysis is completed, validation of certain results by qPCR or Northern blot may be convenient. Even when the transcript levels have been validated, they do not quantitatively correlate to the protein levels in about 75% cases [132]. Unfortunately, transcriptome analysis is the only feasible omics approach for sand fly-derived promastigotes so far due to sample amount requirements for translatome and proteome analysis (see below). The number of qualitative RNA-protein level coincidences (up-regulation, down-regulation, and constant expression at both levels) in Lahav et al. [132] datasets is about 60%. This suggests that at least one third of the changes in transcript levels will not be reflected in protein levels. Groups of functionally related genes showing transcript level variation in the same sense (up-regulation or down-regulation) in the biological process under study will be more likely reflected at the protein level. This is also variable depending on the life cycle stages analyzed. For example, lower RNA-protein correlation has been observed across organisms under stress situations, fundamentally the differentiation processes of procyclics to metacyclics and metacyclics to amastigotes (reviewed in [133]). Messenger RNA changes not correlated to protein levels may be also important for regulation of steady-state transcript levels. Mature RNA can be immediately used for protein synthesis or be stabilized and indefinitely kept translationally inactive (reviewed in [134]). Modulation of translational efficiency is an additional gene expression regulation mechanism [135].

Four DGE analyses of *L. infantum* promastigotes obtained from experimentally infected *P. perniciosus* within the vector [7, 25, 26, 69] and one of *L. major* from *P. duboscqi* [125] have been performed (Table 1). An slRNA-seq analysis of heterogeneous populations has also been published [69]. The outcomes of these studies are considerably different fundamentally because the comparisons are not equivalent. First, *L. infantum* is responsible for

zoonotic visceral leishmaniasis in the Mediterranean basin and South America, whereas *L. major* is responsible for cutaneous leishmaniasis in the Old World. Their different affinity for sand fly vector species and in key developmental processes (e.g. attachment of nectomonads to the gut epithelium; see above) is a probable cause of obtaining mismatched DGE. Second, the samples compared and most comparisons themselves are not equivalent. For example, intracellular *L. infantum* amastigotes obtained *in vitro* from the myeloid human U937 cell line [26] are not equivalent to intracellular *L. major* amastigotes obtained from mice footpad lesions (LmAM) [125]. As it could be expected, the number of ≥2-fold differentially expressed genes (DEG) was 2.4 times greater in the latter, where more complex biological samples represented not only the parasite and the host cell themselves, but also the complex interactions with other immunological components. In both cases, DEG data were referred to *L. infantum* (LiPro-Pper) and *L. major* sand fly metacyclic promastigotes (LmSFMP). In the first case [26], they were isolated from the anterior pole of the PSG plug in contact with the SV because this location is enriched in metacyclics and their infectivity was checked by using the *in vitro* infection model (see above). Haptomonad promastigotes are also present in any residual material carried over from the SV structure (Figure 1B). In the second case, procyclics, nectomonads and metacyclics were isolated from different guts and processed individually, assuming that the populations were homogeneous after 2, 4 and 15 days of development, respectively. The whole guts were macerated and promastigote populations quantified with a hemocytometer and morphology examined. Only samples that were supposed to have >90% stage homogeneity were included for analysis [125]. However, squeezing whole guts does not necessarily guarantee homogeneity of populations even when timing is expanded, because different parasite forms are always remaining in the gut. For example, Killick-Kendrick et al. [27] did not find more than 10% metacyclics of *L. infantum* in the gut of *P. perniciosus* even 8-15 days after bloodfeeding from infected dogs. As mentioned above, this is dependent on the parasite-vector pair. In summary, all populations analyzed in the studies listed in Table 1 are homogeneous, with the exception of the study comparing heterogeneous populations on purpose [69]; but complete sample homogeneity is impossible to reach nowadays. An alternative analysis strategy is single-cell

genomics. Unfortunately, molecular markers are not available for metacyclic promastigotes, which are the result of metacyclogenesis. HASP and SHERP are metacyclogenesis markers (i.e. they are expressed not only in metacyclic promastigotes but also in intermediate stages) in *L. major* [103]. For these reasons, comparisons of LiPro-Stat with LiPro-Pper and LiPro-PNA$^-$ with LiPro-Pper [7, 25] are not equivalent to comparisons of LmSFMP with sand fly procyclics (LmSFPP) [125] or culture metacyclics (LmCMP) *vs.* log phase promastigotes (LmPro-Log) [136]. For example, amino acid transporters aATP11 were up-regulated in LmSFMP *vs.* LmSFPP and in nectomonad promastigotes (LmSFNP) *vs.* LmSFPP [125], but it was not observed in LiPro-Pper *vs.* LiPro-Stat possibly because LiPro-Stat populations could contain nectomonad-, leptomonad- and metacyclic-like forms [21]. Consistently, no aATP11 was differentially regulated when comparing LiPro-Pper and LiPro-Stat either [25]. Not only the experimental design is different in order to answer different biological questions, but also, the parasite-vector models are different in many instances. For example, only one kind of promastigote-sand fly gut interaction is clearly known so far, which is the LPG-galectin binding mechanism, only demonstrated in the *L. major-P. papatasi* and *L. major-P. duboscqi* pairs (reviewed in [23]). Another example is the gut microbiota, which has been shown to favor promastigote differentiation in *Lu. longipalpis* [137] but may be different in other sand fly species. In summary, generalization across *Leishmania*-sand fly models should be cautiously considered case-by-case, and different experimental settings should be taken into account when comparing DGE studies. An example of correct generalization is the HASP/SHERP cluster, gp63, and autophagy genes in *L. major* and *L. infantum* (see next section).

