PLOS Pathogens

Divergent metabolism between Trypanosoma congolense and Trypanosoma brucei results in differential drug sensitivity
--Manuscript Draft--

Manuscript Number:	PPATHOGENS-D-21-00223
Full Title:	Divergent metabolism between Trypanosoma congolense and Trypanosoma brucei results in differential drug sensitivity
Short Title:	Divergent metabolism between Trypanosoma congolense and Trypanosoma brucei
Article Type:	Research Article
Section/Category:	Parasitology
Keywords:	Trypanosoma congolense; Trypanosoma brucei; Animal African Trypanosomiasis; metabolism; drug target identification
Abstract:	Animal African Trypanosomiasis (AAT) is a debilitating livestock disease prevalent across sub-Saharan Africa, a main cause of which is the protozoan parasite Trypanosoma congolense . In comparison to the well-studied T. brucei , there is a major paucity of knowledge regarding the biology of T. congolense . Here, we use a combination of omics technologies and novel genetic tools to characterise core metabolism in T. congolense mammalian-infective bloodstream-form parasites, and test whether metabolic differences compared to T. brucei impact upon drug sensitivity. Like T. brucei , glycolysis plays a major part in T. congolense energy metabolism. However, the rate of glucose uptake is significantly reduced in T. congolense , with cells remaining viable when cultured in concentrations as low as 2 mM. Instead of pyruvate, the primary glycolytic endpoints are succinate, malate and acetate. Comparative transcriptomics analysis showed higher levels of activity associated with the mitochondrial pyruvate dehydrogenase complex and acetate generation, and the glycosomal succinate shunt in T. congolense . However, based on omics analysis and chemical inhibition, there does not appear to be significant levels of oxidative phosphorylation. Stable-isotope labelling of glucose enabled the comparison of carbon usage between T. brucei and T. congolense , highlighting differences in nucleotide and fatty acid metabolism. To validate the metabolic similarities and differences, both species were treated with pharmacological inhibitors, confirming a lack of essential electron transport chain activity in T. congolense, but increased sensitivity to inhibition of mitochondrial pyruvate import. Strikingly, T. congolense exhibited significant resistance to inhibitors of fatty acid synthesis, including a 780-fold greater EC $_{50}$ against the lipase and fatty acid synthase inhibitor Orlistat, compared to T. brucei . These data highlight that bloodstream form T. congolense diverges from T. brucei in key areas of metabolism, with several features that are intermediate between bloodstream- and insect-stage T. brucei . These results have implications for drug development, mechanisms of drug resistance and host-pathogen interactions.
Additional Information:	
Question	Response

and **will appear in the published article** if the submission is accepted. Please make sure it is accurate.

* typeset

analysis, decision to publish, or preparation of the manuscript.

Competing Interests

Use the instructions below to enter a competing interest statement for this submission. On behalf of all authors, disclose any competing interests that could be perceived to bias this work—acknowledging all financial support and any other relevant financial or non-financial competing interests.

This statement **will appear in the published article** if the submission is accepted. Please make sure it is accurate. View published research articles from *PLOS Pathogens* for specific examples.

The authors have declared that no competing interests exist.

This statement is required for submission and **will appear in the published article** if the submission is accepted. Please make sure it is accurate and that any funding sources listed in your Funding Information later in the submission form are also declared in your Financial Disclosure statement.

Data Availability

Authors are required to make all data underlying the findings described fully available, without restriction, and from the time of publication. PLOS allows rare exceptions to address legal and ethical concerns. See the PLOS Data Policy and FAQ for detailed information.

A Data Availability Statement describing where the data can be found is required at submission. Your answers to this question constitute the Data Availability Statement and **will be published in the article**, if accepted.

Important: Stating 'data available on request from the author' is not sufficient. If your data are only available upon request, select 'No' for the first question and explain your exceptional situation in the text box.

Yes - all data are fully available without restriction

Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?	
Describe where the data may be found in full sentences. If you are copying our sample text, replace any instances of XXX with the appropriate details. • If the data are **held or will be held in a public repository**, include URLs, accession numbers or DOIs. If this information will only be available after acceptance, indicate this by ticking the box below. For example: *All XXX files are available from the XXX database (accession number(s) XXX, XXX.).* • If the data are all contained **within the manuscript and/or Supporting Information files**, enter the following: *All relevant data are within the manuscript and its Supporting Information files.* • If neither of these applies but you are able to provide **details of access elsewhere**, with or without limitations, please do so. For example: *Data cannot be shared publicly because of [XXX]. Data are available from the XXX Institutional Data Access / Ethics Committee (contact via XXX) for researchers who meet the criteria for access to confidential data.* *The data underlying the results presented in the study are available from (include the name of the third party and contact information or URL).* • This text is appropriate if the data are owned by a third party and authors do not have permission to share the data. * typeset	RNA-seq data is deposited at GEO (accession number: GSE165290; URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165290). Metabolomics data is deposited at MetaboLights (accession number: MTBLS2372; URL: www.ebi.ac.uk/metabolights/MTBLS2372). All other relevant data are within the manuscript and its Supporting Information files.
Additional data availability information:	

Divergent metabolism between *Trypanosoma congolense* and *Trypanosoma brucei* results in differential drug sensitivity

P. C. Steketee[1*], E. A. Dickie[2], J. Iremonger[1], K. Crouch[2], E. Paxton[1], S. Jayaraman[1], O. A. Alfituri[1], G. Awuah-Mensah[3], R. Ritchie[2], A. Schnaufer[4], H. P. de Koning[5], C. Gadelha[3], B. Wickstead[3], M. P. Barrett[2,6] and L. J. Morrison[1]

[1]The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, UK

[2]Wellcome Centre for Integrative Parasitology, Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, UK

[3]School of Life Sciences, University of Nottingham, Nottingham, UK

[4]Institute of Immunology and Infection Research, University of Edinburgh, Edinburgh, UK

[5]Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, UK

[6]Glasgow Polyomics, University of Glasgow, UK

*Corresponding author: Pieter.Steketee@ed.ac.uk

Abstract

Animal African Trypanosomiasis (AAT) is a debilitating livestock disease prevalent across sub-Saharan Africa, a main cause of which is the protozoan parasite *Trypanosoma congolense*. In comparison to the well-studied *T. brucei*, there is a major paucity of knowledge regarding the biology of *T. congolense*. Here, we use a combination of omics technologies and novel genetic tools to characterise core metabolism in *T. congolense* mammalian-infective bloodstream-form parasites, and test whether metabolic differences compared to *T. brucei* impact upon drug sensitivity. Like *T. brucei*, glycolysis plays a major part in *T. congolense* energy metabolism. However, the rate of glucose uptake is significantly reduced in *T. congolense*, with cells remaining viable when cultured in concentrations as low as 2 mM. Instead of pyruvate, the primary glycolytic endpoints are succinate, malate and acetate. Comparative transcriptomics analysis showed higher levels of activity associated with the mitochondrial pyruvate dehydrogenase complex and acetate generation, and the glycosomal succinate shunt in *T. congolense*. However, based on omics analysis and chemical inhibition, there does not appear to be significant levels of oxidative phosphorylation. Stable-isotope labelling of glucose enabled the comparison of carbon usage between *T. brucei* and *T. congolense*, highlighting differences in nucleotide and fatty acid metabolism. To validate the metabolic similarities and differences, both species were treated with pharmacological inhibitors, confirming a lack of essential electron transport chain activity in *T. congolense,* but increased sensitivity to inhibition of mitochondrial pyruvate import. Strikingly, *T. congolense* exhibited significant resistance to inhibitors of fatty acid synthesis, including a 780-fold greater EC_{50} against the lipase and fatty acid synthase inhibitor Orlistat, compared to *T. brucei*. These data highlight that bloodstream form *T. congolense* diverges from *T. brucei* in key areas of metabolism, with several features that are intermediate between bloodstream- and insect-stage *T. brucei*. These results have implications for drug development, mechanisms of drug resistance and host-pathogen interactions.

