Chapter 4
Using Metabolomic Approaches to Characterize the Human Pathogen *Leishmania* in Macrophages

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1 *Leishmania* and Leishmaniasis

Leishmania spp., are protozoan parasites that belong to the order *Kinetoplastida* (Phylum *Euglenozoa*). The *Kinetoplastida* encompass a large group of flagellated protists including the uniflagellated *Trypanosomatidae,* which all have parasitic life styles, and the biflagellated *Bodonidae* which are typically free living. The family *Trypanosomatidae* comprises the medically important genera *Trypanosoma*, which includes *Trypanosoma brucei* and *Trypanosoma cruzi*, the causative agent of human African trypanosomiasis and Chagas disease, respectively, as well as *Leishmania* spp., which are the cause of a spectrum of diseases collectively termed the leishmaniases (Stuart et al. 2008). *T. brucei*, *T. cruzi,* and *Leishmania* spp., all rely on an insect vector as well as a mammalian host to complete their complex lifecycle. While *T. brucei* and *T. cruzi* are transmitted by the tsetse fly and triatomine bugs, respectively, *Leishmania* spp., are transmitted by phlebotomine sandflies.

The pathology of leishmanial diseases range from localized self-healing cutaneous or diffuse cutaneous infections (CL and DCL, respectively) to mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL, also called kala-azar) (Pearson and Sousa 1996). The pathology can be linked to different species—e.g., *Leishmania donovani*, *Leishmania infantum,* and *Leishmania chagasi* cause VL, while *Leishmania major*, *Leishmania mexicana*, *Leishmania amazonensis,* and *Leishmania aethiopica* cause CL or DCL, and *Leishmania braziliensis*, *Leishmania peruviana,*

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and *Leishmania guyanensis* cause MCL. CL is characterized by open sores around the site of the sandfly bite, which can take years to heal and leave disfiguring scars. Furthermore, CL can recur years after patients have seemingly healed from the initial infection (Marovich et al. 2001; Gangneux et al. 2007). DCL is a more severe form of CL that results in the formation of several hundred nodules or ulcers. The marring lesions and disfiguring scars caused by CL and DCL can result in stigmatization and lead to social exclusion and economic disadvantage (Kassi et al. 2008). MCL results from the dissemination of parasites to mucosal membranes around the mouth and nose and, in some cases, to the genital or optical mucosa (Huna-Baron et al. 2000). This severe form of leishmaniasis can result in devastating destruction and deformation of the face with high risk of secondary infections. VL, or kala-azar, occurs when parasites disseminate to the bone marrow, liver, and spleen, resulting in anemia, fever, weightloss, and enlargement of liver and spleen. If left untreated, VL leads to death in nearly 100 % of cases within a two-year period (WHO.int).

Leishmaniasis is endemic in 88 countries throughout the tropics, subtropics, and the Mediterranean Basin with over 350 million people at risk of infection (WHO.int). Disease prevalence is estimated at 12 million people with more than 2 million new infections occurring annually (WHO.int). Mortalities from VL are increasing worldwide and currently stand at >20,000 deaths annually, making it the second deadliest parasitic disease after malaria (WHO.int; Desjeux 2004; Reithinger 2008; Alvar et al. 2012). The current war in Syria and instability in the Middle East have led to increased incidence and spread of leishmaniasis in the area due to a number of factors including greater refugee migration and an inability to access staff and facilities for diagnosis and treatment (Alawieh et al. 2014).

2 Treatment of Leishmaniasis

Current antileishmanial treatments suffer from one or more major limitations including high toxicity/severe side effects (amphotericin, pentamidine, paromomycin, miltefosine and sitamaquine), the requirement for long-term/parenteral administration (pentavalent antimonials, amphotericin B and paromomycin), high cost (liposomal amphotericin B), variable efficacy (e.g. species specificity, pentavalent antimonials, imidazole and pentamidine), and resistance or likely development of resistance (pentavalent antimonials and miltefosine) (Bouchard et al. 1982; Rangel et al. 1996; Seifert et al. 2003, 2007; Ouellette et al. 2004; Olliaro et al. 2005; Croft et al. 2006; Sindermann and Engel 2006; Bhattacharya et al. 2008; Sundar et al. 2007; Davidson et al. 2009; Moore and Lockwood 2010; Chakravarty and Sundar 2010; Seifert 2011; Freitas-Junior et al. 2012). While combination treatments are being used to reduce the emergence of resistant strains (van Griensven et al. 2010), new drugs which overcome the limitations of the current drugs are needed urgently (Freitas-Junior et al. 2012). Furthermore, despite the fact that humans can generate strong protective immunity against *Leishmania*

infection/reinfection (Evans and Kedzierski 2012), no efficacious defined vaccine for preventing human leishmaniasis has been developed to date (Handman 2001; de Oliveira et al. 2009; Kedzierski 2010; McCall et al. 2013; Joshi et al. 2014; Kumar and Engwerda 2014).

3 The Lifecycle of *Leishmania*

Leishmania differentiate through several morphologically and physiologically distinct stages during their complex digenetic lifecycle in their insect vector and animal hosts (Fig. 1). The major developmental stage in the sandfly vector (*Phlebotomus* and *Lutzomyia*) is the motile promastigote, which possesses a single long flagellum that emerges from the anterior flagellar pocket. Sandflies become a vector following the uptake of infected cells or free *Leishmania* parasites (typically low, usually 10–100 parasites) when feeding on a mammalian host (Anjili et al. 2006). Ingested parasites initially differentiate to promastigotes, which undergo a period of rapid proliferation, exploiting the nutrient-rich milieu of the blood meal as it is progressively digested by the sandfly's hydrolases (Pimenta et al. 1997). Following the breakdown of the peritrophic membrane that encapsulates the initial blood meal, the promastigote differentiates through several developmental stages which are distinguished by markedly different replication rates (Gossage et al. 2003; Dostalova and Volf 2012) including the nondividing metacyclic stage that accumulate in the sandfly foregut. This stage exhibits a similar physiology to nondividing (stationary phase) promastigotes (Pro^{stat}) in in vitro culture and appears to be highly virulent and preadapted for life in the mammalian host (Sacks and Perkins 1984, 1985; da Silva and Sacks 1987; Sacks and da Silva 1987; Sacks 1989). The accumulation of large clusters or aggregates of these metacyclic promastigotes at the sandfly mouthparts (specifically the stomodeal valve) causes an alteration in sandfly feeding behavior (e.g. repeated probing of the skin), damage to the stomodeal valve, and regurgitation of the parasite bolus, which may enhance transmission of the parasite to the host (Killick-Kendrick et al. 1977; Beach et al. 1984, 1985; Bates 2007; Rogers and Bates 2007, Schlein et al. 1992; Volf et al. 2004).

The number of *Leishmania* transmitted during a blood meal is typically low (<600 parasites) but can, in some cases, be as high as 100,000 cells (Kimblin et al. 2008). Infectious metacyclics are transmitted along with sandfly saliva and a highly immunogenic polysaccharide gel secreted by the promastigote (Titus and Ribeiro 1988; Bates 2007). Injected promastigotes are initially phagocytosed by polymorphonuclear leucocytes (PMNs) that are rapidly recruited to the sandfly bite site (van Zandbergen et al. 2004). These PMNs undergo apoptosis within a few days and the cellular debris (including released parasites) and/or intact PMNs containing parasites, are phagocytosed by a wave of macrophages that are recruited to the damaged tissue. Macrophages are the primary host cells of *Leishmania* (Handman and Bullen 2002) and it has been proposed that the initial infection of PMNs (in which the