The across-experiment comparison of LmSFMP/LmSFPP and LmCMP/LmPro-Log [125] is presumably robust even when the technical RNA-seq approach is not exactly the same, as supported by the methodological study on meta-analysis of RNA-seq expression data by Sudmant et al. [138]. Only 26 DEG were claimed to differ between both datasets, but actually, the number of genes differentially expressed ≥2-fold at a statistical level of significance $\alpha = 0.05$ is 398 in LmSFMP/LmSFPP [125] and only 108 in the case of LmCMP/LmPro-Log [136], of which 72 are not coincident. In the case of *L. infantum*, the number of DEGs found in the

direct comparison of LiPro-Pper with LiPro-PNA$^-$ was 285 at the cutoff expression values mentioned above [25], comparable to the number of LiPro-Pper/LiPro-Stat DEGs [7]. Most DEGs were different between both *L. infantum* datasets, which reflects the above mentioned differences found in infectivity between these promastigote populations (LiPro-Pper> LiPro-PNA$^-$ >LiPro-Stat). All *L. major* and *L. infantum* datasets are different because different stages have been compared in each case. For example, the LmSFMP/Lm-SFPP DGE analysis is not comparable to the LiPro-Pper/LiPro-Stat study because cultures in stationary phase mostly contain nectomonads and metacyclics [21] and probably low amounts of procyclics and leptomonads. In an slRNA-seq analysis of *L. infantum* comparing heterogeneous populations of sand fly promastigotes (LisfPro) [69], taken from the whole gut of *P. perniciosus*, with the heterogeneous promastigote populations in axenic culture (LiacPro), we observed ~950 genes up-regulated ≥2-fold, which is 2.0 to 3.6 times higher as expected, compared to the previous DGE datasets about more homogeneous promastigote populations showing approximately 300 DEGs each [7, 25, 125]. Therefore, the DGE rates are relatively not very high in *Leishmania* spp., including homogeneous and heterogeneous populations (maximum ~1,000 DEGs out of ~8,300 genes annotated in the genome sequences). In summary, global concordances and differences between studies on sand fly-derived promastigotes have been found, but comparative interpretation of studies should be cautious considering different biological comparisons, sample source origin and preparation, and technical approaches.

What has transcriptome analysis taught?

The microenvironment influences parasite's differentiation [7, 125]. Steady-state transcript level changes of the glucose-6-phosphate N-acetyltransferase, the cytochrome oxidase subunit VI, the vacuolar proton-translocating pyrophosphatase, and the amastin superfamily genes when comparing promastigotes with amastigotes (all decreasing in amastigotes except for the amastins) were observed when promastigotes were obtained from the sand fly's SV [26] and from cultures [8]. However, most DEGs between LiPro-Stat and amastigotes are not coincident with DEGs between LiPro-Pper and amastigotes. Up-regulation of several amastin superfamily genes in metacyclics from the sand fly with respect to metacyclics from culture in *L. infantum*

553 [25] and with respect to sand fly procyclics in *L. major* [125] is additional evidence supporting
554 the pre-adaptation hypothesis [8, 13, 26, 139-142], which consists of promastigote preparation
555 in advance towards life within the host phagocytes, i.e. the amastigote stage. The highest levels
556 of amastin transcripts are found in amastigotes when compared to both sand fly-derived
557 promastigotes [26, 125] and cultured promastigotes [8].

558 Cell cycle-related genes are generally down-regulated in LmSFMP and LmSFNP
559 compared to LmSFPP and LmAM, which is in agreement with the replicative or non-replicative
560 status of these stages [125].Steady-state transcript level comparisons between procyclic and
561 metacyclic promastigotes in the sand fly gut (LmSFMP *vs.* LmSFPP) [125] and in culture
562 (LmCMP *vs.* LmCPP) [136] resulted in relatively similar results because few differences were
563 found between both studies. This includes transporters (pteridine transporter, nucleoside
564 transporter 1, glucose transporters lmgt1 and lmgt2, amino acid transporters, and ABC10),
565 signaling molecules (phosphoprotein phosphatase, and protein kinase LmjF.26.2570) calpain-
566 like cysteine peptidase LmjF.30.2040, inosine guanosine nucleoside hydrolase, P27 protein,
567 H2B and H4 histones, 4E-interacting protein LmjF.25.2450, the membrane-bound acid
568 phosphatase 2 (MBAP2), and several hypothetical protein-encoding transcripts.

569 Many genes involved in metacyclogenesis (see below) are highly up-regulated in
570 heterogeneous populations of sand fly-derived promastigotes (LisfPro) compared to cultured
571 promastigotes (LiacPro) [69] but not in more homogeneous metacyclic populations (LiPro-Stat
572 *vs.* LiPro-Pper, LiPro-PNA- *vs.* LiPro-Pper, and LmSFMP/LmSFPP *vs.* LmCMP/LmPro-Log)
573 [7, 25, 125]. Comparing *L. infantum* heterogeneous populations composed of all promastigote
574 development forms from the sand fly (whole gut preparations) and culture (growth curve
575 mixtures), we also observed gp63 and autophagy genes up-regulated [69], as well as the
576 HASP/SHERP cluster. As mentioned above, these genes are essential for metacyclogenesis at
577 least in *L. major*. In fact, Inbar et al. [125] results are in agreement because gp63 and autophagy
578 gene up-regulation was found in LmSFNP. In addition, they found that LPG3, a gene essential
579 for biosynthesis and assembly of GPI-anchored glycoconjugates, reaches its expression peak in
580 LmSFPP. Sand fly-derived populations enriched in metacyclics (LiPro-Pper) are more infective

than stationary phase cultures (LiPro-Stat) and metacyclics obtained from those populations (LiPro-PNA$^-$) [7, 25]. Autophagy, gp63, and HASP/SHERP gene cluster up-regulation in sand fly-derived promastigotes compared to cultured promastigotes supports that metacyclogenesis is more successful in the sand fly gut than in culture. Therefore, the microenvironment exerts an important influence in differentiation [7].

SHERP is essential for metacyclogenesis in *L. major* [103]. Inbar et al. [125] revealed evidence supporting this statement which consists of SHERP up-regulation in LmSFNP and LmSFMP, reaching maximum levels in LmSFMP. *L. infantum* transcriptome analysis is also in agreement with the role in metacyclogenesis, but SHERP transcripts are less abundant in LiPro-Pper than in LiPro-Stat [7], indicating that the levels are higher in nectomonads and leptomonads in culture (major forms within the stationary phase compared to metacyclics) than in sand fly-derived metacyclics. SHERP is not differentially expressed between LiPro-Pper and LiPro-PNA$^-$ indicating that different microenvironments do no influence SHERP expression in *L. infantum* [25]. Stationary phase promastigote cultures mostly contain nectomonad promastigotes [21], whereas most promastigotes derived from the sand fly's SV and isolated using the PNA negative selection method are metacyclic. HASPA1 is also down-regulated in LiPro-Pper *vs.* LiPro-Stat [7], leading to the same conclusion about metacyclogenesis because this is also an essential gene for this process (see above). *L. infantum* sfPro *vs.* acPro (heterogeneous populations) transcriptome analysis is also consistent with the previous studies because SHERP is up-regulated in sfPro (i.e., metacyclogenesis taking place more extensively in sand fly than in culture). Interspecies comparison should be cautious, as previously mentioned. SHERP data are concordant between *L. major* and *L. infantum* with the previously established idea about essentiality for metacyclogenesis, but simultaneously, transcriptome analysis has revealed specific differences.