Author summary

Animal African Trypanosomiasis (AAT), also known as Nagana, is a devastating disease affecting livestock across sub-Saharan Africa. AAT is primarily caused by the parasite *Trypanosoma congolense*, yet our biological knowledge about this pathogen is poor, especially compared to the related species *T. brucei*. Understanding core metabolism of *T. congolense* is crucial in order to identify new drug targets, and develop novel chemotherapeutics. In this work, we addressed the lack of knowledge concerning *T. congolense* by carrying out a comprehensive analysis of core metabolism, and comparing the data to *T. brucei*. We then used the findings of metabolic differences to predict differential drug sensitivity. We show that unlike *T. brucei*, where glucose metabolism leads to high levels of pyruvate excretion, *T. congolense* metabolises glucose to other end-products, namely succinate, malate and acetate. Moreover, there are pronounced differences in the way *T. congolense* uses glucose to feed into other areas of metabolism. Further analysis also suggests that *T. congolense* prefers to scavenge lipids and fatty acids, rather than de novo biosynthesis of these essential factors. To validate these findings, we show that *T. congolense* is differentially susceptible to drugs compared to *T. brucei*, and in particular *T. congolense* is significantly less sensitive to inhibitors of fatty acid synthesis. Our study provides a foundation of functional metabolic knowledge on *T. congolense*, with key insights into how this parasite differs from *T. brucei*, and highlights potential areas that are exploitable for pharmacological intervention.

Introduction

The hemoflagellate protozoan parasite *Trypanosoma congolense* is a primary causative agent of animal African trypanosomiasis (AAT), which can also be caused by *T. vivax* and *T. brucei* [1]. AAT accounts for livestock deaths in excess of 3 million annually with up to 120 million cattle at risk [2-4], making AAT one of the most important livestock diseases across sub-Saharan Africa. Current methods of AAT control centre around chemotherapy and prophylaxis (reviewed in [3]), but the very few available veterinary trypanocidal drugs have been used

72 extensively for decades, resulting in resistance and inadequate protection against AAT [5-7].

73 As such, there is a dire need for the development of new and improved chemotherapeutics to

74 manage AAT [3, 8].

75 Most of our biological understanding of African trypanosomes derives from studies on *T.*

76 *brucei*, subspecies of which, *T. b. gambiense* and *T. b. rhodesiense,* cause Human African

77 Trypanosomiasis (HAT) [9]. The ability to culture both procyclic (PCF; tsetse fly) and

78 bloodstream (BSF; mammalian) forms of *T. brucei in vitro*, combined with its tractability with

79 respect to genetic manipulation, have enabled extensive study of this species on a molecular

80 level [10, 11]. In stark contrast, very few *T. congolense* strains are amenable to continuous

81 bloodstream form (BSF) culture, with a single strain (IL3000) used in most studies [12]. Whilst

82 genetic modification is possible in *T. congolense* PCF stage, routine BSF transfection has only

83 recently become possible [13-15]. Additionally, although *T. congolense* exhibits a superficially

84 similar morphology and life cycle to *T. brucei* [16, 17], emerging evidence increasingly

85 suggests that *T. brucei, T. congolense* and *T. vivax* exhibit some profound differences at the

86 genomic level [18-22], including in genes and phenotypes of direct relevance to infection

87 biology and disease epidemiology. However, there is a lack of understanding to what extent

88 these genetic differences translate into biological differences, including with respect to

89 metabolism.

90 Understanding metabolism is critical to identifying how pathogens survive and thrive in the

91 varying host environments they encounter, as well as being a means of elucidating drug

92 targets, modes of drug action and mechanisms of drug resistance [23-25]. *T. brucei*

93 metabolism has been extensively studied, aided by the application of technologies such as

94 liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR)

95 spectroscopy (reviewed in detail by [26, 27]), which enable global profiling of the cellular

96 metabolome.

97 The BSF stage of *T. brucei* utilizes the high levels of glucose available in the mammalian

98 bloodstream, and depends almost exclusively on the glycolytic pathway to generate ATP [28].

99   The first seven steps of glycolysis are encompassed by a specialized organelle, the

100   glycosome, which maintains its own ATP/ADP and NAD/NADH balance, allowing glycolysis

101   to proceed at an extraordinarily high rate in comparison to most other eukaryotic cells [29].

102   The endpoint of glycolysis, pyruvate, is a waste product of *T. brucei*, and the majority is

103   excreted from the cell in large quantities. However, small amounts of pyruvate are further

104   metabolized in the mitochondrion to acetate by pyruvate dehydrogenase (PDH) and

105   acetate:succinate CoA transferase (ASCT), a secondary, yet essential pathway [30]. The

106   acetate generated from this pathway is utilized, at least partially, for the *de novo* synthesis of

107   fatty acids [31]. Indeed, both BSF and PCF *T. brucei* are highly sensitive to the lipase and fatty

108   acid synthase inhibitor Orlistat [32].

109   Conversely, in the absence of blood meals, glucose is scarce in the tsetse fly midgut [33], and

110   the main energy source of PCF *T. brucei* is L-proline, the catabolism of which leads to

111   production of acetate, succinate and L-alanine through a more developed and active

112   mitochondrion (including an active respiratory chain capable of generating ATP, which is

113   inactive in BSF *T. brucei* [34]). Until recently, it was thought that PCF *T. brucei* did not exhibit

114   active TCA metabolism, although recent data have shown that TCA intermediates such as

115   succinate and 2-oxoglutarate can stimulate PCF *T. brucei* growth [35-37].

116   Among the glycolytic enzymes, *T. brucei* expresses three isoforms of phosphoglycerate

117   kinase (PGK), which catalyze the conversion of 1,3-bisphosphoglycerate to 3-

118   phosphoglycerate [38]. These are developmentally regulated, with the major isoform in BSF

119   parasites present in the glycosome (PGK-C), whilst the primary PCF isoform is found in the

120   cytosol (PGK-B) [39]. The localization of PGK-B in the PCF cytosol is thought to result in an

121   ATP/ADP imbalance in the glycosome, which is rectified by upregulating the glycosomal

122   "succinate shunt", a pathway that includes the ATP-generating phospho*enol*pyruvate

123   carboxykinase (PEPCK)- and pyruvate phosphate dikinase (PPDK)-mediated conversion of

124   phospho*enol*pyruvate (PEP) to oxaloacetate and pyruvate respectively [39, 40]. The succinate

125 shunt, combined with amino acid metabolism, results in the excretion of high levels of

126 succinate in PCF *T. brucei* [41].

127 Stable isotope labelling data has revealed that BSF *T. brucei* utilize D-glucose to a greater

128 extent than first realized, with heavy carbons disseminating into amino acid, lipid and

129 nucleotide metabolism [42]. This study also showed that some of the succinate and malate

130 excreted from BSF parasites originates from glycolysis and, unexpectedly, inhibition of

131 PEPCK is lethal at this life-cycle stage [42]. It has also been shown that acetate production is

132 essential to BSF *T. brucei*, in particular for the synthesis of fatty acids (FAs) [30]. However,

133 acetate excretion, as well as that of succinate and malate, is negligible in BSF *T. brucei*

134 compared to that of pyruvate and L-alanine.