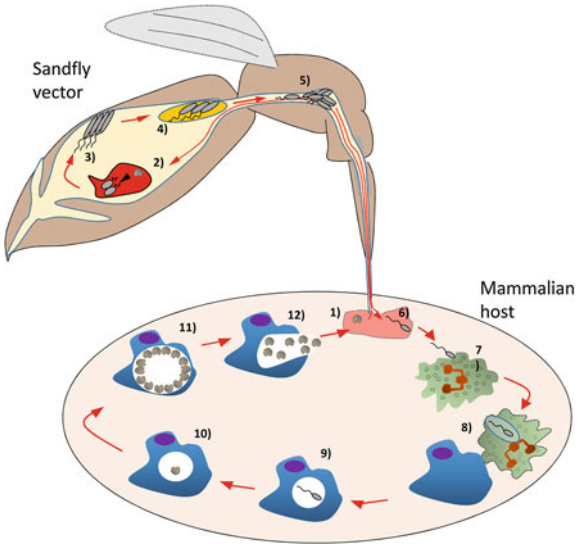


Fig. 1 *Leishmania* undergo a complex digenetic lifecycle developing within the sandfly vector and the mammalian host. Transmission occurs when an infected sandfly takes a blood meal. Amastigotes/infected macrophages are taken up by the sandfly during a blood meal (*1*) (Anjili et al. 2006) and are initially enclosed within a digestive peritrophic sac in the sandfly gut (*red*) (Pimenta et al. 1997). Amastigotes differentiate to flagellated procyclic promastigotes (*2*) which undergo multiple rounds of division before slowing their replication and differentiating into highly motile, but nondividing, nectomonads which escape the peritrophic sac (Gossage et al. 2003; Dostalova and Volf 2012). Nectomonads adhere to the mid-gut wall (*3*) and develop into leptomonads in the thoracic mid-gut (*4*). Leptomonads undergo further rounds of rapid replication and are immobilized in a gel-like matrix which is secreted by this life-cycle stage. Leptomonads differentiate into either nondividing haptomonads, which form a plug by attaching to each other and the stomodeal valve (*5*), or nondividing metacyclics, which are highly infectious and are transmitted during a blood meal (*6*) (Sacks and Perkins 1984, 1985; da Silva and Sacks 1987; Sacks and da Silva 1987; Sacks 1989). In the mammalian host, metacyclic promastigotes are phagocytosed by polymorphic neutrophils (PMN), which are first to reach the site of inflammation following the sandfly bite (*7*, *8*) (Laskay et al. 2003; van Zandbergen et al. 2004). PMNs have a short life span and macrophages phagocytose released parasites and/or infected apoptotic PMNs (*8*, *9*) (Laskay et al. 2003; van Zandbergen et al. 2004; Ritter et al. 2009). Within the phagolysosome of macrophages, *Leishmania* differentiate into the amastigote stage, adapt to their new milieu (*10*) and start replicating (*11*) (Kaye and Scott 2011). Amastigotes are released when heavily infected macrophages rupture (*12*) allowing infection of other cells (Noronha et al. 2000). The lifecycle begins again when sandflies ingest free amastigotes or infected cells during a blood meal (*1*)

parasites do not replicate) represents a 'Trojan Horse' strategy for targeting macrophages, the preferred host cell for these pathogens (Laskay et al. 2003; van Zandbergen et al. 2004, 2007). Alternatively, extracellular *Leishmania* may be taken up incidentally during the phagocytosis of necrotic PMNs (the 'Trojan rabbit' model) (Ritter et al. 2009). Following uptake by macrophages and the maturation of the vacuole into a phagolysosomal compartment, promastigotes differentiate to

small, aflagellate amastigotes which re-enter a proliferative state that is associated with progression of the disease (Kaye and Scott 2011). Further recruitment of naïve macrophages to the site of infection leads to the development of granulomatous lesions that are predominantly composed of infected and uninfected macrophages, as well as other populations of monocytes, PMNs, dendritic cells, and lymphocytes (Amaral et al. 2000; Souza-Lemos et al. 2011). Infected macrophages, or even extracellular amastigotes, can also disseminate to other tissues and establish infection in lymph nodes, mucosal membranes (MCL), or the spleen and liver (VL) (Ridley et al. 1989; McElrath et al. 1988). Subpopulations of *Leishmania*-infected macrophages in lymph nodes are thought to be responsible for long-term persistence of the pathogen, but are inadequately characterized (Dereure et al. 2003).

Macrophages are specialized phagocytic cells that are a vital part of the innate and adaptive immune system. These cells are responsible for internalizing and then killing microbes within the phagolysosome which has a low pH, a complex array of hydrolytic enzymes, and is the target of key microbicidal processes such as the oxidative burst and nitric oxide (NO) synthesis (Russell et al. 2009). However, a number of bacterial (*Coxiella brunetti*, *Listeria monocytogenes*, *Shigella* spp., *Mycobacterium tuberculosis*), fungal (*Cryptococcus neoformans*), and protozoan pathogens (*Leishmania* spp., *T. cruzi* and *Toxoplasma gondii*) actively target macrophages (Dermine and Desjardins 1999). While most of these organisms (including *Leishmania* promastigotes) exhibit mechanisms to escape the phagolysosome or prevent its maturation (Desjardins and Descoteaux 1997; Dermine et al. 2000, 2005; Moradin and Descoteaux 2012), *Leishmania* amastigotes and *Coxiella burnetti* have evolved to withstand the harsh conditions encountered within the mature phagolysosome (Dermine and Desjardins 1999; Thi et al. 2012). In order to survive the acidic pH within the phagolysosome, *Leishmania* amastigotes express proton pumps to maintain a near-neutral intracellular pH (Glaser et al. 1988; Zilberstein et al. 1989; Grigore and Meade 2006). The mechanisms by which *Leishmania* amastigotes withstand the high concentration of proteases within the phagolysosome are poorly understood (Prina et al. 1990). Possibly, *Leishmania* cell surface glycoconjugates play a role in acid resistance and/or *Leishmania* membrane proteins are resistant to proteolysis (Chang and Fong 1983; Prina et al. 1990). Prina et al. (1990) proposed that the enlarged phagolysosomes observed in macrophages infected with *L. mexicana* and related species are beneficial to the parasite as they result in dilution of the lysosomal proteases. Due to the difficulty in studying intracellular amastigotes, most studies rely on the axenic differentiation of promastigotes to amastigotes (Ama^{axenic}) which can be achieved by lowering the pH of the culture medium and increasing the temperature or through depletion of iron in the culture medium to mimic the conditions of the host macrophage (Saar et al. 1998; Mittra et al. 2013).

4 Characterizing *Leishmania* Metabolism: Insights Offered by '-Omics'Approaches

The paucity of good treatment options and no vaccine means that there is an urgent need to identify new therapeutic targets in *Leishmania*. Many of these targets will ideally be specific to parasite metabolism, targeting pathways critical for the survival and proliferation of the intracellular amastigote stage. Understanding the interplay of host and parasite metabolism is critical in identifying targets that will not affect the host metabolism. Finally, by mapping parasite metabolism it is anticipated that the mode of action (MOA) of current chemotherapies will be revealed and new insights into drug resistance mechanisms gained. The metabolism of *Leishmania* has been studied for several decades using a range of approaches and techniques. Here we discuss these techniques, the insights they have provided into *Leishmania* metabolism as well as their limitations.

5 Genome Studies and *Leishmania* Metabolism Mapping

Genome sequencing has revealed many new potential drug targets in *Leishmania*. To date, the genomes of several *Leishmania* species have been sequenced (Ivens et al. 2005; Peacock et al. 2007; Downing et al. 2011; Real et al. 2013) providing important new insights into the biology of these parasites, including the identification of novel surface proteins, protein kinases, and phosphatases that could be involved in signaling pathways, differentiation and parasite stress responses, as well as many metabolic pathways (Myler et al. 2000; Worthey et al. 2003; Ivens et al. 2005). Genome-wide annotations have led to the development of curated biochemical pathways databases, such as LeishCyc and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Doyle et al. 2009; Saunders et al. 2012). Furthermore, genome-wide flux balance models of *Leishmania* metabolism give unique insight into crucial reactions, predicted auxotrophies and minimal culture media components, potentially lethal or growth-reducing gene deletions, and novel drug target identification (Chavali et al. 2008, 2012; Silva et al. 2015; Subramanian et al. 2015). These tools are particularly useful in providing a means to contextualize and map metabolomics data and are also valuable in hypothesis-driven metabolomic analyses.

However, as is the case with other protozoan genomes, more than half of the genes in the *Leishmania* genomes have not yet been assigned a function (Pawar et al. 2014), highlighting the importance of pairing genomic data with functional gene analysis tools such as metabolomics. Another striking feature to emerge from these analyses is the extraordinarily high level of gene synteny identified by the comparative analysis of *T. brucei*, *T. cruzi,* and *L. major* genomes (El-Sayed et al. 2005). About 6150 genes were found to be shared between these species, while the authors identified about 900 *Leishmania*-specific genes. Furthermore, a comparison

of *L. major*, *L. braziliensis,* and *L. infantum* genomes, identified only about 80 genes that are restricted to an individual species despite the early divergence of these species and the distinct clinical manifestation observed during infection with these species (Peacock et al. 2007). This indicates that there are only few species-specific genes which contribute to the different biology of each species, indicating that species and strain-specific differences may result from gene dosage (copy number) (Rogers et al. 2011). Additionally, it should be noted that the genome provides insights into the overall metabolic capacity of *Leishmania,* but allows no conclusion about the significance of metabolic pathways in specific developmental stages and given conditions (e.g. nutrient restriction, drug treatment etc.). For example, enzymes/pathways which may be crucial for survival in the sandfly vector might be dispensable during mammalian host infection and vice versa.

Finally, models of *Leishmania* metabolism may not adequately account for niche/species/stage-specific changes to enzyme compartmentalization which in turn will affect metabolic flux. For example, *Leishmania* and other kinetoplastids maintain an unusual peroxisome-like organelle termed the glycosome (Haanstra et al. 2016). This organelle contains the enzymes of several metabolic pathways including glycolysis, gluconeogenesis, the pentose phosphate pathway (PPP), fatty acid β-oxidation, purine salvage, and pyrimidine synthesis as well as hypothetical proteins with unknown function (Michels et al. 2006; Jamdhade et al. 2015). The localization of enzymes within the glycosome has repercussions on metabolic flux and is thought to provide a means of regulation in the absence of traditional feedback loops. For example, *Leishmania* hexokinase and phosphofructokinase lack conventional activity regulation which could cause the toxic accumulation of hexose phosphates (Bakker et al. 2000; Michels et al. 2006). Indeed, the relocalization of glycolytic enzymes, such as the phosphoglucokinase and the triose phosphate isomerase, to the cytoplasm proved to be fatal for *T. brucei* (Blattner et al. 1998; Helfert et al. 2001). Furthermore, the number and enzymic content of the glycosome changes during the promastigote to amastigote differentiation thereby affecting the metabolic regulation and capacity of the parasite (Cull et al. 2014).