Genes involved in fatty acid biosynthetic processes are up-regulated in sand fly-derived metacyclics in both *L. infantum* and *L. major* [7, 125] but the highest levels of these transcripts are reached in LmSFNP. According to DGE, glucose catabolism may be more pronounced not only in LmSFPP than in LmSFMP [125], but also in cultured than in sand fly-derived

promastigotes (LiPro-Stat *vs.* LiPro-Pper) [7]. Certain amino acid biosynthesis processes seem more active in culture according to DGE [7]. Genes involved in ATP synthesis-coupled proton transport are up-regulated in sand fly metacyclics (LiPro-Pper *vs.* LiPro-Stat and LiPro-Pper *vs.* LiPro-PNA$^-$). According to relative infectivity (LiPro-Pper>LiPro-PNA$^-$>LiPro-Stat), sand fly metacyclics are "more metacyclic" than culture metacyclics. These findings are consistent with the considerable energy requirements for high motility ascribed to metacyclic promastigotes [14].

Confrontation of the transcriptomes and infectivity of sand fly-derived promastigotes with cultured promastigotes [7] is in agreement with the principle of non-equivalence of stationary phase promastigotes supported by Gossage et al. [14]. Both transcriptomes showed moderate correlation in gene expression and 286 DEGs, and infectivity was ~30-50% higher in LiPro-Pper. On the basis of these results, it was postulated that the adequacy of axenic promastigotes may depend on each particular experimental aims and design [7]. The characteristic transcriptome profiles found in LmSFPP, LmSFNP, and LmSFMP [38] are presumably a consequence of their adaptation to the different microenvironments in the vector as well. In fact, 72 out of the 108 DEGs found in LmCM/LmPro-Log [136] were not found among the 398 DEGs found in LmSFMP/LmSFPP, as stated above. Inbar et al. [125] performed LmSFMP *vs.* LmSFPP differential expression analysis and compared data with an analogous experiment using cultured parasites (LmCMP *vs.* LmCPP) [136]. Both studies were performed using the same RNA-seq procedure. These data are not comparable to LiPro-Pper *vs.* LiPro-PNA$^-$ promastigotes because this is a direct comparison [25] and these populations are not normalized to their corresponding past procyclic promastigote forms. In other words, directly comparing sand fly-derived and culture-derived metacyclics does not correspond to comparing the differences between metacyclics and procyclics in both environments, unless procyclics from culture were exactly equal than procyclics in the sand fly, which is very unlikely. Different isolation methods may also influence the results (see the previous section).

A considerable number of the DEGs are involved in signal transduction and gene expression regulation at the post-transcriptional, translational and post-translational levels

between cultured and sand fly-derived promastigotes [7, 25]. However, the biological implications of these findings remain unknown (see below). The finding which consists of translational efficiency being lower in differentiated non-dividing metacyclic epimastigotes than in undifferentiated dividing *Trypanosoma cruzi* epimastigotes [143, 144] should guide interpretation.

Promastigotes constitutively secrete exosomes to the sand fly gut lumen. Co-inoculation of cultured *L. major* promastigotes with sand fly gut-derived *L. major* exosomes leads to greater footpad lesions in mice [145]. These exosomes contain gp63 and other virulence factors [146-150]. These studies indicate that parasite's exosome content has signaling-inducing and immunomodulatory activities. Exosomes are secreted from multivesicular bodies (MVBs) and the flagellar pocket. Protein content of culture- and sand fly-derived promastigote exosomes is very similar [145]: gp63, which is secreted in the midgut and contributes to egestion [151]; HSP70 [152] and HSP83 [145]; calpain-like cysteine peptidases [153]; tryparedoxin peroxidase [154]; and surface antigen proteins [155]. Transcripts encoding for these proteins were also found increased in sfPro *vs.* acPro [69].

Unanswered questions about development and metacyclogenesis within the sand fly gut

Metacyclic promastigotes are defined by morphology, but their molecular features are not entirely known. PNA separation is effective to obtain highly infective promastigotes because PNA^- promastigotes are more infective than PNA^+ in both *L. major* [22] and *L. infantum* [24], but the subpopulations obtained by this procedure may not be entirely equivalent in other species. A major LPG role in parasite-vector interaction is well defined only for *L. major*, whereas the parasite-interaction mechanisms remain unknown in all other species. LPG-independent promastigote development has been demonstrated in permissive vector species (see above). However, highly infective (therefore metacyclic) promastigotes isolated using the PNA^- negative selection procedure is possible in *L. infantum* [24, 118, 156], which usually develops in permissive vectors such as *P. perniciosus*. Alternative unknown mechanisms participate in

recognition because LPG is not strictly required for development, and the importance of this molecule is relegated to *L. major* only [31]. However, it is produced in all *Leishmania* species. Unknown PG receptors recognize the LPG in the sand fly gut [115, 117], which has at least an additional function acting as a shield against proteolytic activity during the first *L. major* development stages (see above), and presumably in *L. infantum* because both contain the key repeated [Gal-Man-PO_4] motif in the LPG structure [114]. Variation of the LPG structure (see above) at the last stages towards the metacyclic stage makes negative selection with PNA possible in both species. Surprisingly, PNA^- and PNA^+ subpopulations could be isolated in the monoxenous parasite *C. fasciculata* [121] a fact of unknown meaning suggesting that PG-derivatives capable of agglutinating with the PNA may have more than one function. Studying LPG function in *C. fasciculata* may lead to raising other approaches for searching LPG interactions and alternative functions in different *Leishmania* species. High-throughput comparative metabolomics approaches may be useful to answer these questions, but not transcriptomics approaches. Bearing these considerations in mind, we suggest that the role of the modified LPG at this stage may not be necessarily the same between species as already shown for the unmodified LPG at earlier stages. Consequently, we postulate that the "metacyclic status" of PNA^- from *L. infantum* may not be necessarily the same as for PNA^- from *L. major*, as the molecular markers and infection mechanisms may be different depending on the species. This is not surprising because each species complex causes different pathology, and accurate measurements comparing metacyclic promastigote infectivity of each species are not possible. The peanut lectin has different affinity for LPG from a distinct origin, as different substitutions of the molecule disaccharide backbone are found depending on the species (see above). In any case, *L. major* [22] and *L. infantum* PNA^- promastigotes [24] have been demonstrated to be more infective than PNA^+ promastigotes.