135 In contrast to *T. brucei*, the literature on metabolism in *T. congolense* is scarce. More than half

136 a century ago it was suggested that BSF *T. congolense* has a significantly lower rate of

137 glucose consumption compared to BSF *T. brucei* [43]. Furthermore, pyruvate is not the main

138 glycolytic end product and instead, acetate and succinate are excreted at high levels,

139 indicative of metabolism more akin to PCF *T. brucei* [43]. Further work has revealed additional

140 differences that support this hypothesis [44-46]. For example, BSF *T. congolense* primarily

141 expresses cytosolic PGK-C, rather than glycosomal PGK-B [46]. Microscopy has also

142 revealed a more developed mitochondrion in BSF *T. congolense*, with visible cristae,

143 suggesting that mitochondrial energy metabolism could play a more prominent role in BSF *T.*

144 *congolense* [47]. The high levels of acetate excretion first shown by Agosin & Von Brand [43]

145 are consistent with this hypothesis. However, other studies have shown that BSF *T.*

146 *congolense* is sensitive to inhibitors of Trypanosome Alternative Oxidase (TAO), including

147 salicylhydroxamide (SHAM); and is insensitive to cyanide, suggesting that, as for BSF *T.*

148 *brucei*, TAO (Gene ID: TcIL3000.A.H_000015900) is the sole terminal oxidase, responsible

149 for reoxidising glycerol 3-phosphate, in BSF *T. congolense* [48-51]. Notably, nitroblue

150 tetrazolium staining indicates the presence of NADH dehydrogenase (complex I) activity in

151 BSF *T. congolense* [48]. However, to date, no studies have assessed BSF *T. congolense*

152 sensitivity to chemical inhibition of the electron transport chain, or the F_1F_o-ATPase.

153 Post-genomic technologies allow for the generation of large datasets that enable analysis of

154 cellular processes on a systems scale, including metabolomics and transcriptomics.

155 Integration of these data can provide a detailed snapshot of cell metabolism at the transcript

156 and metabolite levels and help to dissect differences between species or conditions in

157 unprecedented detail [52]. Furthermore, this knowledge can aid in predication and

158 understanding of drug efficacy and mode of action.

159 This study aimed to generate the first comprehensive overview of the metabolome of BSF *T.*

160 *congolense* IL3000 parasites, allowing a global metabolic comparison of differences between

161 *T. congolense* and *T. brucei*. Glycolytic metabolism in BSF *T. congolense* appears to be

162 similar to PCF *T. brucei*, particularly in terms of metabolic outputs and gene expression.

163 However, there are pronounced differences in parasite reliance on exogenous amino acids as

164 well as carbon dissemination into pathways involved in nucleotide and lipid metabolism, as

165 shown by stable isotope-labelled metabolomics. Using these data, we further validated these

166 metabolic differences in *T. congolense* by pharmacological inhibition, which highlighted

167 increased sensitivity to inhibition of mitochondrial pyruvate uptake, as well as significant

168 resistance to inhibition of fatty acid synthesis, tested using inhibitors of fatty acid synthase and

169 acetyl-coA synthetase. Taken together, these results suggest that *T. congolense* and *T. brucei*

170 differ in some fundamental aspects of their core metabolism, which has important implications

171 in terms drug sensitivity, and therefore, development of novel chemotherapeutics.

Results

Comparative RNA-sequencing of *T. congolense* and *T. brucei*

174 To permit direct comparison of BSF *T. congolense* and *T. brucei* at the transcriptome level,

175 RNAseq analysis was carried out on parasites cultured *in vitro* and trypanosome samples

176 isolated from infected mice at first peak parasitaemia (*ex vivo*) (Fig 1). Samples were prepared

177 using *T. congolense* (strain IL3000, *in vitro* and *ex vivo*) and pleomorphic *T. brucei* (strain

178 STIB 247; *in vitro* and *ex vivo*), to assess similarities and differences between trypanosomes

179 grown in culture and those from an infection (Fig 1A and 1B), and to compare and contrast

180 the transcriptome across the species (Fig 1C and 1D). Sequencing data were aligned to the

181 respective genome sequence with a mean overall alignment rate of $88.0 \pm 2.3\%$ and $94.1 \pm$

182 0.7% for *T. brucei* and *T. congolense* reads, respectively. Resultant files were sorted and

183 filtered for quality, and to minimize artefacts from multigene families, only uniquely aligned

184 reads were used for downstream analyses. Read counts were normalised using transcripts

185 per million (TPM) [53]. Orthologues were inferred between the species using Orthofinder [54],

186 in order to directly compare TPM values for 1-to-1 orthologues, as well as sum-of-TPM values

187 for groups containing families of paralogues (e.g. hexose transporters). These normalised

188 read counts are henceforth referred to as orthoTPM values (S1 Table). The Orthofinder

189 dataset (S2 Table) consisted of 6,677 orthogroups (denoted with the prefix "TbTc"), of which

190 5,398 (80.84%) were 1-to-1 orthologues. The Orthofinder tool was also used to predict genes

191 only present in one of the two species (S2 Table). There are several metabolic genes that are

192 not present in the *T. congolense* genome, including putative delta-4 and delta-6 desaturases

193 (Tb927.10.7100 & Tb11.v5.0580), a succinate dehydrogenase subunit (SDH11;

194 Tb927.8.6890) and guanine deaminase (Tb927.5.4560, Tb05.5K5.200 & Tb11.v5.0409), in

195 addition to mitochondrial pyruvate carrier 1 (MPC1; Tb927.9.3780) (S2 Table).

196 Differences between four sample groups were assessed based on orthoTPM values (Fig 1;

197 full dataset in S1 Table). There was a strong intra-species correlation between the *in vitro* and

198 *ex vivo* conditions at the transcriptome level (Pearson correlation coefficient, *T. congolense*

199 (ρ) = 0.765, Fig 1A; *T. brucei* ρ = 0.803, Fig 1B), showing that *in vitro*-derived BSF *T.*

200 *congolense* and *T. brucei* closely resemble parasites isolated from infections at the

201 transcriptome level. However, correlations between species even in the same condition were

202 lower, implying transcriptional differences between the species (*ex vivo*: ρ = 0.651, Fig 1C; *in*

203 *vitro*: ρ = 0.687, Fig 1D).

To compare data from this study to BSF *T. congolense* transcriptomics data generated by Silvester *et al.* (generated at ascending and peak parasitaemia [55]), TPM values for each annotated *T. congolense* gene were compared directly (S1 Fig, S3 Table). There was good correlation between both *in vitro* and *ex vivo T. congolense* BSF datasets and the data from Silvester *et al.* ($\rho > 0.8$, S1 Fig), with the highest correlation being between the *ex vivo* and ascending data as expected ($\rho = 0.897$, S1 Fig), albeit the correlation between the 'ascending' and 'peak parasitaemia' in Silvester *et al.* was higher ($\rho = 0.979$, S1 Fig).

T. congolense metabolite consumption and output

Global metabolite (metabolomics) analysis of *in vitro* culture supernatant samples provides a detailed insight into the metabolic inputs and outputs of cultured cells [56]. However, high levels of medium components can often mask subtle but significant changes in culture medium composition over time. To counteract this, a modified culture medium was designed for *T. congolense* strain IL3000, based on previous published medium formulations (Steketee's *congolense* medium, SCM-3; for details see Materials and Methods) [14, 15].

A time course was initiated in SCM-3. BSF *T. congolense* IL3000 cells during exponential growth phase were inoculated into fresh medium (0 h time point). Culture supernatant samples were collected at 0, 8, 24, 32, 48 and 56 hours (n = 4 at each time point) and metabolites extracted for LC-MS analysis.

A total of 290 putative metabolites were detected across all samples (207 after removing putative metabolites that did not map to metabolic pathways, e.g. peptides and medium components), of which 37 were matched to an authentic standard to confidently predict their identity (S4 Table).

80 of the 207 putative metabolites were significantly altered across the dataset (false discovery rate-adjusted $P < 0.05$; one-way repeated measures ANOVA; Fig 2A and S4 Table). To analyse metabolites undergoing similar changes, K-means clustering with Pearson correlation coefficient as the similarity metric was used, highlighting seven clusters with two clusters of

230 particular interest: one containing metabolites that accumulated over time, and the other

231 containing metabolites depleted over time (Fig. 2A). Log$_2$ fold change (Log$_2$ FC) between the

232 first and final time points (0 and 56 h, respectively) was also calculated for each metabolite

233 (S4 Table).