6 Transcriptomic and Proteomic Studies

Unlike the situation in other eukaryotes, protein-encoding genes in *Leishmania* lack introns and are organized and constitutively transcribed as large polycistronic clusters (Myler et al. 2000; Worthey et al. 2003; Ivens et al. 2005). Transcription is not regulated by transcription factors and the polycistronic mRNA is subsequently processed into individual mRNAs by transsplicing and polyadenylation (Ivens et al. 2005). As a result of this unusual mode of gene expression, relative levels of mRNA expression remain remarkably constant across different life-cycle stages under different growth conditions (Leifso et al. 2007). Consequently, transcriptomic studies have not been particularly fruitful in identifying enzymes or metabolic pathways that

are activated (or repressed) in amastigotes following their uptake by macrophages, as has been the case in some other bacterial or fungal pathogens (Haile and Papadopoulou 2007). However, there are a limited number of examples where stage-specific changes in protein expression are regulated at the level of mRNA stability. Examples include some of the lysosomal cysteine proteases, as well as some enzymes involved in metabolism and protein synthesis (Saxena et al. 2007). A recent analysis of the *L. major* transcriptome reported that the polycistronic transcripts can be highly heterogeneous in length which may lead to differences in stability, further complicating the interpretation of transcriptomic data (Rastrojo et al. 2013). Lastly, a general limitation of transcriptome analyses is the potentially poor correlation between mRNA and protein levels or between transcript levels and enzymatic activity as observed in other organisms (Miyamoto et al. 2001; Maier et al. 2009; Hoppe 2012; Vogel and Marcotte 2012).

Proteomic approaches have been used to identify differentially expressed proteins in promastigote and amastigote stages, and have given some crucial insights into the metabolism of the two life-cycle stages (Handman et al. 1995; Thiel and Bruchhaus 2001; El Fakhry et al. 2002; Bente et al. 2003; Nugent et al. 2004; Walker et al. 2006; Rosenzweig et al. 2008a, b). Promastigote to amastigote differentiation is associated with a global reduction in protein synthesis but increased expression of some amastigote specific proteins such as A2, an ATP-dependent RNA helicase, the amastin family of proteins, lysosomal cysteine proteinases, some protein chaperones and histones (Handman et al. 1995; Carvalho et al. 2002; Barhoumi et al. 2006; Nasereddin et al. 2010). Comparing the proteome of cultured promastigotes and Ama^{axenic}, Rosenzweig et al. (2008a) reported significant differences in the levels of some metabolic enzymes including increases in enzymes involved in gluconeogenesis, β-oxidation, amino acid catabolism, the tricarboxylic acid (TCA) cycle, and the mitochondrial respiratory chain, suggesting stage-specific remodeling of amastigote metabolism. A number of other studies have identified proteins that are differentially expressed in the distinct life-cycle stages (El Fakhry et al. 2002; Bente et al. 2003; Nugent et al. 2004; Walker et al. 2006). However, the identified differences were surprisingly small and interpretation of these analyses are complicated by the fact that many changes in protein expression are inconsistent across different species and/or the possibility that at some of these changes may be attributed to stage-specific changes in the size or complement of mitochondria and glycosomes in the different developmental stages.

More broadly, while protein levels are commonly considered to provide a measure for the activity of an enzyme/pathway, this correlation is not always observed (Miyamoto et al. 2001). Furthermore, relatively few *Leishmania* proteins have been functionally characterized and there is an increasing number of reports of parasite proteins that have divergent or repurposed roles from those inferred from homology alignments (Oppenheim et al. 2014). For example, *Leishmania* hexokinase functions as a metabolic enzyme in glycosomal glycolysis, as well as heme receptor in the flagellar pocket and mitochondrion, while enolase 'moonlights' as a microtubule-binding protein (Krishnamurthy et al. 2005; Quinones et al. 2007;

Vanegas et al. 2007; Collingridge et al. 2010; Tonkin et al. 2015), complicating the interpretation of proteomics analyses.

These studies suggest that *Leishmania* may be more dependent on posttranslational regulatory mechanisms during differentiation or adaptation to changing environmental circumstance than other eukaryotes. Indeed gene families for proteins involved in protein posttranslational modifications (kinases, phosphatases, etc.) are commonly amplified in these parasites. In particular, phosphoproteomic studies have revealed major changes in protein phosphorylation during promastigote to amastigote differentiation (Rosenzweig et al. 2008b; Morales et al. 2010a; Tsigankov et al. 2013, 2014). Interestingly, some of the most abundant proteins to be phosphorylated during differentiation were heat-shock proteins which are otherwise constitutively expressed (Morales et al. 2010a). Rosenzweig et al. (2008b) utilized isobaric tags for relative and absolute quantification/liquid chromatography-mass spectrometry/mass spectrometry (iTRAQ/LC-MS/MS) to characterize a number of posttranslational modifications in *Leishmania* promastigotes and Ama^{axenic}. The *Leishmania* genomes also encode large numbers of protein kinases and phosphatases, several of which have been shown to be essential for differentiation and/or amastigote survival (Ivens et al. 2005; Morales et al. 2010b; Cayla et al. 2014). To date, however, no complete signaling pathways in *Leishmania* (from surface receptor/sensor to downstream effector(s)) have been delineated and the function of most of these posttranslational modifications remains poorly defined. Nonetheless, studies on proteins involved in regulating mitochondrial proteins through the covalent attachment of ubiquitin-like proteins (Gannavaram et al. 2011, 2012) suggest that this will be a rewarding endeavor. In a recent study, Goldman-Pinkovich et al. (2016) described a coordinated arginine deprivation response (ADR) that is activated in response to low arginine levels which are expected to occur in the macrophage phagolysosome. *L. donovani* responded to low external arginine levels by rapidly upregulating the arginine transporter, LdAAP3. This response was dependent on expression of the mitogen activated protein kinase 2 (MAPK2)-dependent signaling pathway (Goldman-Pinkovich et al. 2016). Similarly, stage-specific regulation of expression of the major plasma membrane glucose transporters in *L. mexicana* was found to be regulated by ubiquitination of the cytoplasmic tails of the transporter proteins and their internalization and degradation in the lysosome (Vince et al. 2011).

7 Insights into *Leishmania* Metabolism using Metabolomics

Further advances in our understanding of *Leishmania* adaptive responses in both the sandfly and mammalian hosts will be dependent on the use of complementary approaches, such as metabolomics. Metabolomics is increasingly being used alone or in combination with other '-omics' approaches to identify new or unanticipated

metabolic pathways and to characterize metabolic networks in microbial pathogens (Holmes 2010; Creek and Barrett 2014; McConville 2014; McConville et al. 2015; Saunders et al. 2015; Lau et al. 2015; Kim and Creek 2015; Kloehn et al. 2016). The metabolome of a cell/tissue/organism can be considered the major downstream phenotype of changes in the transcriptome and proteome, or the most upstream input into cellular processes from the environment. The later point is particularly relevant when considering pathogens which are highly responsive to changes in the nutrient levels within their specific host niches. Trindade et al. (2016) demonstrated this in a recent study as they identified a major reservoir of *T. brucei* cells in adipose tissue. In comparison with parasites in the bloodstream and central nervous system, the adipose tissue form (ATF) exhibited a distinct metabolism, as it is adapted to its niche by utilizing myristate as a major carbon source through β-oxidation. Furthermore, metabolomics has proven to be particularly useful in drug target discovery research and is expected to become increasingly valuable due to the rapid refinement of existing approaches and the development of new analytical techniques (Rabinowitz et al. 2011). To date in *Leishmania* spp., metabolomics approaches have been employed to characterize mutants, elucidate the MOA of drugs and mechanisms of resistance, and to describe the nutritional requirements and central carbon metabolism of different parasite developmental stages as well as different species (Naderer et al. 2006; De Souza et al. 2006; Creek and Barrett 2014; Vincent et al. 2014; Rojo et al. 2015; Saunders et al. 2015; Arjmand et al. 2016; Westrop et al. 2015). Though beyond the scope of this review, metabolomics has also been used to investigate changes to the host cell upon infection (Lamour et al. 2012; Moreira et al. 2015).