When comparing the heterogeneous populations LisfPro and LiacPro by slRNA-seq, a group of genes directly involved in metacyclogenesis were found to be highly up-regulated (≥4-fold) [69], which suggests that they are required during most stages of the developmental process within the sand fly gut compared to culture, not just at the last developmental stages.

This includes 5 out of 14 autophagy genes, 4 out of 8 gp63 genes, the HASP protein cluster (HASPA1, HASPA2, HASPB, respectively LinJ.23.1200, LinJ.23.1220, and LinJ.23.1240), 1 out of 3 membrane-bound acid phosphatases (LinJ.28.2850), all three apical membrane antigen 1 (ama1, LinJ.30.1470, LinJ.30.1480, and LinJ.30.1490) proteins, and the META domain-containing protein (META2, LinJ.17.0970) gene. Both small hydrophilic surface protein-encoding gene copies (SHERP, LinJ.23.1210, and LinJ.23.1230) are not included in the LisfPro *vs.* LiacPro differentially expressed gene set according to the 2-fold threshold value imposed, but still show statistically significant ~1.5-fold higher levels in sfPro *vs.* acPro [69]. Whereas SHERP is clearly up-regulated in *L. major* metacyclics (LmSFMP *vs.* LmSFPP and LmCMP *vs.* LmPro-Log), and to a lower extent in nectomonads (LmSFNP *vs.* LmSFPP) [125, 136], different expression profiles supporting an over-expression maximum in nectomonads (LiPro-Pper *vs.* LiPro-Stat) [7] (see the reasons in the previous section), were observed in *L. infantum*. While the specific SHERP expression profiles are different, both are concordant with SHERP essentiality in metacyclogenesis [103]. Cultured and sand fly-derived *L. infantum* and *L. major* metacyclics differentially regulate SHERP expression (LiPro-Pper *vs.* LiPro-PNA^{-}, and comparison between LmSFMP *vs.* LmSFPP and LmCMP *vs.* LmCPP). Interestingly, both SHERP genes are up-regulated in LiPro-Stat *vs.* LiPro-Log of this species according to microarray analysis [8] and further confirmation by qPCR in two independent works [24, 157]. This is equivalent to state that the set of nectomonads, leptomonads, and metacyclics up-regulate SHERP compared to procyclics. SHERP is a good metacyclogenesis marker but not a metacyclics marker because it is over-expressed in more than one promastigote form (nectomonads and metacyclics). The data suggest that the SHERP gene expression patterns are similar between *L. major* and *L. infantum*, except for the promastigote form reaching the maximum expression levels, which peak earlier in *L. infantum* than in *L. major*. This would not be surprising whenever confirmed in the future, given the different biological affinity for vectors and different developmental processes of both species, resulting in different disease progression in mammalian hosts. These observations are in agreement with the fact that metacyclic promastigote features and behavior may vary between species and are not entirely

known. For example, they are highly infective, or more infective than other promastigote forms, but how much? Which molecules are true markers of metacyclics in each species?

The META1 gene was described to be expressed specifically at the metacyclic stage in culture, but the high-throughput DGE studies of *L. infantum* and *L. major* have not confirmed this result at the transcript level in sand fly-derived promastigotes [7, 25, 125]. As mentioned before and discussed below, studies at the protein level like Western blot or proteomic approaches are not viable so far. About half of the genes annotated in the *Leishmania* spp. genomes encode for hypothetical proteins, most of unknown biological role in the parasite. These observations provide an idea of how little is known about development within the sand fly vector.

Elucidation of processes involving the unknown relationship between external stimuli from the microenvironment, the parasite's uncharacterized sensing and intracellular signaling mechanisms and the unusual gene expression regulation mechanisms found in these organisms (reviewed in [134, 158]) may probably help to further understand promastigote development within the sand fly gut. For these purposes, elucidation of signal transduction pathways and the underlying mechanisms affecting gene expression regulation is essential because more crucial genes in development may be found.

Translatome and proteome analysis: a major challenge

In an experiment combining DGE analysis by means of DNA microarrays and quantitative proteomics with polysome profiling in *L. donovani*, Lahav et al. [132] observed that gene expression regulation is performed at the post-transcriptional, translational and post-translational levels, leading to find that only 25% transcript levels were quantitatively correlated with the corresponding protein levels, as mentioned above. Therefore, DGE at the translational and post-translational levels is more directly related to physiological changes of the different life cycle stages than at the post-transcriptional level. A complete picture of DGE would be provided by combined transcriptome, translatome and proteome analysis. Polysome profiling is an approach for measuring translational efficiency which consists of separation of mRNA-ribosome complexes (polysomes) according to their molecular weight by means of density

748 gradient centrifugation for subsequent quantification of the fractions and high-throughput
749 analysis of the mRNA molecules in each fraction. The procedure requires about ~4 $x10^8$ cells
750 (50mL at an OD_{600nm}=0.6) in the case of *Saccharomyces cerevisiae* [159]. As the average cell
751 volume of this yeast species is ~900μm^3 and the average volume of a *Leishmania* spp. cell is
752 ~65-75μm^3, about 10 times more promastigotes or amastigotes would be required in principle.
753 Ribosome profiling is a more specific high-throughput approach for measurement of
754 translational efficiency. Protection of mRNA sequences by ribosomes is quantified by means of
755 NGS from a ribosome footprinting library combined with a fragmented mRNA library [160].
756 The first ribosome profiling studies in trypanosomatids have revealed that changes in protein
757 production between slender bloodstream and procyclic stages of *T. brucei* are more extensive
758 than indicated by transcriptome profiling [135, 161]. In these approaches, at least 10^9 parasites
759 per sample were used to generate the ribosome footprinting and the fragmented mRNA library.
760 Jensen et al. [135] also mapped the 5' ends of mRNAs by means of slRNA-seq. The same
761 general finding was reported for *T. cruzi* [144], where a higher amount of parasites was used.
762 Consequently, ribosome profiling is not viable for studies in *Leishmania* spp. promastigotes
763 obtained from the sand fly so far. In fact, as high as ~10^4 infected sand flies would be required
764 to obtain enough promastigotes for a replicate of a ribosome profiling experiment, and many
765 more sand flies would be required for ribosome profiling of more homogeneous populations, for
766 example, ~10^6 for metacyclics.
767 Typical samples for proteome analysis require ~1-2$x10^8$ *Leishmania* spp. cells for both
768 two-dimension electrophoresis-based strategies [162] and quantitative proteomics strategies
769 [163]. Although this is about 1/10 to 1/5 the amounts required for translatome analysis, still
770 numbers indicate that proteome analysis is not possible for sand fly-derived promastigotes
771 either. Even Western blot semiquantitative analysis of single protein levels has not been tested
772 so far and would be very challenging, if not impossible. Despite the approach is very sensitive,
773 the challenge is to obtain sufficient sample and equalize amounts across samples in order to
774 make them comparable. Consequently, only transcript levels can be analyzed so far. Although
775 transcriptome analysis is very informative and many strategies based on this approach can be