234 Glucose, the primary energy source for *T. brucei*, whilst clearly consumed, was not fully

235 depleted after 56 hours in *T. congolense* culture (Log$_2$ FC: -0.76; Fig 2A and 3A), in contrast

236 to *T. brucei*, where 10 mM glucose is consumed by the same time-point [56]. Ribose,

237 glucosamine, inosine and threonine were similarly depleted in *T. congolense* culture (Log$_2$ FC:

238 -0.78, -0.97, -2.82 and -0.89, respectively).

239 In contrast, a number of metabolites accumulated in the medium (Fig 2A). The most significant

240 of these were guanine (Log$_2$ FC: 6.34; Fig 2A and 5A), succinate (Log$_2$ FC: 5.60; Fig 2A & 3B)

241 and (S)-malate (malate, Log$_2$ FC: 1.37; Fig 2A and 3B). Interestingly, pyruvate (Log$_2$ FC: 0.24;

242 Fig 3B) was not excreted at the high levels relative to starting concentration, in contrast to BSF

243 *T. brucei* culture, where pyruvate secretion is consistently observed in both HMI-11 and in

244 Creek's Minimal medium (CMM) [56]. Instead, succinate and malate appear to be the primary

245 glycolytic outputs from BSF *T. congolense*, which is similar to PCF *T. brucei*. Elevated levels

246 of 2-oxoglutarate and a metabolite putatively identified as 2-oxoglutaramate were observed,

247 which potentially originate from alanine aminotransferase activity using L-glutamate and L-

248 glutamine, respectively, as substrates [42, 57]. Moreover, a significant build-up of *N*6-Acetyl-

249 L-lysine (Log$_2$ FC: 6.30) was observed (Fig 2B). Whilst the low molecular weight of acetate

250 means it could not be detected by the LC-MS platform used here, concentrations of this

251 molecule were measured directly using an acetate assay in samples taken at the same time

252 points from four independent cultures, which confirmed high levels of acetate excretion by

253 BSF *T. congolense* (Fig 3F).

254 Other notable observations included the depletion of several putative lysophosphatidylcholine

255 species at 56 hours (Fig 2A; S4 Table), as seen in *T. brucei* [56], coincident with increased

256 medium levels of *sn*-glycero-3-phosphocholine, choline and choline phosphate, indicating

257 lyso-phospholipase activity where the charged headgroup moiety of a lyso-species is cleaved

258 from its bound fatty acid [58]. In addition, tryptophan (Log$_2$ FC: -0.74; Fig 6B; S4 Table) was

259 significantly consumed ($P = 0.042$), in contrast with cysteine (Log$_2$ FC: -0.07; $P > 0.05$), despite

260 the latter being essential to *T. brucei* [59] (S4 Table).

261 The Log$_2$ metabolite fold changes after 56 hours of culture of *T. congolense* were compared

262 to those of *T. brucei* grown in HMI-11 (Fig 2B) [56]. A total of 90 metabolites were identified in

263 both datasets, with some showing divergence between the two species (Fig 2B). Several

264 metabolites only accumulated in *T. brucei* supernatant, in particular pyruvate, D-glycerate, 2-

265 oxoglutarate and 12-hydroxydodecanoic acid (Fig 2B). Conversely, succinate, *N*6-acetyl-L-

266 lysine, 4-hydroxy-4-methylglutamate, *N*6,*N*6,*N*6-trimethyl-L-lysine and choline only

267 accumulated in *T. congolense* supernatant (Fig 2B). Whilst cystine (Fig 2B; 12) was depleted

268 in *T. brucei* samples, this metabolite remained unchanged in those from *T. congolense*.

269 In summary, whilst core elements of metabolism have been conserved between BSF *T.*

270 *congolense* and *T. brucei*, several pronounced differences in *T. congolense* metabolism were

271 identified based solely on metabolic input and output in *in vitro* culture. An integrated analysis

272 of the metabolomic and transcriptomic datasets was then undertaken in order to further define

273 the metabolic differences between the two species.

Energy metabolism

275 As described above, RNA sequencing and culture supernatant metabolomics provided initial

276 indications that *T. congolense* energy metabolism, specifically with respect to glucose usage,

277 diverges substantially from that characterized in *T. brucei* BSFs (simplified map of glycolysis

278 depicted in Fig 3G).

279 To dissect metabolic differences at the transcriptome level, pathway analysis was carried out

280 using the TrypanoCyc database [60], which contains 186 manually curated pathways covering

281 422 genes or groups of multi-copy genes (S5 Table). These analyses showed broadly similar

282 levels of gene expression of glycolytic components between BSF *T. brucei* and *T. congolense*

283 (Fig 3G and 3I). However, the *T. brucei ex vivo* samples displayed a more distinct expression

284 profile, with low transcript abundances for most glycolytic components compared to all sample

285 groups. This is most likely the result of cells being sampled near peak parasitaemia, and as

286 the pleomorphic strain STIB 247 was used, having a higher proportion of tsetse-transmissible,

287 quiescent short stumpy forms – consistent with this there was elevated expression of stumpy

288 markers such as the PAD array (TbTc_0074), PIP39 (TbTc_0700) and reduced expression of

289 RBP10 (TbTc_0619) (S1 Table) [61-63].

290 Transcripts associated with gluconeogenesis, the succinate shunt, and the acetate generation

291 pathway were upregulated in BSF *T. congolense* under both *in vitro* and *ex vivo* conditions

292 compared to BSF *T. brucei*. Key examples of this are pyruvate phosphate dikinase (PPDK),

293 phosphoenolpyruvate carboxykinase (PEPCK), glycosomal malate dehydrogenase (gMDH)

294 and two subunits of pyruvate dehydrogenase (PDH) (Fig 3I). PPDK was previously reported

295 to be expressed in BSF *T. congolense*, but not BSF *T. brucei* [44], and it may be assumed

296 that the enzyme serves a similar function in BSF *T. congolense* as it does in PCF *T. brucei*; in

297 a mainly glycolytic role to maintain ATP/ADP balance in the glycosome. The high levels of

298 glycosomal MDH expression in BSF *T. congolense* contrasts with BSF *T. brucei*, where gMDH

299 expression is reported to be mostly absent, and cytosolic MDH (cMDH) is the major isoform

300 [64]. The RNAseq analysis also supports a previous study showing high levels of glycerol

301 kinase expression in BSF *T. congolense* [45]. The most recent PacBio assembly of the *T.*

302 *congolense* reference genome indicates that the parasite encodes five copies of PEPCK in

303 tandem array (TcIL3000.A.H_000300300, TcIL3000.A.H_000300400,

304 TcIL3000.A.H_000300500, TcIL3000.A.H_000300600 & TcIL3000.A.H_000300700;

305 compared to one copy in *T. brucei* – Tb927.2.4210; [65]), whilst there are only three copies of

306 glycerol kinase in *T. congolense* (compared to five in *T. brucei* TREU 927).

307 To confirm that the elevated levels of succinate and malate seen in *T. congolense* spent

308 medium samples originated from glucose, LC-MS analysis using ^{13}C-U-D-glucose was carried

309 out on intracellular metabolites from cell pellet extracts. Stable isotope analysis has provided

310   valuable insights into *T. brucei* central carbon metabolism [42], and generating *T. congolense*

311   datasets enabled comparative analysis of glucose catabolism (albeit with an unavoidable

312   difference in medium supplementation of goat serum for *T. congolense*, rather than foetal

313   bovine serum for *T. brucei*).