8 Characterization of Genetic Knockouts using Metabolite Profiling

Notwithstanding the technical difficulties of deleting genes in a diploid organism such as *Leishmania*, a significant number of genetically defined mutants have been generated, which have provided new insights into the metabolic requirements of intracellular amastigotes (McConville et al. 2007). To date, metabolomic profiling in *Leishmania* spp., has predominantly been used in a highly targeted fashion to validate the deletion of the desired target gene, complementing detailed molecular biology, biochemistry, and virulence data (Naderer et al. 2006; Saunders et al. 2012; Naderer et al., 2015). For example, Naderer et al. (2006) deleted the gene encoding the gluconeogenic enzyme, fructose 1,6-bisphosphatase (FBPase) in *L. major*. In contrast to wild-type cells, these parasites were unable to utilize gluconeogenic carbon sources (glycerol) to synthesize hexose sugars and mannogen, a unique storage polysaccharide composed of β1,2-linked mannose residues. While the *L. major* ΔFBPase promastigotes grew normally in rich culture medium, they were unable to replicate in macrophages and displayed highly attenuated virulence

in mice suggesting that gluconeogenesis is essential for *Leishmania* survival in the macrophage, possibly due to low levels of hexoses in the phagolysosome. Paradoxically, other genetic studies have shown that *Leishmania* spp., are also dependent on the uptake of sugars (Burchmore et al. 2003; Naderer et al. 2010). An abundant source of sugars in the macrophages phagolysosome are likely to be aminosugars (*N*-acetyl-glucosamine and glucosamine) derived from the breakdown of glycosaminoglycans which are internalized by macrophages (Naderer et al. 2010, 2015) which would provide amastigotes with a source of both carbon skeletons and nitrogen groups. The significance of aminosugar uptake and utilization in amastigote metabolism was supported by the finding that deletion of the gene encoding the enzyme glucosamine 6-phosphate deaminase (GND), which converts glucosamine (GlcN) to fructose 6-phosphate, resulted in severe loss of virulence in mice and the capacity to grow in macrophages, while loss of the enzyme N-acetylglucosamine acetyltransferase (GNAT), which converts these parasites to GlcN auxotrophs had no effect on intracellular growth or virulence (Naderer et al. 2015).

In the future, it is anticipated that metabolite profiling will be increasingly used to analyze entire pathways (or nodes) as, for example, demonstrated in its recent application to analysis of the polyamine pathway in *L. amazonensis* (Castilho-Martins et al. 2015). An untargeted (or at least more extensive) metabolite profiling of gene knock-out lines and wild-type cells is particularly recommended in *Leishmania* spp., in order to prevent overlooking unforeseen (side-) effects of the gene depletion. Due to the complexity and interconnectivity of the metabolic network, as well as our still limited understanding of the metabolic network and metabolite-protein interactions, unexpected effects of gene depletions are likely to be relatively common (Hellerstein 2003).

Untargeted metabolomics may also be combined with functional genomics to assign identities to a large number of unknown/hypothetical *Leishmania* genes. Although this approach has not been systematically applied to *Leishmania* spp., such approaches have been useful in identifying pathways and annotating genes in other, more intensely studied organisms such as *Saccharomyces cerevisiae* (Raamsdonk et al. 2001) and *Arabidopsis thaliana* (Fukushima et al. 2014). Indeed, the development of a robust and high-throughput means to characterize mutants will become increasingly important as new technologies, such as the CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 system (Sollelis et al. 2015; Zhang and Matlashewski 2015), are applied to *Leishmania* making gene disruption easier and feasible in large-scale assays. Extensive annotation of metabolic phenotypes and perturbations will help to refine existing in silico generated metabolic networks and the usefulness of these models for predicting lethality, auxotrophy, and drug susceptibility. When combined with traditional phenotypic data of a given mutant (e.g. growth rate, virulence, viability of each life cycle stage) metabolomics is a powerful way to characterize a mutant and potentially offers a means to ascribe gene function in a high-throughput manner.

9 Studying a Drug's Mode of Action and the Development of Resistance in *Leishmania*

Metabolomics represents a powerful tool to understand both the MOA of drugs as well as how drug resistance occurs (Vincent et al. 2014). In the case of anti-*Leishmania* chemotherapies, this is especially important as the MOA of several frontline drugs remains poorly defined (Croft and Coombs 2003) and the drug efficacy is highly variable dependent on the *Leishmania* species and host genetics (Rangel et al. 1996; Schriefer et al. 2008).

Pentavalent and trivalent antimonials (Sb^V, Sb^{III}) have been used to treat VL as well as CL, since the 1960s. Pentavalent antimonials remain effective in 95 % of patients in many areas (Seifert 2011), however, the widespread use of the drugs for several decades has given rise to resistant strains in hyperendemic areas in India (Croft et al. 2006; Ashutosh et al. 2007). Aside from increasing resistance, a major drawback of pentavalent antimonials is their severe cardiotoxicity, which is observed in as many as 10 % of patients (Sundar et al. 1998; Sundar and Chakravarty 2010), and the requirement of parenteral administration as no oral preparation is available. Despite the use of antimonials to treat leishmaniasis (VL and CL) for about 60 years, the compounds' MOA remains unclear (Haldar et al. 2011). Several studies have employed metabolomics in order to determine the effect of drugs on metabolism and understand how resistance occurs (Canuto et al. 2012; t'Kindt et al. 2010; Berg et al. 2013). Most of these studies have employed an untargeted approach to quantify as many metabolites as possible, although the technology employed (capillary electrophoresis-electron spray ionization-time of flight-mass spectrometry (CE-ESI-TOF-MS), LC-MS (Orbitrap)), as well as the *Leishmania* species (*L. infantum*, *L. donovani*), and isolates differ between the publications. t'Kindt et al. (2010) profiled (untreated) antimonial susceptible and resistant clones of *L. donovani*, identifying major differences in phospholipid and sphingolipid metabolism and pools of amino acid/amino acid derivatives. Berg et al. (2013) conducted a more comprehensive analysis, profiling the metabolism of three *L. donovani* clinical isolates that differed in their susceptibility to antimony. Rapidly dividing and nondividing promastigotes (Pro^{log} and Pro^{stat} respectively) were analyzed revealing significant differences in several metabolic pathways including arginine metabolism, cysteine transsulfuration pathway, acylglycines, indole acrylate, glycerophospholipids, and amino acid levels. From these studies it was inferred that drug (Sb^{III}) resistance may be linked to (1) increased protection from drug-induced oxidative stress via greater thiol production and tryptophan degradation, (2) changes to mitochondrial metabolism, (3) increased membrane fluidity (protecting the cell from host-derived oxidants and/or affect drug uptake/export), and (4) accumulation of nonessential amino acids as alternative carbon source for use upon host cell invasion. More recently, Rojo et al. (2015) used a multiplatform approach (LC-MS, CE-MS and gas chromatography (GC)-MS) to increase the diversity of metabolites detected in antimony susceptible and resistant *L. infantum*. Consistent with other metabolomics experiments, a depletion

of urea cycle and polyamine biosynthetic pathway intermediates was observed when sensitive promastigotes were treated with Sb^{III} which was not apparent (or reversed) in resistant parasites. Treated parasites also appeared to have a disturbed TCA cycle, with several important intermediates (malate and anaplerotic amino acids, aspartate and glutamate) significantly reduced, as well as changes to parasite membrane composition.

The alkylphosphocoline, miltefosine, was the first oral drug to be licensed for use against leishmaniasis. It is used to treat VL on the Indian subcontinent and CL in South America. Miltefosine was initially developed as an anticancer drug in the 1980s (Eibl and Unger 1990) and was later repurposed as an antileishmanial drug. Drug repurposing/repositioning has been suggested to be an important cost- and time-saving strategy for the identification of new antiparasitic drugs (Andrews et al. 2014). Given that miltefosine is thought to affect lipid metabolism and membrane composition, Vincent et al. (2014) compared miltefosine treated/untreated *L. infantum* promastigotes using a lipidomics approach. Changes to intracellular lipid metabolism were observed as an increase in the abundance of short alkanes, although no change in membrane lipids was detected. These finding contradict earlier studies, which detailed changes to fatty acid metabolism, sterol pathways, and phospholipid levels (Rakotomanga et al. 2004, 2007) although comparison between the two studies is complicated by differences in the experimental design (Vincent et al. 2014). Vincent et al. (2014) also observed an increase in the abundance of selected sugars and DNA damage (released nucleotide fragments). Canuto et al. (2014) performed a more extensive analysis comparing miltefosine-treated/untreated and resistant lines of *L. donovani* while also broadening the metabolite base by employing three different platform technologies (LC-MS, GC-MS and CE-MS). Upon miltefosine treatment, a decrease in the abundance of several key intermediates in the arginine/polyamine pathway (arginine, ornithine and citrulline) was observed in susceptible lines. This suggests that the parasites, due to a decreased trypanothione availability, are particularly susceptible to oxidative stress (potentially induced by miltefosine). In resistant parasites, the abundance of these metabolites, as well as spermidine and intermediates of trypanothione biosynthesis, was increased. The amino acid profile also differed in susceptible and resistant lines. In the resistant line, the abundance of most amino acids was increased, while in the susceptible line many amino acids showed a moderate to strong decrease in abundance upon exposure to the drug suggesting some degree of amino acid starvation. The importance of amino acids as a potential carbon source is detailed below.