developed (e.g. DGE of *knock out* or *knock in* promastigote cell lines within the sand fly vector) leading to significant biological findings, the absence of low input translatome and proteome approaches implies that many physiological aspects of promastigote development within the sand fly gut will remain unexplored for a long time.

Concluding remarks

Metacyclic promastigotes are distinguished by morphology (rapid swimming forms with an elongated flagellum) and high infectivity. No molecular markers are available. Metacyclics can be isolated by negative selection with PNA, as confirmed by infection experiments. Caution should be exercised when using cultured promastigotes depending on the experimental design, and when comparing studies. Transcriptome analysis has revealed the crucial microenvironmental role in parasite development in the sand fly gut because substantial differences and moderate correlation between cultured and sand fly-derived promastigotes have been found. In fact, sand fly-derived metacyclics are more infective than metacyclics in culture, and genes involved in metacyclogenesis such as the HASP/SHERP cluster, the gp63 metalloprotease family, and autophagy genes are over-expressed in sand fly metacyclic promastigotes compared to cultured promastigotes. Differential expression of several genes involved in gene expression regulation, signaling, and metabolic processes between sand fly-derived and cultured promastigotes supports an important microenvironmental influence differentiation. Elucidating signal transduction pathways in these parasites may substantially improve understanding of the relationships between promastigotes and the different microenvironments in the sand fly gut (Table 2). Unfortunately, translatome and proteome analysis is not feasible in promastigotes obtained from the sand fly gut so far.

The main outstanding questions are: i) What are the molecular features of the different *Leishmania* spp. promastigote forms? ii) Are the multiple roles of the LPG different between species causing different types of leishmaniasis? iii) Are there truly stage-specific markers? iv) Are they different between species? v) How different are canonical signal transduction cascades and those of *Leishmania* spp.? vi) Are there developmentally regulated changes in trans-

splicing? If so, what implications would they have? vii) How can relative protein levels be analyzed in sand fly-derived promastigotes?

Key Papers

Alcolea PJ, Alonso A, Gomez MJ, Postigo M, Molina R, et al. (2014) Stage-specific differential gene expression in Leishmania infantum: from the foregut of Phlebotomus perniciosus to the human phagocyte. BMC Genomics 15: 849.

Alcolea PJ, Alonso A, Dominguez M, Parro V, Jimenez M, et al. (2016) Influence of the Microenvironment in the Transcriptome of Leishmania infantum Promastigotes: Sand Fly versus Culture. PLoS Negl Trop Dis 10: e0004693.

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Figure captions

Figure 1. Isolation of metacyclic promastigotes from the sand fly gut. (A) Promastigote stages during development within the sand fly gut. Adapted from [14]. (B) Location of metacyclic promastigotes in the anterior pole of the PSG-promastigote plug in contact with the stomodeal valve (SV). Reproduced from [7]. (C) *In vitro* infectivity of sand fly-derived metacyclic promastigotes (LiPro-Pper) compared to metacyclic promastigotes from culture (LiPro-PNA$^-$) in the human cell line U937. Reproduced from [25].

Figure 2. Strategies for DGE analysis of sand fly-derived promastigotes. Only transcriptomics strategies are feasible to date for DGE analysis for very low input samples such as sand fly-derived promastigotes. In slRNA-seq strategies, the SL sequence is used in second strand cDNA synthesis (#), thus increasing specificity when analyzing samples containing genetic material from the host. A cross-hybridization control should be included in microarray experiments to avoid biased results due to noise of the host genetic material. The RNA-seq strategies allow for multiplexed analysis by including indexing sequences during PCR amplification (†). Mapping to genome and alignment to transcript annotations is required during microarray hybridization experiments only when the DNA probes spotted on the slides have not been identified before the experiment (*). An example is shotgun genome DNA microarrays, where only the clones of interest containing DEGs are sequenced and aligned to identify those genes [24]. aRNA: amplified RNA.

Tables

Table 1. Transcriptome studies and sample abbreviations. Original abbreviations have been used. Ama, amastigotes; Li, *L. infantum*; Lm, *L. major*; Pro, promastigotes; Stat, stationary phase; Log, logarithmic phase.

Ref.	Stages	Microenvironment	Comparisons	Approach
[24]	PNA^+ *vs.* PNA^- Stat Pro	Culture	LiPro-PNA^+ *vs.* LiPro-PNA^-	Microarrays
[25]	SV-derived *vs.* PNA^- Pro	*P. perniciosus* gut *vs.* culture	LiPro-Pper *vs.* LiPro-PNA^-	Microarrays
[7]	SV-derived *vs.* Stat Pro	*P. perniciosus* gut *vs.* culture	LiPro-Pper *vs.* LiPro-Stat	Microarrays
[26]	SV-derived Pro *vs.* Ama	*P. perniciosus* gut *vs.* human cell line	LiPro-Pper *vs.* LiAma	Microarrays
[125]	Nectomonad *vs.* procyclic Pro	*P. duboscqi* gut	LmSFNP *vs.* LmSFPP	RNA-seq
	Metacyclic *vs.* procyclic Pro	*P. duboscqi* gut	LmSFMP *vs.* LmSFPP	
	Ama *vs.* procyclic/metacyclic Pro	*P. duboscqi* gut *vs.* BALB/c mice footpad lesions	LmAM *vs.* LmSFPP/LmSFMP	
[69]	All gut *vs.* culture forms	*P. perniciosus* whole gut *vs.* culture mixtures	LisfPro *vs.* LiacPro	RNA-seq
[136]	Procyclic *vs.* metacyclic Pro	Culture	LmCPP *vs.* LmCMP	RNA-seq
[8]	Log *vs.* Stat Pro	Culture	LiPro-Log *vs.* LiPro-Stat	Microarrays

Table 2. Functional genomics in sand fly-derived promastigotes: main findings.