314   BSF *T. congolense* was grown for 48 hours in a custom medium (SCM-6; S6 Table),

315   containing a total D-glucose concentration of 10 mM in a 1:1 ratio of D-glucose:[13]C-U-D-

316   glucose. Following metabolite extraction, LC-MS analysis was undertaken and the majority of

317   glycolytic intermediates were detected, including ^{13}C-labels (Fig 3H). Moreover, labelling ratios

318   of downstream metabolites were largely similar to that of intracellular glucose, and the number

319   of carbons found to be labelled in each metabolite matched that which would be expected in

320   the BSF *T. brucei* glycolytic pathway (i.e. three ^{13}C atoms in all metabolites downstream of

321   glyceraldehyde 3-phosphate and glycerol-3-phosphate). Similar to *T. brucei*, a high

322   percentage of 3-carbon labelled fructose-1,6-bisphosphate (FBP) (34.8%) was observed in *T.*

323   *congolense* (Fig 3H), probably a result of the "reverse" aldolase reaction occurring in the

324   glycosome [42]. Importantly, two-carbon labelling was observed in several acetylated

325   compounds (N-acetylornithine & N-acetyl-L-lysine; Fig 3H), confirming that acetyl groups used

326   to generate these metabolites originate from D-glucose. Although acetyl-CoA, the product of

327   pyruvate oxidation, was not detected for technical reasons, labelling of acetylated metabolites

328   indicate that glucose-derived pyruvate is used to generate acetyl-CoA and subsequently

329   acetate in the mitochondrion, similar to other trypanosomatids. Taken together, these data

330   indicate that the flow of carbon atoms for glycolytic components in *T. congolense* is very similar

331   to that in *T. brucei*. However, the metabolic outputs differ drastically from BSF *T. brucei* and

332   appear to be more similar to PCF *T. brucei*.

333   To determine whether the elevated succinate in supernatants originated from glucose

334   catabolism, metabolite labelling was corrected for the 1:1 (50%) ratio of natural glucose to [13]C-

335   U-D-glucose, which equated to a mean percentage labelling of 43.1% (the value is less than

336   50% due to D-glucose in the serum). All glycolytic metabolites up to pyruvate showed >90%

337 labelling when corrected (for glucose 6-phosphate and fructose-1,6-bisphosphate, both 3-

338 carbon and 6-carbon labels were taken into account), although glycerol and glycerol 3-

339 phosphate exhibited 57.2% and 64.4% labelling, respectively, as these metabolites can also

340 be obtained from catabolism of lipid precursors. Moreover, 40.1% (93.0% corrected) labelling

341 was detected in L-alanine, suggesting that the alanine aminotransferase reaction that utilizes

342 pyruvate to generate 2-oxoglutarate and L-alanine in both BSF and PCF *T. brucei*, also occurs

343 in BSF *T. congolense* [42, 66]. For both succinate and malate, 3 carbons are derived from

344 glucose and these metabolites showed 33.6% (78.1% corrected) and 26.0% (60.3%

345 corrected) labelling, respectively. These results suggest that glucose is not the only source of

346 intracellular succinate and malate in *T. congolense*. However, these values were higher than

347 those reported in *T. brucei* (70% and 52% for malate and succinate, respectively [42]).

348 Whilst PCF *T. brucei* exhibit citric acid (TCA) cycle activity, this pathway is not used to

349 catabolize glucose [35]. Similarly, no citric acid cycle intermediate isotopologues (e.g. citrate)

350 were found when BSF *T. congolense* were incubated with ^{13}C-U-D-glucose, although small

351 amounts of 2-carbon labelled succinate and malate were observed (Fig 3H). This is similar to

352 BSF *T. brucei* [42], indicating that, like *T. brucei*, *T. congolense* does not appear to couple

353 glycolysis to TCA metabolism and instead directs high amounts of pyruvate through PDH into

354 acetyl-CoA and acetate. Taken together, these data suggest that BSF *T. congolense* both

355 from *in vitro* cultures and *in vivo* infection metabolically resemble an intermediate between

356 BSF and PCF *T. brucei*, with moderate glycolytic capacity and significant levels of succinate

357 shunt activity (glycosomal, rather than mitochondrial; S1 Table) as well as a highly active

358 mitochondrial acetate generating pathway.

359 Previous work has shown that reduction of glucose concentrations in BSF *T. brucei* culture

360 from 10 mM to 5 mM leads to decreased cellular motility, reduction in growth and cell body

361 rounding morphology within 8 hours [67]. Given that glucose was not substantially depleted in

362 *T. congolense* cultures after 56 h, we tested the effect of reduced glucose concentrations on

363 *T. congolense* viability. Unlike *T. brucei*, *T. congolense* was able to maintain a growth rate

equal to controls at concentrations as low as 2 mM (Fig 4A) when continuously passaged with no observable change in morphology or motility. To test whether glycolysis was essential in *T. congolense*, cells were incubated with D-glucose in addition to varying concentrations of 2-deoxy-D-glucose (2DG), which can be internalised, but not metabolised further than 2-deoxy-D-glucose 6-phosphate, thereby inhibiting glycolysis and pentose phosphate pathway metabolism (Fig 4B). Incubation of *T. congolense* in medium supplemented with 2DG (in addition to 10 mM glucose) led to growth defects in a dose dependent manner, likely due to 2DG being outcompeted by glucose at lower concentrations (Fig 4B). Although the growth defect was minor in the presence of 1 mM 2DG, there was a more pronounced reduction with 5 mM 2DG. When equimolar concentrations of glucose and 2DG were used, growth was repressed and cell death occurred within 48 hours (Fig 4B). *T. congolense* viability was also tested in SCM-6 in the presence of N-acetyl-D-glucosamine (GlcNAc), a sugar that inhibits glucose uptake [68] (S2 Fig). In the presence of 60 mM GlcNAc with 10 mM glucose, there was a moderate, yet significant ($P < 0.0001$ at 96 h, t-test of cell densities) growth defect in *T. congolense* (S2 Fig). Viability was further reduced when the same concentration GlcNAc was used alongside 2 mM glucose ($P < 0.0001$ at 96 h, t-test of cell densities), the lowest concentration *T. congolense* could tolerate (S2 Fig). The rate of glucose consumption was measured by assaying glucose concentrations in cell culture supplemented with 4 mM glucose, and shown to be 47.17 ± 27.91 nmol^{-1} min^{-1} 10^8 cells in *T. congolense*, significantly lower than the rate (132.18 ± 16.31 nmol^{-1} min^{-1} 10^8 cells) in *T. brucei* (n = 4, $P = 0.0039$; t-test).

To further probe glycolytic metabolism in BSF *T. congolense*, several targets were selected for RNAi-mediated knock-down, using a tetracycline-inducible *T. congolense* line expressing T7 polymerase and Tet repressor under puromycin selection (TcoSM [69]). Given that the majority of both malate and succinate appear to originate from glucose catabolism, the effect of reducing expression levels of two proteins involved in the succinate shunt, PEPCK and PPDK (TcIL3000.A.H_000922100 – both expressed at high levels in *T. congolense*; Fig 3I),

was tested in separate experiments (Fig 4C, D). RNAi was induced by addition of 1 µg/mL tetracycline, and cell growth in culture and transcript abundance measured by qPCR were monitored every 24 hours (Fig 4C, D). Creek *et al* showed that PEPCK is essential in BSF *T. brucei*, even though the levels of succinate generated through this pathway are negligible [42]. In BSF *T. congolense*, RNAi targeting the five copies of PEPCK reduced overall *PEPCK* transcript abundance by approximately 50% (mean transcript levels of 60%, 46% and 63% compared to uninduced controls at 24, 48 and 72 h post-induction, respectively; Fig 4E), leading to a small but non-significant reduction in growth rate (Fig 4C; $P = 0.0689$, *t*-test at the 96 h time-point). PPDK expression is not detected in BSF *T. brucei* but is expressed in the PCF stage [44]. Knock-down of PPDK in *T. congolense* did not affect parasite viability or growth rate (Fig 4D), although similar levels of transcript knockdown were observed (mean transcript levels of 67%, 64% and 50% compared to uninduced controls at 24, 48 and 72 h post-induction, respectively; Fig 4F).