Metabolomics has also been used to explore changes in the metabolism of single and combined therapy-resistant (CTR) *L. donovani* lines (Berg et al. 2015). Combination therapies are commonly used in the field with the aim of shortening treatment time, improving efficacy, and delaying the emergence of resistance (Croft and Olliaro 2011). Researchers compared the metabolite profiles, acquired by LC-MS, of lines resistant to amphotericin B-, miltefosine-, antimonial (SB^{III})-, paromomycin-, and combinations thereof, in order to identify unique and common metabolic features. Comparing CTR lines with their singly resistant counterparts

revealed that changes in the metabolome were highly varied. For some combinations, the changes were not additive (amphotericin-B/paromomycin, amphotericin/miltefosine and amphotericin-B/antimonial), while in other cases changes to the metabolome were greater than what would be predicted from singly resistant lines (miltefosine/paromomycin and antimonial/paromomycin). Quantitative analysis of amphotericin B/antimonial (AS) and antimonial/paromomycin (SP) resistant lines identified several shared changes in metabolites of proline biosynthesis and the transsulfuration pathway which the researchers suggest is indicative of increased protection against oxidative stress in these CTR lines (proline as a general stress-response metabolite and free radical scavenger, products of transsulfuration pathway feeding into trypanothione biosynthesis). Changes in the abundance of lipid and sterol pathway intermediates were also observed in the tested CTR lines suggesting alterations in membrane composition. Importantly, the researchers sought to validate these conclusions using functional assays (susceptibility to drug-induced and extracellular reactive oxygen stress, genomic DNA damage and membrane fluidity).

As with many other metabolomics studies in *Leishmania*, the bulk of MOA/resistance experiments have been undertaken only in the promastigote stage (Vincent and Barrett 2015). Ultimately, however, any conclusions made about a given drug's MOA or proposed mechanism of resistance need to be verified in the disease causing amastigote stage, such as those generated in vitro (Ama^{axenic}) or isolated from in vitro infected macrophages ($Ama^{M\Phi}$) or from murine lesions (Ama^{lesion}) (Vincent and Barrett 2015). As detailed in the following sections, each *Leishmania* developmental stage is characterized by unique metabolic features and growth rate which may result in significantly different drug efficacy, MOA, and mechanisms of resistance. Furthermore, the host environment may additionally alter a drug's effectiveness by altering the parasite's environment (e.g. nutrient restriction and stress induced by host microbicidal responses), modulate a drug's availability (e.g. membrane permeability and prodrug catabolism), or indirectly kill the amastigote by inhibiting targets in the macrophage host (e.g. immunomodulatoy effects). Indeed, compound screening experiments have revealed significant differences in the sensitivity of promastigote and amastigote stages to many drugs (De Muylder et al. 2011; De Rycker et al. 2013). These findings indicate that understanding the nutritional environment and metabolism of the host cell is also critically important when delineating a drug's MOA and the parasite's development of resistance. For example, it has been suggested that the accumulation of amino acids in drug-resistant lines preserves the parasite's fitness for the invasion and proliferation in the nutritionally limited environment of the host macrophage by providing an alternative carbon source (Vermeersch et al. 2009; Berg et al. 2013; Canuto et al. 2014). Finally, care needs to be taken when studying field isolates so that detected genetic differences can be aligned with drug susceptibility/resistance measures, virulence, and metabolomics data.

10 Metabolomics Reveals Key Differences Between *Leishmania* Developmental Stages

10.1 Footprinting Approaches

As discussed above, *Leishmania* parasites progress through several different developmental stages during their lifecycle. Many of these developmental stages can be generated in vitro by manipulating temperature and culture conditions allowing detailed studies on parasite metabolism in the absence of the confounding influence of host metabolism (with the caveat mentioned above that in vitro conditions may be an inadequate model for the in vivo environment). A number of studies have measured changes in the culture supernatant (footprinting (Kell et al. 2005)) of these different axenic parasite stages to infer the operation of specific pathways in central carbon metabolism and overall metabolic fluxes (Hart and Coombs 1982; Rainey and MacKenzie 1991; Castilla et al. 1995). Hart and Coombs measured changes in the sugars, amino acids, and fatty acids in the medium of axenically-derived *L. mexicana* promastigotes and amastigotes using a variety of analytical approaches, including enzyme assays, gas chromatography, and radioassays (Hart and Coombs 1982). Their results suggested that promastigotes have a highly glycolytic metabolism with significant amino acid uptake, while Ama^{axenic} exhibit a shift toward greater reliance on fatty acid utilization via β-oxidation (with a concordant decrease in glucose uptake). These conclusions were supported by the increased sensitivity of Ama^{axenic} to the β-oxidation inhibitors, 4-pentenoate, and 2-mercaptoacetate (Hart and Coombs 1982). The researchers also measured several metabolic end products—succinate, acetate, alanine, and CO_2—with the amount secreted varying depending on the developmental stage assayed. In a complementary approach, Rainey and MacKenzie (1991) used ^{13}C nuclear magnetic resonance (NMR) spectroscopy to analyze the end products of *Leishmania pifanio* metabolism. Analysis of the positional isotopologs provided direct evidence for operation of the glycosomal succinate fermentation (GSF) pathway in *Leishmania* in which glycolytic phosphoenolpyruvate is imported back into the glycosomes and reduced to succinate via a series of reactions that replicate those in the TCA cycle. These studies supported a model for promastigote metabolism in which glucose is catabolized via glycolysis to generate phosphoenolpyruvate (PEP) and pyruvate, which are either converted to succinate (via the GSF) or alanine (via alanine transaminase) and secreted or further catabolized in the TCA cycle to generate CO_2, and reducing equivalents for oxidative phosphorylation (Fig. 2). While amastigotes also utilize glucose, they do so at a decreased rate, which coincided with increased use of fatty acids, although these studies did not quantitate the relative contribution of these different carbon sources. Notably, *Leishmania* lack a functional glyoxylate cycle and are therefore unable to generate C_4 precursors for sugar hexose synthesis, suggesting that any increase in fatty acid β-oxidation would primarily be used to sustain ATP synthesis or to top-up (anaplerosis) TCA cycle intermediates.

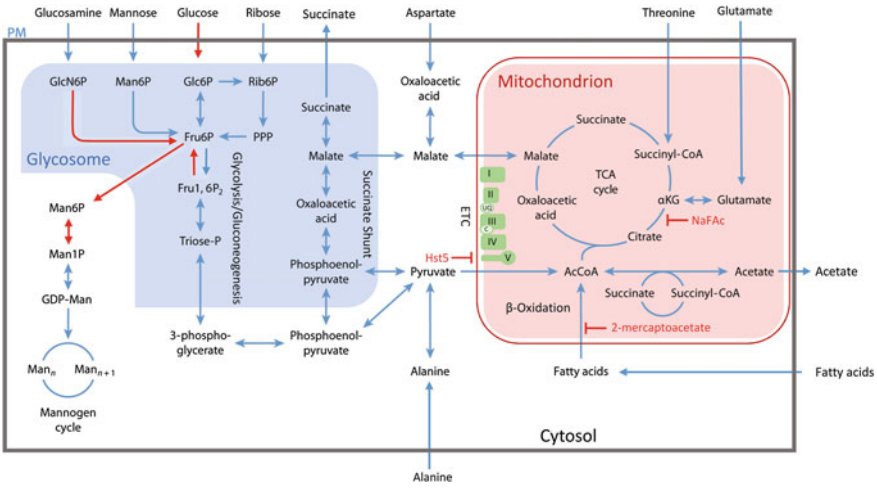


Fig. 2 Central carbon metabolism in *Leishmania*. The schematic shows the acquisition of carbon sources and key metabolic pathways. Glycolysis and gluconeogenesis as well as the GSF pathway occur in specialized peroxisomes termed glycosomes, while the TCA cycle and β-oxidation occur in mitochondria (enzymes for β-oxidation also localize to the glycosomes but mitochondria are likely the main site of β-oxidation). The major carbohydrate reserve material of *Leishmania* is comprised of short oligosaccharides (4–40 mannose residues long), termed mannogen which accumulates in the cytosol and may play a major role in regulating substrate availability for gluconeogenesis and glycolysis. The role of several enzymes/pathways in *Leishmania* metabolism was assessed by generating gene knock-outs or using metabolic inhibitors. Essential pathways are indicated by *red arrows*. In many cases, the effects of gene depletions or metabolic inhibitors on *Leishmania* viability differ between the promastigote and amastigote stage (see text), highlighting the distinct metabolism of the two life-cycle stages. For example parasites lacking the gluconeogenic enzyme FBPase grow normally as promastigotes in rich media but display attenuated virulence in macrophages and mice. Other pathways appear to be essential in both life-cycle stages—e.g., Luque-Ortega et al. (2008) showed that inhibition of the ATP synthase in *Leishmania* through histatin 5 is lethal for promastigotes and amastigotes. The salvage of some essential metabolites such as purine and vitamins are discussed elsewhere (e.g. McConville and Naderer 2011). *AcCoA* Acetylcoenzyme A; *C* Cytochrome C; *ETC* Electron transport chain; *Fru6P* Fructose-6-phosphate; *Fru1,6P*$_2$, Fructose-1,6-bisphosphate; *GDP-Man* Guanosine diphosphate mannose; *Glc6P* Glucose-6-phosphate; *GlcN6P* Glucosamine-6-phosphate; *Hst5* Histatin 5; *Man6P* Mannose-6-phosphate; *Man1P* Mannose-1-phosphate; *NaFAc* Sodium fluoroacetate; *KG* Ketoglutarate; *Man*$_n$ Mannogen oligosaccharides; *NAD* Nicotinamide dinucleotide; *PM* Plasma membrane; *PPP* Pentose phosphate pathway; *Rib5P* Ribose-5-phosphate; *TCA* Tricarboxylic acid; *Triose-P* Triose phosphate; *UQ* Ubiquinone; *I* Complex I (NADH dehydrogenase); *II* Complex II (fumarate reductase); *III* Complex III (cytochrome bc1 complex); *IV* Complex IV (cytochrome c oxidase); *V* Complex V (ATP synthase)