Ref.	Main findings
[7, 125]	The microenvironment influences parasite's differentiation.
[7, 25]	Sand fly-derived promastigotes from the stomodeal valve are more infective than stationary phase and PNA^- cultured promastigotes. Approximately 300 genes are differentially regulated.
[69, 125]	Autophagy, gp63, and HASP/SHERP cluster genes are up-regulated during metacyclogenesis (nectomonad and metacyclic promastigotes). These findings confirm that these genes are metacyclogenesis markers.
[125]	Pteridine, glucose, nucleoside and amino acid transporter genes are up-regulated in *L. major* sand fly-derived *vs.* cultured metacyclics.
[125]	Calpain-like cysteine peptidase, membrane-bound acid phosphatase 2, and several signaling molecule-encoding genes are up-regulated in *L. major* sand fly-derived *vs.* cultured metacyclics.
[7, 25, 26]	Many signal transduction genes are differentially expressed between cultured and sand fly-derived promastigotes.
[7, 25, 26, 66]	Most signal transduction mechanisms are unknown in *Leishmania* parasites. Therefore, changes between sand fly- and culture-derived promastigotes are unknown.
[69, 125]	Several genes involved in fatty acid biosynthetic processes are up-regulated in sand fly-derived *L. major* and *L. infantum* promastigotes.
[145]	Promastigotes secrete exosomes to the sand fly gut lumen. Co-inoculation with *L. major* promastigotes leads to magnified footpad lesions in mice.
[145]	Protein content of culture- and sand fly-derived promastigote exosomes is very similar.
[146-150]	gp63 and other virulence factors are present in exosomes.
[69, 145, 151-155]	Several proteins contained in promastigote exosomes (gp63, HSP70, HSP83, calpain-like cysteine peptidases, surface antigen proteins, etc.) are up-regulated in whole gut sand fly-derived promastigotes.

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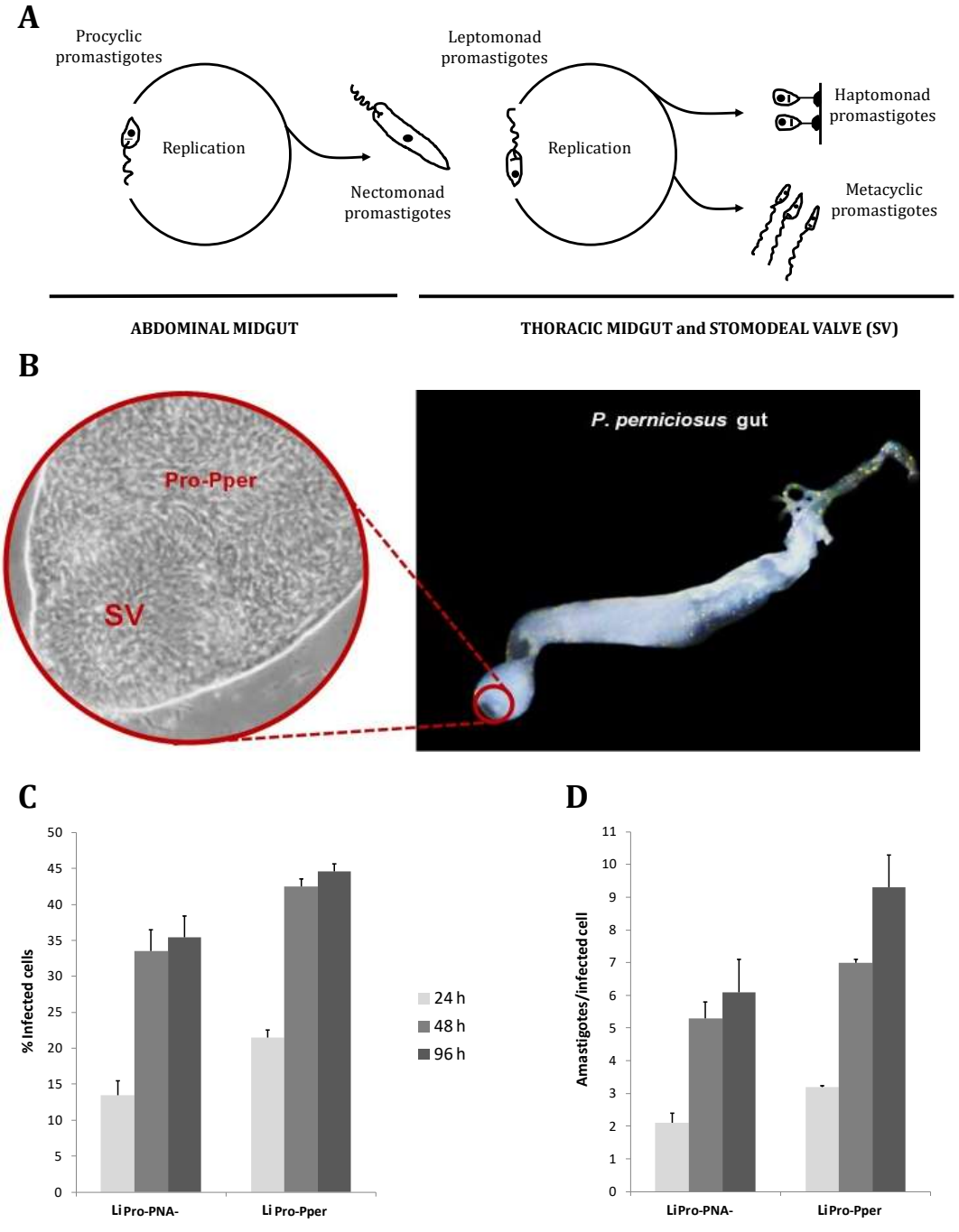
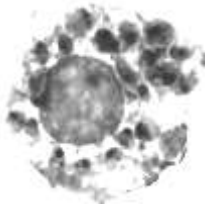