RNAi was also used to knock down expression of the hexose transporter (HT) array, specifically those matching the THT1 and THT2 array in *T. brucei* (TcIL3000.A.H_000260500, TcIL3000.A.H_000260600, TcIL3000.A.H_000794500, TcIL3000.A.H_000794600, TcIL3000.A.H_000794700), which has been shown to significantly restrict growth of BSF *T. brucei* [70]. Whilst growth rate was unaffected in BSF *T. congolense* (Fig 4G), induction of HT RNAi led to a reduction in transcript abundance at all time points (mean transcript levels of 83%, 75%, 68% and 65% compared to uninduced controls at 24, 48, 72 and 96 h post-induction, respectively; Fig 4H). Glucose uptake was decreased (mean reduction of 37% in uptake compared to uninduced controls after 72 h; Fig 4I), suggesting that either lower levels of glucose are sufficient for energy generation in *T. congolense*, or the parasite can utilize other carbon sources for ATP production. These alternatives sources could include serum components such as fatty acids or amino acids, both of which trypanosomatids have been reported to utilise [71, 72].

417 PCF *T. brucei* express most components of the electron transport chain (ETC) to generate

418 ATP through oxidative phosphorylation, in contrast to BSF *T. brucei*, which do not detectably

419 express any ETC components with the exception of the reversed F_1F_o-ATPase and alternative

420 oxidase [73]. As mentioned previously, BSF *T. congolense* is thought to express a complex I

421 NADH dehydrogenase, but it is not known whether BSF *T. congolense* has capacity for

422 oxidative phosphorylation. Transcriptomics analysis of the ETC was attempted, using a gene

423 list generated by Zikova and colleagues [73], but no significant patterns could be discerned

424 (S1 Table, S3 Fig).

Nucleotide metabolism

426 Metabolomic analysis of BSF *T. congolense* culture supernatants indicated a significant

427 uptake of exogenous ribose, a contributor to nucleotide metabolism via uptake, or via the

428 pentose phosphate pathway (PPP; Fig 5A and Fig 2A). Whilst guanosine was not detected in

429 the supernatant, significant accumulation of guanine (Fig 5B) was observed, suggesting either

430 excretion of this metabolite, or, hydrolysis of guanosine through parasite-secreted

431 hydrolases/nucleosidases (previously identified in BSF *T. brucei* secretomes [74, 75]). This

432 mechanism would enable uptake of guanine and other nucleobases through nucleobase

433 transporters, for which multiple orthologues have been identified in the *T. congolense* genome

434 [18] through homology with known *T. brucei* nucleobase transporters TbNT8.1 and TbNBT1

435 [76, 77]. In addition, there was an accumulation of xanthine, a product of xanthosine

436 hydrolysis, and depletion of inosine, an important nucleoside composed of hypoxanthine and

437 ribose (Fig 5C and 5D). The nucleoside cytidine and the nucleobase hypoxanthine were also

438 detected, but appeared to remain unchanged during the time course, although the latter was

439 a medium supplement potentially added in excess (S4 Table). It is noteworthy that only a

440 single nucleoside transporter gene (TbTc_1072; *T. congolense* gene IDs:

441 TcIL3000.A.H_000665800 and the pseudogene TcIL3000.A.H_000679300; S2 Table) can be

442 identified in *T. congolense*, a syntenic homologue of TbNT10 [18], functionally characterized

443 as a P1-type purine nucleoside transporter [78], and is thus unlikely to transport cytidine [79].

444   Purine salvage is an essential process in trypanosomatids, as they lack the *de novo* synthesis

445   pathway for the purine ring [80], and previous analysis of cell pellets to investigate intracellular

446   nucleotide metabolism utilizing ^{13}C-U-D-glucose in BSF *T. brucei* showed purine salvage

447   pathways incorporating 5-carbon labelled ribose derived from glucose [42] (Fig. 5F). Whilst

448   the ribose incorporated into these nucleosides originates almost exclusively from glucose in

449   *T. brucei* (Fig 5F), *T. congolense* appears to use far less glucose-derived ribose to make

450   purine nucleosides such as adenosine, guanosine and inosine (Fig 5F).

451   Transcriptomics analyses indicated upregulation of genes associated with generation of

452   adenosine nucleotides (Fig 5G; red vertical bar), especially in *ex vivo T. congolense*, as well

453   as hypoxanthine-guanine phosphoribosyltransferase and uracil phosphoribosyltransferase.

454   Upregulation of nucleoside hydrolases and phosphoribosyltransferases supports previous

455   theories based upon genome content that *T. congolense* has a capacity for nucleobase uptake

456   [18].

457   The purines guanosine and inosine, which incorporate glucose-derived ribose in *T. brucei*,

458   were almost entirely unlabelled in *T. congolense* (Fig 5F). However, the phosphorylated

459   nucleosides GMP, GDP and GTP all incorporate glucose-derived carbon atoms, presumably

460   through ribose. Given the labelling patterns seen in adenosine, one possible explanation could

461   be conversion of AMP to inosine monophosphate (IMP; adenosine monophosphate

462   deaminase; TbTc_0145), IMP to xanthosine monophosphate (IMP dehydrogenase;

463   TbTc_1648) and XMP to GMP (GMP synthase; TbTc_1452). However, only one of these

464   enzymes, GMP synthase, was expressed at higher abundance in *T. congolense* (Log$_2$ fold

465   change: 1.56 and 2.02 for *ex vivo* and *in vitro*, respectively). Overall, incorporation of glucose-

466   derived carbons into purine nucleosides is reduced in *T. congolense* compared to *T. brucei*. It

467   should be noted that in both experiments, there was no ribose supplementation in the media

468   Of the pyrimidines, uracil and its derivatives were detected during the glucose labelling

469   experiment (S4 Fig). Uracil is known to be the main pyrimidine salvaged by other

470   kinetoplastids including *T. brucei* [81-83]. Whilst the majority of the uridine, UMP, UDP and

471   UTP pools incorporate glucose-derived ribose (five ^{13}C labels), 5-carbon isotopologues of

472   these pyrimidines were reduced in abundance in *T. congolense* compared to *T. brucei*.

473   Instead, 2-carbon labelled isotopologues appeared to comprise the majority of uridine, uracil

474   and their nucleotides (S4 Fig).

475   Whilst uracil biosynthesis is not essential in *T. brucei* [84], the uracil pool in *T. congolense*

476   appears to derive almost entirely from glucose, when corrected for 50% glucose labelling (76%

477   in *T. congolense* vs 44% in *T. brucei* [42]; S4 Fig), suggesting that this species predominantly

478   synthesizes uracil from orotate to UMP (orotate phosphoribosyltransferase/orotidine 5-

479   phosphate decarboxylase; TbTc_0735) and from UMP to uracil (uracil

480   phosphoribosyltransferase; TbTc_4220), as can occur in *T. brucei* [42]. Both these genes are

481   expressed at higher abundance in *T. congolense*, both *in vitro* and *ex vivo,* compared to *T.*

482   *brucei* (Fig 5G, S1 Table), which could explain the increased isotopologue labelling. Uridine

483   nucleosides (UMP, UDP, UTP) all show a similar pattern, with significant 2-carbon labelling,

484   as well as moderate levels of 5-carbon labelling from incorporation of glucose-derived ribose

485   (S4 Fig).

486   These data indicate that, at least under the growth conditions used here, BSF *T. congolense*

487   favours purine nucleoside/nucleotide synthesis from nucleobases with a reduced dependence

488   on glucose-derived ribose 5-phosphate, in addition to *de novo* synthesis of orotate, uracil and

489   uridine nucleosides. However, the difference in serum requirements for the two organisms is

490   a confounding factor to the interpretation of this difference.