10.2 Intracellular Metabolite Levels

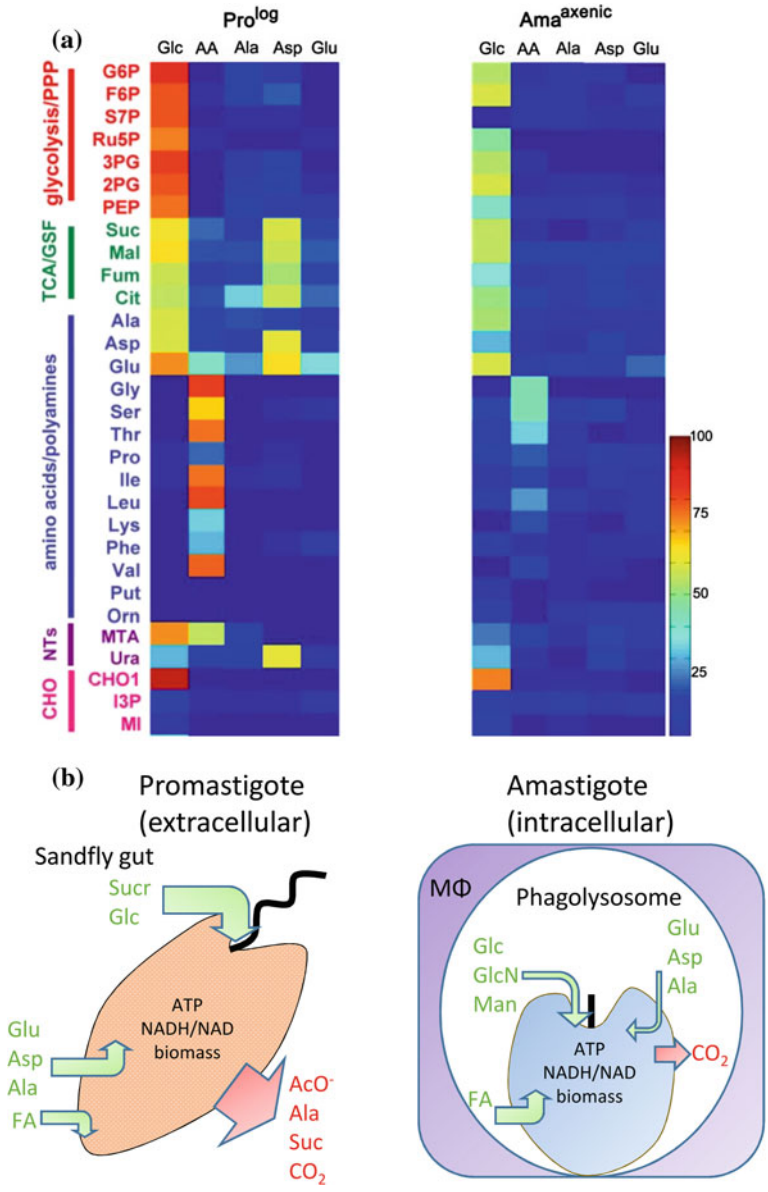
While metabolic footprinting has provided important insights into the distinct metabolism of the *Leishmania* life-cycle stages, the information gained from analyzing extracellular metabolites is limited. An alternative approach is to measure

changes in intracellular metabolite pools. Several studies on *Leishmania* promastigotes and amastigotes using NMR spectroscopy (Rainey et al. 1991; Gupta et al. 1999; Arjmand et al. 2016) identified a range of amino acids (Ala, Arg, Glu, Gln, Gly, Ser, Val, Iso/Leu), sugars (mannose), organic acids (lactate, succinate), and other metabolites (acetate, creatinine, β-hydroxybutyrate, glycerol 3-phosphate, glycerol, a-glycerophosphoryl choline, acetoacetate) that differed between the two developmental stages, reinforcing the importance of glucose catabolism in the promastigote stage and a shift to fatty acid/lipid catabolism in the amasitogote stage. In fact, Berg et al. (2015) proposed that the switch from a promastigote-like metabolism (highly glycolytic) to the amastigote-like metabolism (increased reliance on the TCA cycle and β-oxidation for energy generation) occurs gradually, as Pro^{stat} exhibit decreased levels of amino acids and sugar phosphates, which more closely resembles the metabolite profile of Ama^{axenic} than Pro^{log}. Gupta et al. (2001) compared the profiles of Ama^{axenic} with that of Ama^{lesion} and found some striking differences suggesting that Ama^{axenic} may represent an intermediate stage between Pro^{stat} and Ama^{lesion}. While many studies have suggested that Ama^{axenic} closely resemble Ama^{lesion} with regards to morphology, protein expression, infectivity, etc., differences between Ama^{axenic} and Ama^{lesion} potentially reflect inconsistencies in the nutritional conditions of the culture medium and lesion environments. These findings underscore the importance of undertaking future metabolomics work (and proteomics/transcriptomics work) in vivo where possible (see below), alternatively using purified extracted Ama^{lesion} or improving culture conditions to more closely resemble the in vivo milieu.

While these approaches point to the operations of specific pathways, they provide little information on overall flux or relative partitioning between different arms of central carbon metabolism. In particular, they do not allow distinction between the possibilities that changes in metabolite levels can be driven by changes in synthesis/degradation, and uptake/secretion. Even when uptake data are considered, it can be unclear whether a substrate is used for energy generation or metabolite biosynthesis. Given the dynamic nature of metabolite pools, stable isotope (e.g. ^{13}C, ^{2}H, ^{15}N) labeling approaches, provide a powerful means to measure metabolic flux.

10.3 Flux Analyses

A detailed analysis of metabolic networks of *L. mexicana* promastigotes and amastigotes was recently undertaken using comprehensive ^{13}C-stable isotope labeling (Saunders et al. 2014). All major developmental stages (Pro^{log}, Pro^{stat}, Ama^{axenic} isolated Ama^{lesion}) were labeled in defined medium with ^{13}C-labeled glucose, ^{13}C-amino acids, and ^{13}C-fatty acids and the incorporation of tracer carbons into a targeted list of 30 intracellular metabolites was quantitated by GC-MS. Both Ama^{axenic} and Ama^{lesion} were found to exhibit a distinct stringent metabolic response that was characterized by decreased glucose and amino acid uptake and more efficient utilization of these carbon sources (Saunders et al. 2014) (Fig. 3).



This glucose-sparing metabolism of amastigotes stands in stark contrast to the glucose-wasting metabolism observed in rapidly dividing Pro^{log} as well as nondividing Pro^{stat}, which were characterized by high rates of glucose uptake and secretion of partly oxidized end products (succinate, alanine, acetate) (Saunders et al. 2014). Interestingly, both nondividing (Pro^{stat}) as well as replicating promastigotes (Pro^{log}) exhibited a glucose-wasting metabolism, indicating that the

◀ **Fig. 3** Differences in the central carbon metabolism of *Leishmania* life-cycle stages. **a** ^{13}C-labeling experiments were carried out to determine differences in the central carbon metabolism and carbon source utilization of the *Leishmania* insect stage (Pro^{log}) and the disease causing amastigote stage (Ama^{axenic}) (Saunders et al. 2014). Following incubation of Pro^{log} and Ama^{axenic} in media containing U-^{13}C-glucose (Glc), U-^{13}C-amino acid mix (AA), U-^{13}C-alanine (Ala), U-^{13}C-asparate (Asp), or U-^{13}C glutamate (Glu), the ^{13}C-enrichment was quantified in numerous metabolites using GC-MS. **b** The study revealed marked differences between the two life-cycle stages—promastigotes exhibit a glucose-wasting metabolism which is characterized by rapid uptake and utilization of glucose as well as secretion of organic acids and alanine (*green arrows* indicate uptake of metabolites, *red arrows* indicate secretion of end products). In contrast, amastigotes display a glucose sparing metabolism and show drastically reduced rates of glucose as well as amino acid uptake and utilization together. Both stages scavenge fatty acids, but promastigotes primarily use these for membrane synthesis while amastigotes oxidize acquired fatty acids. *G6P* Glucose 6-phosphate; *F6P* Fructose 6-phosphate; *S7P* Seduheptulose 7-phosphate; *Ru5P* Ribulose 5-phosphate; *3PG* 3-phosphoglycerate; *2PG* 2-phosphoglycerate; *PEP* Phosphoenolpyruvate; *Suc* Succinate; *Mal* Malate; *Fum* Fumarate; *Cit* Citrate; *Ala* Alanine; *Asp* Aspartate; *Glu* Glutamate; *Gly* Glycine; *Ser* Serine; *Thr* Threonine; *Pro* Proline; *Ile* Isoleucine; *Leu* Leucine; *Lys* Lysine; *Phe* Phenylalanine; *Val* Valine; *Put* Putrescine; *Orn* Ornithine; *MTA* 5-methylthioadenosine; *Ura* Uracil; *CHO1* Mannogen; *I3P* Inositol 3-phosphate; *MI Myo*-inositol; *G3P* Glycerol 3-phosphate; *Sucr* Sucrose; *Glc* Glucose; *FA* Fatty acids; AcO^- Acetate; *GlcN* Glucosamine; *Man* Mannose; *ATP* Adenosine triphosphate; *NAD* Nicotinamide adenine dinucleotide. The figures are adapted from Saunders et al. (2014) and McConville et al. (2015)

stringent response was not just a consequence of reduced replication but rather a hard-wired response that is induced during amastigote differentiation. Amastigotes also exhibited increased fatty acid β-oxidation and increased reliance on the TCA cycle metabolism (Saunders et al. 2014), consistent with results from earlier proteomic analyses and metabolomics analysis (see above).