A
Procyclic promastigotes
Replication
Nectomonad promastigotes
Leptomonad promastigotes
Replication
Haptomonad promastigotes
Metacyclic promastigotes
ABDOMINAL MIDGUT
THORACIC MIDGUT and STOMODEAL VALVE (SV)
B
Pro-Pper
SV
P. perniciosus gut
C
% Infected cells
50
45
40
35
30
25
20
15
10
5
0
LiPro-PNA-
LiPro-Pper
24 h
48 h
96 h
D
Amastigotes/infected cell
11
10
9
8
7
6
5
4
3
2
1
0
LiPro-PNA-
LiPro-Pper
24 h
48 h
96 h



Dissection

Infection ⟷ **Promastigote pools**

RNA

Single stranded cDNA

Double stranded cDNA

IVT

aRNA

Biotinylated aRNA

Cy5/Cy3-labelled cDNA

Hybridization

Scan

Single stranded cDNA

Double stranded cDNA#

PCR†

Library

Fragmentation

NGS

Mapping to genome

Assignment to transcripts

DGE*

DNA microarrays

RNA-Seq

SUPPLEMENTARY FILE 1. Development of promastigotes within the sand fly gut. The axenic culture model: strengths and limitations.

Development of promastigotes within the sand fly gut.

According to the model of Gossage et al. (Gossage, et al., 2003) based on time course flow cytometry analysis, the life cycle of *Leishmania* spp. is completed in three dividing phases separated by non-dividing or transmission stages. One of them is replication of amastigotes within phagolysosomes of mammalian phagocytes. Thereafter, the bloodmeal phase takes place within the abdominal midgut of the sand fly and consists of replication of immature or procyclic promastigotes followed by differentiation to nectomonad promastigotes. This is valid for suprapylarian species, which are grouped within the subgenus *Leishmania*, whereas promastigotes of peripylarian species (subgenus *Viannia*) begin development in the hindgut (Lainson and Shaw, 1987). Nectomonads are non-dividing forms with an elongated flagellum and migrate towards the thoracic midgut. During the sugarmeal phase, they become leptomonads, which are able to divide. A few leptomonad promastigotes differentiate to metacyclic promastigotes, which is the highly infective stage (Figure 1A). Other forms like haptomonads and paramastigotes have been reported. This terminology is useful for basic understanding of development, but Gossage et al. (Gossage, et al., 2003) urge for finding molecular markers which may help in defining these stages more precisely. In the case of *Leishmania* spp., the term "metacyclic" has been defined as the infective form or the end product of development of promastigotes within the sand fly vector (Sacks and Kamhawi, 2001), small rapid-swimming forms with an elongated flagellum differentiated from leptomonads (Gossage, et al., 2003). The term was first used to designate non-dividing infective trypomastigotes of *Trypanosoma brucei* (Fairbairn and Burtt, 1946) injected by the tsetse fly (*Glossina* spp.) during bloodmeal intakes. Likewise, the term metacyclics was used for trypomastigotes of *T. cruzi* (Grignaschi, 1954) contained in feces of triatomine bugs (Reduviidae: Triatominae) that enter the bloodstream of the human host through the bite. Development of trypomastigotes within the vector gut is successfully mimicked in culture in

both cases, leading to metacyclic trypomastigotes which can be preserved (Cunningham and Harley, 1962) and are phenotypically well defined (Tyler and Engman, 2001). Development of *Leishmania* spp. promastigotes is successfully mimicked in culture also (see below), and the term "metacyclic" was adopted for a small fraction of the population becoming highly infective in both axenic culture and sand fly gut. There is no evidence supporting preservation of *Leishmania* spp. metacyclic promastigotes, and they rapidly transform in other stage when the environmental conditions change. For example, metacyclics immediately de-differentiate back to procyclics with each culture passage (i.e. when thawed) or when passed through established laboratory colonies of sand flies (Moreno, et al., 2007). Also, most metacyclics are lysed by complement during the first 3 min in contact with normal human serum and only the remaining ~5-15% (Dominguez, et al., 2002) would be able to interact with a phagocyte and potentially be internalized. For these reasons, metacyclic promastigotes have not been precisely and unequivocally characterized yet. Gossage et al. (Gossage, et al., 2003) highlighted the absence of parasite-sand fly interactions in axenic culture and warned about improper usage of the terms procyclics and metacyclics when identified with logarithmic and stationary phase promastigotes, respectively.

Bates (Bates, 2007), Dostálová and Volf (Dostalova and Volf, 2012) reviewed promastigote-sand fly interactions during development and the hypotheses about the mechanisms of transmission of metacyclic promastigotes. During the bloodmeal phase, blood is digested within the chitinous peritrophic matrix whereas embedded procyclic promastigotes proliferate (Secundino, et al., 2005). Then, nectomonads accumulate at the anterior part of the matrix and are able to escape (Schlein, et al., 1991, Shakarian and Dwyer, 2000) thanks to the chitinase secreted by the gut epithelium (Coutinho-Abreu, et al., 2010, Ramalho-Ortigao, et al., 2005). The ability of nectomonads to migrate forward and firmly attach to the microvilli of the gut epithelium contributes to explain why the sand fly is a true vector because promastigotes are not lost during defecation and continue their developmental process. One of the attachment mechanisms in *L. major* within *P. papatasi* is the interaction of the lipophosphoglycan (LPG) to gut epithelium galectins. However, the presence of LPG-receptors in other sand fly species

remains unclear, and LPG-independent development has been reported. In fact, LPG composition is variable across species. The LPG together with certain proteophosphoglycans (PPG) may have a major role in resistance to proteolysis within the gut also (reviewed in (Dostalova and Volf, 2012)). Once nectomonads reach the stomodeal valve (SV), located in the anterior part of the thoracic midgut, they become leptomonads and divide (Gossage, et al., 2003). Leptomonads produce the promastigote secretory gel (PSG) (Rogers, et al., 2002), mainly composed of filamentous PPG (Ilg, et al., 1996), which also let them bind to the epithelium to some extent. A small fraction of leptomonads become haptomonad promastigotes (Killick-Kendrick, et al., 1974), which attach tightly to the epithelium through hemidesmosome-like structures (Vickerman and Tetley, 1990, Wakid and Bates, 2004) probably priming formation of the PSG plug (Bates, 2007) and/or favouring blockage (Schlein, et al., 1992, Volf, et al., 2004), while some others differentiate to metacyclic promastigotes (Rogers, et al., 2002) in a process called metacyclogenesis and defined as the transformation of poorly infective to highly infective promastigotes (da Silva and Sacks, 1987, Muskus and Marin Villa, 2002). According to the "blocked fly hypothesis", the PSG plug blocks the stomodeal valve until it is removed by regurgitation during blood meal intakes (Rogers, et al., 2004), being leptomonads embedded in the plug and most metacyclics located in its poles (Bates, 2007). A different hypothesis was passive inoculation of only promastigotes found in the proboscis (Adler and Theodor, 1935, Beach, et al., 1984, Killick-Kendrick, et al., 1977). Both hypotheses are not mutually exclusive because both mechanisms may participate in transmission (Bates, 2007). In fact, low-dose and high-dose bite patterns have been observed and may be correlated to the respective transmission mechanisms mentioned (Kimblin, et al., 2008). In addition, chitinase-mediated damage was also observed in the stomodeal valve (Schlein, et al., 1991), which favours the regurgitation hypothesis, including participation of the pharyngeal and cibarial pumps (Schlein, et al., 1992, Volf, et al., 2004). A few metacyclic promastigotes are released from the pole of the PSG plug when it contacts blood being ingested, which is explained by the high solubility of PSG (reviewed in (Rogers, 2012)). Egestion of PSG and sand fly saliva together with metacyclic promastigotes probably plays a role in the initial steps of infection