Amino acid metabolism

491

492   It is well established that trypanosomatid parasites scavenge amino acids, key nutrients for

493   survival, from their hosts [85, 86]. Therefore, comparative analyses of *T. congolense* and *T.*

494   *brucei* amino acid metabolism were undertaken. Whilst the majority of amino acids were

495   detected during the supernatant time course, relative abundances in the medium did not vary

496   greatly after 56 hours of *in vitro* culture (Fig 6A–C, S4 Table). The greatest reductions were

497   observed in threonine (Log$_2$ FC after 56 hours: -0.89; Fig 6A), tryptophan (Log$_2$ FC: -0.74; Fig

498 6B), glutamine (Log$_2$ FC: -0.39), asparagine (Log$_2$ FC: -0.35) and phenylalanine (Log$_2$ FC: -

499 0.35). Interestingly, cysteine, an essential factor for the *in vitro* culture of *T. brucei*, was not

500 significantly consumed by 56 hours (Log$_2$ FC: -0.07; Fig 6C). However, at least low-level

501 exogenous cysteine is still required to sustain parasite growth *in vitro*, as viability was

502 significantly affected in the absence of cysteine (for both 1.5 mM and 1 mM vs 0 mM cysteine,

503 *P* < 0.0001, *t*-test of cells densities at 96 h; S5 Fig). Experiments were carried out to test the

504 essentiality of all other individual amino acids (with the exception of glutamine, known to be

505 an important amino donor in trypanosomatid metabolism). Using the minimal medium SCM-6,

506 cell viability was monitored for 72 hours in the absence of specific amino acids. Removal of

507 the following amino acids from culture medium led to defects in growth over 72 hours:

508 asparagine, histidine, isoleucine, leucine, methionine, proline, serine, tyrosine and valine (Fig

509 6D–G). Whilst aspartate appeared to be depleted in spent culture supernatants (S4 Table),

510 this also occurred in the medium only control. Furthermore, removal of aspartate did not lead

511 to reduced cell viability or growth rate in culture (Fig 6F). Long term culture was impossible

512 without the addition of phenylalanine and threonine, leading to a final culture formulation,

513 SCM-7 (S6 Table) containing a total of 14 amino acids. Therefore, BSF *T. congolense* appears

514 to require a higher number of amino acids than BSF *T. brucei*, at least *in vitro*, with CMM

515 containing only 8 amino acids in total, including cysteine and glutamine [56]. To further probe

516 amino acid metabolism, pathway analysis was carried out on the transcriptome (S6 Fig) and

517 metabolome (Fig 6; S6, S7 Fig).

518 BSF *T. brucei* utilizes exogenous L-glutamine as the primary source of intracellular glutamate

519 and 2-oxoglutarate and produce significant levels of glutamine-derived succinate [42, 85] (Fig

520 6I). Given the high levels of succinate excreted by *T. congolense*, stable isotope labelling was

521 used to determine the contribution of L-glutamine to this pool. *T. congolense* was incubated

522 for 48 hours with 1 mM ^{13}C-U-L-glutamine and cell pellets analysed by LC-MS. Results

523 indicated the presence of biochemical activities consistent with those observed in *T. brucei*.

524 Significant glutamine-derived carbon labelling was detected after 48 h incubation for succinate

(41.3%, 48.5% corrected), glutamate (76.1%, 89.2% corrected), 2-oxoglutarate (80.5%, 94.3% corrected) and succinate semialdehyde (94.7% corrected; Fig 6I). As would be anticipated, labelling of glutathione (86.1%) and trypanothione (98.4%) from glutamine through glutamate were also observed (S7 Fig). No labelling of malate or aspartate was seen in this study, despite the use of high concentrations of ^{13}C-U-L-glutamine compared to the equivalent study performed in *T. brucei* with a 50:50 ratio of ^{13}C-U-L-glutamine [85].

The apparent essentiality of several amino acids was also investigated using stable isotope labelling. Proline is an essential carbon source for PCF but not BSF *T. brucei* [87]. However, removal of proline from BSF *T. congolense* medium led to reduced growth (Fig 6F). RNAi-mediated knock-down of proline metabolism (specifically pyrroline-5-carboxylate dehydrogenase, TbP5CDH) in PCF *T. brucei* has highlighted the requirement of proline metabolism for mitochondrial function [87]. Indeed, both P5CDH (TbTc_1695) and proline dehydrogenase (TbTc_1591) expression were upregulated in *ex vivo T. congolense*, compared to *T. brucei*, suggesting that proline catabolism was more active (S1 Table and S6 Fig). However, ^{13}C-U-L-proline labelling showed that this amino acid did not contribute to the biosynthesis of other metabolites (S8 Fig). Therefore, the apparent requirement for proline in BSF *T. congolense* may be for the purposes of polypeptide synthesis only.

As in *T. brucei*, glucose-derived carbon usage was detected in several amino acids in *T. congolense* (S6A Fig). Aspartate (a precursor for pyrimidine nucleotide biosynthesis) and alanine (a by-product of a pyruvate-utilising aminotransferase reaction) (S6A Fig) exhibited 3-carbon isotopologues derived from ^{13}C-U-D-glucose. However, in *T. brucei*, a small proportion of L-asparagine labelling was observed (1.2% 3-carbon labelling) [42], whilst none was observed in *T. congolense* (S6A Fig). The metabolism of asparagine has not been studied in African trypanosomes; given the reduction of cell growth in the absence of this amino acid (Fig 6F), labelling with ^{13}C-U-L-asparagine was performed, but no other labelled metabolites were detected (S8 Fig). This indicates that, as with proline, protein synthesis is the sole role of asparagine in *T. congolense*. The reduced expression of asparagine synthetase (TbTc_4894;

552 TcIL3000.A.H_000497800), which converts aspartate to asparagine (S6 Fig), suggests that

553 BSF *T. congolense* may rely upon scavenging of exogenous asparagine.

554 Serine was also shown to be essential to *T. congolense* (Fig 6F), in contrast to minimal

555 culturing requirements for *T. brucei* [56]. ^{13}C-U-L-serine labelling indicated that *T. congolense*

556 L-serine metabolism mirrors that of *T. brucei* in several aspects, such as *de novo* sphingolipid

557 biosynthesis, with 70.0% 2-carbon labelling of sphinganine and downstream labelling of

558 ceramide and sphingomyelin species (S8 Fig). Similarly, phosphatidylserine decarboxylase

559 activity was evidenced at both transcript and metabolite levels, with 40.1% 2-carbon labelling

560 of glycerol-phospho-ethanolamine (S1 Table; S8 Fig). However, L-serine also has a minor role

561 in S-adenosyl-L-homocysteine detoxification, where serine-derived carbon ultimately

562 contributes to cysteine biosynthesis. In *T. congolense*, serine-derived carbon labelling can be

563 detected in cystathionine (18.1%) and cysteine (16.7%), through to glutathione (4.1%) and

564 trypanothione disulfide (3-carbon labelled, 6.8%; 6-carbon labelled, 0.02%; S7 Fig). Therefore,

565 the inability to exclude L-serine from *T. congolense in vitro* culture media may primarily be

566 attributable to lipid metabolism and an increased demand for serine-derived cysteine,

567 potentially over exogenously obtained cysteine, depending on bioavailability. Indeed,

568 metabolomics analysis of culture medium indicates that the ability of *T. congolense* to take up

569 cysteine from its environment may be lower than in *T. brucei* (Fig 6C).

570 Although L-cysteine is primarily a source of sulphur for trypanosomatids, we also investigated

571 the carbon contribution of this amino acid in *T. congolense*, and in particular, whether L-

572 cysteine-derived carbon atoms contribute to the biosynthesis of glutathione and trypanothione.

573 ^{13}C-U-L-cysteine stable isotope labelling experiments were performed (S7 and S8 Fig). Direct

574 replacement of the 1.5 mM L-cysteine present in SCM with ^{13}C-U-L-cysteine led to high levels

575 of labelling in glutathione and trypanothione disulfide (S7B Fig). This indicates that *T.*

576 *congolense* can readily take up and metabolize exogenous cysteine, even though abundance

577 of the amino acid is not reduced significantly over 56 hours of parasite *in vitro* culture. Although

578 no clear pattern could be observed in transcriptomic analysis of the trypanothione biosynthesis

579 pathway, both trypanothione synthase (TRYS; TbTc_1359) and trypanothione reductase

580 (TRYR; TbTc_4239) were expressed at high levels in *in vitro T. congolense* cells relative to

581 *ex vivo* cells, indicating that under *in vitro* conditions, cells may be subjected to higher levels

582 of oxidative stress (S7C Fig).