These labeling studies were used to predict pathways essential for amastigote survival in vivo. In particular, the finding that amastigotes stages exhibited negligible rates of glutamate uptake (compared to promastigotes) while the carbon backbones derived from ^{13}C-glucose and ^{13}C-fatty acids were incorporated into glutamate/glutamine, suggested that the TCA cycle may have an important role in generating precursors, such as α-ketoglutarate for glutamate/glutamine synthesis. These amino acids serve as essential amino donors for a number of other essential pathways including aminosugar synthesis, pyrimidine synthesis, and glutathione/trypanothione synthesis. Consistent with the proposal, treatment of infected macrophages with either sodium fluoroacetate (NaFAc, an inhibitor of TCA cycle aconitase enzyme) or methionine sulfoxime (MSO, an inhibitors of glutamine synthetase) resulted in death of intracellular *L. mexicana* amastigotes (Saunders et al. 2014). While all developmental stages exhibited some growth sensitivity to these inhibitors in axenic cultures, particularly when exogenous glutamate or glutamine was absent, Ama^{axenic} were much more sensitive to these inhibitors than Pro^{log}. While Pro^{log} ceased growth during treatment with 5 mM NaFAc, Ama^{axenic} lost viability at 50–100-times lower concentrations. The effect of NaFAc on Ama^{axenic} was only partially rescued by addition of very high concentrations of glutamate (5 mM). Similarly, MSO treatment led to a growth arrest in Pro^{log} which was rescued by supplementation with glutamine. Interestingly, growth

of intracellular $Ama^{M\Phi}$ could not be rescued by addition of exogenous glutamine to cultures suggesting that uptake of this amino acid into the phagolysosome by bulk vesicle flow is inefficient and/or that expression of glutamate amino acid transporters on intracellular amastigote stages is highly repressed. Taken together, these results suggest that amastigotes rely heavily on a functional TCA cycle to produce glutamate and glutamine and appear to downregulate the uptake of these amino acids. This is particularly surprising, as the phagolysosome is the major site of protein degradation and is expected to be rich in amino acids (Saunders et al. 2014).

These ^{13}C-labeling studies have also been used to define the objective function of a constraint-based model of *L. infantum* energy metabolism (i.e. not genome-wide) (Subramanian et al. 2015). The resulting model, consisting of over 230 reactions and 5 cellular compartments, was able to accurately predict in silico the growth phenotypes of previously experimentally generated knock-out mutants (for example, the *L. major* ΔFBPase and *L. mexicana* Δphosphomannomutase mutants, Naderer et al. 2006; Garami et al. 2001). Using the model, 61 single reaction combinations and 10,884 double reaction combinations were predicted that, when knocked-out, are anticipated to be lethal in *L. infantum* and therefore may prove useful drug targets. The model was also used to investigate changes to metabolism under different nutritional environments, for example the researchers observed that when optimum oxygen uptake is achieved, glucose is completely catabolized with succinate from the GSF entering the TCA cycle rather than being secreted. As oxygen uptake decreases, partially oxidized end products (acetate and succinate) are secreted from the cell. As described in the profiling and labeling experiments described above, glucose catabolism is critical even when other alternative carbon sources are abundant (the model predicts co-utilization of nonessential amino acids alters fluxes through glycolysis, glutamate biosynthesis and glycine/serine biosynthesis). In support of this, when glucose availability is restricted, the model predicts no parasite proliferation despite the availability of amino acids. Nonetheless, nonessential amino acids were still important when catabolized in conjunction with glucose, increasing biomass 1–1.5-fold over glucose alone (indeed Subramanian et al. highlight the importance of glutamate in ATP generation). Finally, the researchers created promastigote and amastigote scenarios in their model and were able to predict the large reduction in glycolysis, TCA cycle, ATP synthesis, and amino acid metabolism in the amastigote stage (reduced, but essential, glucose uptake, reduced glutamate uptake and reduced overflow metabolite excretion).

11 Studying *Leishmania* Metabolism in Vivo: Amastigote Proliferation and Macromolecule Turnover

All of the studies described above rely on the analysis of axenically cultured amastigotes or isolated Ama^{lesion} that have been incubated in rich media which may not reflect the nutrient levels in vivo (Saunders et al. 2014). Indeed, the nutrients

present in the phagolysosome as well as their concentrations are largely undefined (Lorenz and Fink 2002; McConville and Naderer 2011), making it difficult to establish culture media which replicates the conditions in vivo. An alternative approach is to use stable isotope labeling in vivo. A number of studies have infused animals with ^{13}C-labeled precursors (glucose or amino acids) in order to measure metabolic dynamics in vivo utilizing NMR spectroscopy or MS (Neurohr et al. 1983; Stromski et al. 1986; Shalwitz et al. 1989; Magkos and Mittendorfer 2009; Maher et al. 2012; Kowalski et al. 2015). However, these experiments are technically complex and limited by several factors. First, ^{13}C-labeled tracers are costly and in vivo analyses require high quantities of the tracer to achieve detectable levels in the analyzed tissue. While short-term labeling with these tracers can be easily achieved through an oral bolus (gavage) of the tracer, the long-term administration of ^{13}C-tracers requires continuous infusion, which is costly and stressful to the animal. Maintaining constant levels of a tracer within the analyzed tissue can also be challenging due to rapid clearance, catabolism, or zonation effects. Additionally, interpreting in vivo ^{13}C tracer data is expected to be particularly challenging when analyzing intracellular parasites, as the tracer is rapidly metabolized by the host with the pathogens potentially taking up a variety of labeled tracer-derived metabolites and intermediates. Hence, long-term ^{13}C-labeling approaches are largely restricted to in vitro applications and are not well suited for the study of intracellular pathogens.

An alternative approach to using ^{13}C-labeled tracers is the use of $^{2}H_2O$ (also known as heavy water, deuterium oxide and D_2O) as the tracer (Hellerstein 2004). $^{2}H_2O$ differs from natural abundance water (H_2O) in that it contains two deuterium atoms (^{2}H) instead of hydrogen (typically available as >99.9 % $^{2}H_2O$, v/v). $^{2}H_2O$ is utilized by many enzymes that include water in their catalytic mechanisms, resulting in the incorporation of ^{2}H into stable C–H/^{2}H bonds in a wide variety of metabolites. This enzymatic $^{2}H_2O$-labeling differs from ^{2}H-exchange that occurs across labile N–H or O–H bonds which is commonly used to identify exposed residues in protein folding studies (Englander et al. 1997). While high concentrations of $^{2}H_2O$ (>20 %, v/v) can be toxic to some organisms because of a solvent isotope effect (where $^{2}H_2O$/^{2}H can interfere with the catalytic efficiency of some enzymes) (Reuter et al. 1985; Takeda et al. 1998), few or no adverse effects are observed when cells/animals are exposed to low (≤15 %, v/v) concentrations (Thomson and Klipfel 1960; Lester et al. 1960; Kushner et al. 1999; Busch et al. 2007; Berry et al. 2015). The incorporation of ^{2}H into different metabolite pools (sugars, amino acids and fatty acids) and downstream macromolecules (DNA, RNA, proteins, lipids and polysaccharides) can be readily quantitated using MS or ^{2}H-NMR spectroscopy directly or after depolymerization of macromolecules of interest (Dufner and Previs 2003). Importantly, $^{2}H_2O$ rapidly equilibrates across all tissues and cells and can be administered to cell cultures or animals safely and easily for weeks or months, making it particularly suitable for measuring processes that have turnover times on the scale of days or longer (Dufner and Previs 2003; Busch et al. 2007). Administration of $^{2}H_2O$ also results in no perturbation to external or intracellular metabolite levels in biological systems, such as occurs

when a bolus of ^{13}C-labeled metabolites is introduced into cultures or animals (Berry et al. 2015). As a result of these features, $^{2}H_2O$-labeling is increasingly being deployed to measure multiple cellular processes in physiology and nutrition including human studies (Landau et al. 1995; Busch et al. 2006, 2007; Murphy 2006). $^{2}H_2O$ cannot replace but may complement ^{13}C tracers as the processes which can be measured using $^{2}H_2O$ are limited. However, several approaches have been developed which allow the quantification of the following processes in vivo using $^{2}H_2O$: Replication/DNA turnover (Neese et al. 2002; Hsieh et al. 2004; Busch et al. 2007; Pouteau et al. 2009), protein synthesis (Busch et al. 2006; Gasier et al. 2010), lipogenesis and cholesterol synthesis (Murphy 2006; Pouteau et al. 2009; Previs et al. 2011), as well as gluconeogenesis (Landau et al. 1995; Antoniewicz et al. 2011).