(Titus and Ribeiro, 1988), including modulation of the immune response (Gomes and Oliveira, 2012, Kamhawi, 2000, Rohousova and Volf, 2006).

Phenotypical features of the different promastigote forms found in the sand fly gut differ between species, which highlights how challenging studying each form is. For example, the binding ability is strictly stage-dependent, as nectomonads and leptomonads are considerably bound to the epithelium according to the different mechanisms mentioned above, whereas procyclic and metacyclics are non-binding forms. Nonetheless, the relative binding ability is variable between different species, and mild binding tendency has been observed in procyclics and metacyclics. For example, nectomonads bind tighter than leptomonads in *L. infantum*, whereas no substantial differences have been observed in the case of *L. mexicana*, and metacyclics bind slightly tighter than procyclics in *L. mexicana* unlike in *L. infantum* (Wilson, et al., 2010).

The axenic culture model: strengths and limitations.

The first axenic culture of *Leishmania* parasites was performed by Nicolle in the Nicolle-Novy-McNeal medium (Row, 1912). Since then, an increasing number of culture media has been developed, leading to easy, fast and highly productive promastigote cultures. As in the sand fly gut, the complex promastigote populations in axenic culture are asynchronous from both the cell cycle and the differentiation status points of view. It is assumed that development within the sand fly gut is mimicked in axenic culture at 26-27ºC in undefined media containing heat inactivated mammalian serum (Berens and Marr, 1978, Lemma and Schiller, 1964, Neal and Miles, 1963, Steiger and Steiger, 1976, Zilberstein, 2008, Zuckerman and Lainson, 1977). In fact, stationary phase promastigotes are infective despite parasite-sand fly interactions are absent. However, infectivity of cultured promastigotes is lower than in the case of metacyclic promastigotes obtained from the sand fly, at least in *L. infantum* and *L. major* (Alcolea, et al., 2016, Alcolea, et al., 2016, Sacks and Perkins, 1984). In fact, infectivity is attenuated as the number of culture passages increases, thus requiring passages through laboratory animals (reviewed in (Zilberstein, 2008)). These observations highlight the importance of the promastigote-sand fly interactions and suggest that adaptation to the culture conditions results in

a progressive loss of the infective properties. As in the sand fly gut, the promastigote populations are heterogeneous in culture and only a small fraction is metacyclic. The most widespread and successful method to isolate subpopulations of metacyclic promastigotes from cultures is based on agglutination properties of the LPG in the presence of the peanut lectin or agglutinin (PNA). During metacyclogenesis, the LPG is modified, which leads to the loss of agglutination capability in the presence of PNA (Sacks, et al., 1985). The modifications consist of addition of α-D-arabinopyranose residues to the D-galactose-containing side chains (McConville, et al., 1990, McConville, et al., 1992). The agglutinating (PNA^+) subpopulation is less infective than the non-agglutinating (PNA^-) subpopulation in both *L. major* and *L. infantum* (Alcolea, et al., 2009, Sacks, et al., 1985). Yet this method is not valid for certain species such as *L. braziliensis*, and it has been clearly shown to be effective only in *L. major* and *L. infantum*, and even dependent on the strain studied (Alcolea, et al., 2009, Louassini, et al., 1998, Rodriguez-Gonzalez, et al., 2006). The minimum agglutinating amount of PNA is variable between *L. infantum* strains starting at 50µg/ml (unpublished result). The different LPG composition throughout species mentioned above explains these observations. Interestingly, PNA^- and PNA^+ forms can be isolated in the monogenetic trypanosomatid *Crithidia fasciculata* (Alcolea, et al., 2014), but the implications for understanding its life cycle remain still unknown.

In vitro infection experiments of the human myeloid U937 cell line with *L. infantum* promastigotes have shown that the peanut lectin non-agglutinating metacyclic subpopulation (LiPro-PNA^-) is more infective than the agglutinating one (LiPro-PNA^+) and the whole population in stationary phase of axenic culture (LiPro-Stat) from where both are isolated (Alcolea, et al., 2009). The same approach has revealed that LiPro-Stat and LiPro-PNA^- are less infective (~50% and ~20-30%, respectively) than promastigotes isolated from the stomodeal valve of the sand fly vector *P. perniciosus* (LiPro-Pper) (Alcolea, et al., 2016, Alcolea, et al., 2016, Alcolea, et al., 2014). Sand fly metacyclics are found in the vicinity of the stomodeal valve (SV), which is a pump located at the forefront of the thoracic midgut. In the case of the *P. perniciosus-L. infantum* vector-parasite pair, the proportion of metacyclic promastigotes in culture (Alcolea, et al., 2016, Alcolea, et al., 2009) and within the sand fly gut [5] is

approximately equal (~10%). Therefore, higher levels of infectivity of sand fly-derived promastigotes isolated from the SV are explained by a more advanced differentiation status (i.e. these promastigotes are more "metacyclic in character"), rather than simply an enrichment in metacyclics.

Considering how challenging working with promastigotes from the gut is, the cost-benefit balance presumably tilts to axenic culture in principle, but this is not as clear when considering results obtained by means of transcriptome analysis. Alternative methods for isolation of metacyclic promastigotes like centrifugation in Percoll gradient have been described, which are out of the scope of this review and provide less pure metacyclic populations.

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