Kloehn et al. (2015) have recently shown that heavy water ($^{2}H_2O$–) labeling approaches can be employed to measure multiple physiological and metabolic processes in both cultured and tissue stages of *Leishmania* (Fig. 4). The labeling of the major life-cycle stages of *L. mexicana* with 5 % (v/v) $^{2}H_2O$ in culture can be used to accurately determine replication rates by measuring the incorporation of deuterium into the deoxyribose moiety of DNA using GC-MS. This approach was subsequently used to measure the growth rate of *L. mexicana* amastigotes in inflammatory lesions in infected BALB/c mice following enrichment of the host's body water with $^{2}H_2O$. Previously, the in vivo growth rate of pathogens was typically inferred from changes in the microbial burden in the relevant tissues as determined by direct enumeration of pathogen levels in tissue biopsies following microtitration and limiting cell dilution assays (Titus et al. 1985; Cotterell et al. 2000) or from measurements of pathogens that have been genetically manipulated to express bioluminescent or fluorescent proteins (Lang et al. 2005; Thalhofer et al. 2010; Millington et al. 2010; Michel et al. 2011). However, these methods only provide a measure of the net changes in microbial burden that reflect multiple parameters in addition to replication rate, such as death rate and pathogen dissemination to other tissues. In contrast, $^{2}H_2O$-labelling allows the measurement of cell turnover in vivo (Busch et al. 2007). Lesion amastigotes were found to have a doubling time of nearly 12 days, consistent with the ^{13}C-labeling studies showing that these stages enter a metabolically quiescent state. Although slow, this rate of doubling can still account for the observed increase in parasite burden in lesion granulomas, assuming parasite death is minimal (Kloehn et al. 2015). Taken together, these analyses indicate that activation of the stringent response may allow *Leishmania* amastigotes to sustain a very slow rate of replication and persist within long-lived macrophages. An intriguing implication of the finding that both parasite and macrophage populations are both long lived is that expansion of lesions and parasite numbers may occur via the slow replication of macrophages and partitioning of amastigote-containing phagolysosomes to each of the daughter cells. Such a mechanism would be consistent with microscopy observations that rarely detect extracellular parasites in granuloma tissues. Collectively, these findings indicate that murine inflammatory lesions constrain *Leishmania* growth but

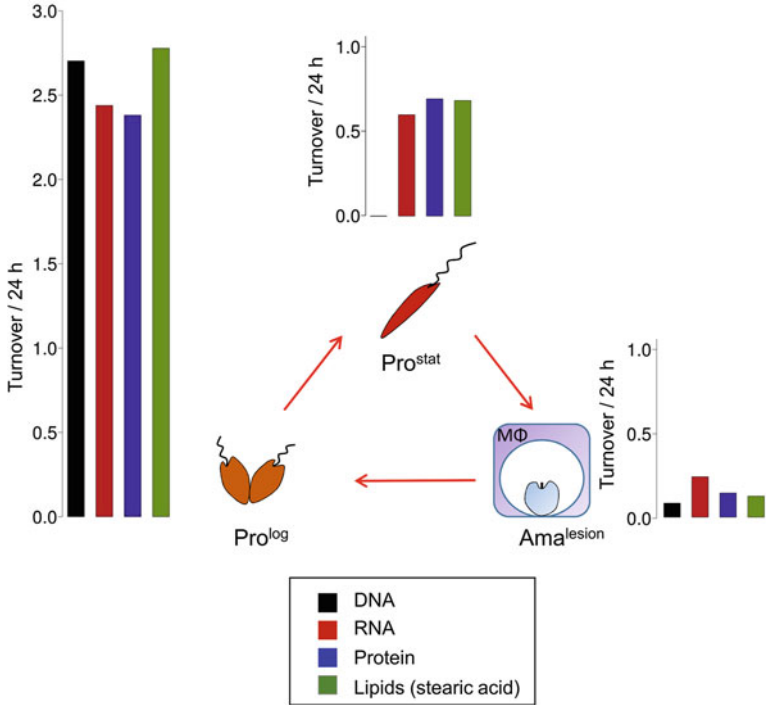


Fig. 4 The physiological state of *Leishmania* life-cycle stages. Differences in the replication rate as well as protein, RNA, and lipid turnover were measured using ^2H_2O-labeling in vitro and in vivo (Kloehn et al. 2015). *Leishmania* Pro^{log} show the fastest turnover rate for all macromolecules indicating rapid replication, protein and RNA, and lipid synthesis. Strikingly, the protein, RNA, and lipid turnover rates of Ama^{lesion} are reduced about 15–30-fold compared to Pro^{log} and are markedly reduced even compared to nondividing Pro^{stat}. These results indicate that *Leishmania* enter a semiquiescent state in the lesion environment which is characterized by slow growth and low activity of other energy intensive cellular processes such as protein synthesis

otherwise provide a highly permissive niche, which allows continuous expansion of the parasite population (Kloehn et al. 2015). This strategy differs from that of other granuloma-inducing pathogens such as *M. tuberculosis* (Munoz-Elias et al. 2005; Gill et al. 2009).

The ^2H_2O-labeling approach was further extended to measure global changes in RNA and protein turnover by measuring ^2H-incorporation into RNA-ribose and protein derived amino acids (Kloehn et al. 2015). As transcription and protein translation represent the most energy consuming processes in cells, these parameters are excellent indicators of the bioenergetic state of a cell. Significant differences were observed in RNA and protein turnover between major life-cycle stages. In particular, Ama^{lesion} were found to have much lower rates of RNA/protein turnover than other stages, including nondividing promastigotes (Kloehn et al. 2015),

providing further support for the notion that amastigotes enter into a distinct state of slow growth and metabolic quiescence.

Several studies on Ama^{axenic} have suggested that global rates of protein synthesis are downregulated, based on measurements of rates of ^{35}S-methionine incorporation and polysome profile analysis (Lahav et al. 2011; Cloutier et al. 2012). Another interesting approach for estimating protein turnover/metabolic activity in Ama^{lesion} in vivo has recently been developed using *L. major* that express a photoconvertible fluorescent protein (Muller et al. 2013). The authors suggest that protein synthesis in situ is reduced due to the suppression of metabolism and cell division by sublethal levels of NO. However, a broader application of this approach is limited given that the fluorescence readout is difficult to calibrate to overall protein turnover rates and also requires the generation of transgenic parasites lines with possible associated virulence reduction (da Silva and Sacks 1987; Moreira et al. 2012; Ali et al. 2013).

Additionally, $^{2}H_2O$-labeling was shown to delineate specific metabolic pathways in culture and in vivo. Analysis of ^{2}H-incorporation into total cellular fatty acid pools in Ama^{lesion} showed that this stage largely relies on the scavenging of fatty acids, in contrast to the situation in promastigotes. However, Ama^{lesion} were still dependent on the synthesis of linoleic acid (C18:2), as shown by the labeling of this fatty acid in parasite extracts but not host cell serum and tissue (Kloehn et al. 2015). Linoleic acid is the major polyunsaturated fatty acid of these stages and is synthesized by desaturation of oleic acid. The enzyme oleate desaturase is absent in mammals, and may therefore be a promising drug target.

12 Conclusion and Outlook

Metabolomic approaches have provided important new insights into the biology of *Leishmania* and will continue to be an essential tool for investigating the metabolism of these parasites given their dependence on posttranslational regulatory processes. In particular, metabolomics has been used to map metabolic networks in different developmental stages and parasite mutant lines, as well as to define the mode of action of drugs and understand resistance mechanisms. Importantly, various $^{13}C/^{2}H$ labeling strategies have now been used to map parasite metabolism and physiology in vitro and in infected tissues. We suggest that metabolomic measurements using noninvasive stable isotope tracers will be increasingly important to understand *Leishmania* metabolism in the mammalian host. Combining metabolomic approaches with other '-omics' approaches as well as molecular biology (e.g. gene depletion), biochemistry (e.g. metabolic inhibitors), and biological data (e.g. virulence) are expected to advance our understanding of *Leishmania* metabolism. Last, phenotypically heterogeneous cells and the mechanism underlying cell-to-cell variability have been identified and studied in a number of bacterial infections (Lewis 2010; Helaine and Holden 2013; Helaine et al. 2014; Bumann 2015; Kopf et al. 2016) but are poorly understood in protozoan infections, despite their crucial

role in development (e.g. *Plasmodium* gametocytes and hypnozoites; *Toxoplasma* bradyzoites, *Leishmania* metacyclics), persistence, and the emergence of drug resistance (Seco-Hidalgo et al. 2015). However, the majority of studies described in this chapter rely on bulk measurement, which provides an average of an entire population of typically $>10^7$ cells masking any variability within the population. Hence, novel single cell metabolomics approaches will be invaluable to identify cell-to-cell heterogeneity in protozoan parasite populations (Zenobi 2013; Seco-Hidalgo et al. 2015).

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