Fatty acid metabolism in *T. congolense*

584 Lipids have a variety of crucial roles in trypanosomes, as a major constituent of membranes

585 and under certain conditions, for energy [72]. BSF *T. brucei* require large quantities of myristic

586 acid in particular, for the synthesis of glycosylphosphatidylinositol (GPI) that anchors the

587 parasite's major surface glycoprotein antigens [88]. To do this, BSF *T. brucei* both synthesises

588 and scavenges myristic acid. Glucose labelling experiments in *T. brucei* have shown that

589 myristic acid is partially synthesized from glucose-derived carbon through acetyl-CoA, using

590 a system of fatty acid elongases [89] (Fig 7A). However, no fatty acid carbon labelling was

591 detected after incubation of *T. congolense* with ^{13}C-U-D-glucose (Fig 7A), unlike *T. brucei* [42].

592 Carbon dissemination was also investigated from threonine, which is used as a source of

593 acetate, and thus, lipids [90] (Fig 7B). Similarly, no saturated lipid carbon labelling was

594 observed, suggesting that *T. congolense* either uses alternative sources of carbon for lipid

595 biosynthesis, or does not rely on acetate as a source of lipids in the same way as *T. brucei*

596 [30].

597 While acetate/acetyl-CoA metabolism is highly active at the level of gene expression in *T.*

598 *congolense* compared to *T. brucei* (Fig 7C), consistent with metabolic data, expression of

599 acetyl-CoA synthetase (TbTc_0318), a key enzyme in lipid biosynthesis from acetate, is

600 reduced in both *ex vivo* and *in vitro T. congolense* (Fig 7C). Furthermore, an acetyl-CoA

601 thioesterase (TbTc_5515) that is involved in ATP synthesis-uncoupled acetate production in

602 PCF *T. brucei* [91] is also expressed at lower levels in *T. congolense* (Fig 7B). Other enzymes

603 involved in fatty acid biosynthesis, namely acetyl-CoA carboxylase (TbTc_0754), β-ketoacyl-

604 CoA synthase (TbTc_3372) and β-ketoacyl-CoA reductase (TbTc_1241), were all expressed

605 at lower abundance in *T. congolense* than *T. brucei*, in particular in *ex vivo* cells (Fig 7C). Of

606   the four elongases, ELO1 (TbTc_0159) and ELO2 (TbTc_1882) were expressed at equal

607   levels in BSF *T. congolense*, compared to BSF *T. brucei* (S1 Table). Whilst expression of

608   ELO3 (TbTc_0235) appeared to be reduced in *T. congolense* (Log$_2$ fold change of -1.98 and

609   -1.62 compared to *T. brucei* for *in vitro* and *ex vivo*, respectively; S1 Table), *T. congolense*

610   cells expressed higher levels of ELO4 (TbTc_0737) in both *in vitro* and *ex vivo* conditions,

611   compared to *T. brucei* (Log2 fold change: 1.39 and 1.38 for *in vitro* and *ex vivo* comparisons,

612   respectively)

613   The variation in observed gene expression associated with the sterol pathway appeared to

614   correlate with sample condition rather than species (Fig 7C). However, *T. congolense*

615   transcripts for genes involved in lanosterol synthesis were reduced, especially under *in vitro*

616   conditions (squalene synthase, SQase, TbTc_2577; squalene monooxygenase, SM,

617   TbTc_3357; lanosterol synthase, LSS, TbTc_4540; Fig 7C).

618   Fatty acid oxidation was recently confirmed to be an energy source for *T. brucei* residing in

619   adipose tissue [72]. Transcripts associated with this pathway were less abundant in *T.*

620   *congolense* compared to *T. brucei* under both conditions (Fig 7C), suggesting this may not be

621   an energy-generating pathway in glucose-rich culture medium, or under the *in vivo* conditions

622   from which they were sampled. However, capacity for ATP generation from fatty acid oxidation

623   should not be ruled out.

Exploiting differences in metabolism for pharmacological intervention

625   Differences in metabolism between *T. congolense* and *T. brucei* have implications for

626   differential drug efficacy between the two species. To validate our findings in key areas of

627   metabolism, pharmacological inhibition was attempted for specific targets in trypanosome

628   metabolism, in order to compare inhibitory concentrations (EC$_{50}$).

629   To assess whether areas of mitochondrial metabolism were required more in BSF *T.*

630   *congolense* than in BSF *T. brucei*, both species were treated with FCCP, an uncoupling agent

631   that depolarises the mitochondrial membrane. However, there was no difference in sensitivity

between the species (EC$_{50}$: 13.0 ± 5.0 µM and 12.6 ± 5.3 µM for *T. brucei* and *T congolense*,

respectively; Table 1). Given both metabolic and transcriptomic data indicated no increased

electron transport chain activity, we also treated with the complex III inhibitor antimycin A,

again with no significant differences seen between the species (Table 1). In addition, there

was no change in sensitivity to azide, an inhibitor of ATP hydrolysis by the F$_1$-ATPase (Table

1). However, *T. congolense* appeared to be less sensitive to rotenone, a complex I NADH

dehydrogenase inhibitor (Table 1). Previous data inferred complex I activity in BSF *T.*

congolense based on nitroblue tetrazolium staining [48]. Rotenone resistance could indicate

NADH dehydrogenase activity of a rotenone-insensitive NADH dehydrogenase, such as the

inner membrane space-facing NDH2 [92]. *T. congolense* also showed enhanced sensitivity to

salicylhydroxamic acid (SHAM), an inhibitor of the trypanosome alternative oxidase (TAO;

Table 1). Taken together, these data indicate that, like *T. brucei*, *T. congolense* does not rely

on oxidative phosphorylation for ATP production, as indicated by transcriptomics analysis, and

that, as previously reported, TAO is the terminal oxidase [48, 51].

Compound	Target	*T. congolense* EC$_{50}$ Mean ± SEM	*T. brucei* EC$_{50}$ Mean ± SEM	Fold change (Tc/Tb)	P value (*t*-test)
Antimycin	Complex III	271.2 ± 143.5 µM	144.2 ± 18.1 µM	1.9	0.4295
FCCP	Uncoupling agent	12.6 ± 5.3 µM	13.0 ± 5.0 µM	1.0	0.9592
Azide	F$_1$-ATPase	432.3 ± 127.9 µM	235.0 ± 6.0 µM	1.8	0.1982
Oligomycin	Complex V (F$_0$ ATPase)	33.9 ± 14.1 nM	197.6 ± 39.0 nM	0.2	0.0169
Rotenone	Complex I	27.4 ± 1.4 µM	7.4 ± 0.9 µM	3.7	0.0003
SHAM	TAO	14.4 ± 0.5 µM	26.0 ± 1.5 µM	0.6	0.0004
UK5099	Pyruvate transport	82.1 ± 8.8 µM	130.0 ± 5.0 µM	0.6	0.0091
ACS inhibitor	Acetyl-CoA synthetase	57.7 ± 15.2 µM	7.1 ± 2.4 µM	8.1	0.0304
Orlistat	Fatty acid synthase/lipases	15.6 ± 2.5 µM	0.02 ± 0.01 µM	780.0	0.0033
Diminazene	Kinetoplast	50.0 ± 5.6 nM	32.0 ± 0.5 nM	1.6	0.0425

Table 1: Comparative analysis of sensitivity to metabolic inhibitors in *T. congolense*

and *T. brucei*. Abbreviations: SHAM, salicylhydroxamic acid; LCFA, long-chain fatty acid

649 Metabolomics and transcriptomics data indicated that *T. congolense* direct pyruvate towards

650 mitochondrial metabolism, with high transcript levels in PDH and enzymes involved in acetate

651 generation, compared to *T. brucei* (Fig 3 and 7). We therefore hypothesised *T. congolense* to

652 be more sensitive to inhibition of mitochondrial pyruvate uptake and to investigate this further,

653 we tested drug sensitivities for UK5099, an inhibitor of mitochondrial pyruvate transport [93].

654 As expected, *T. congolense* (EC$_{50}$: 82.1 µM) was significantly more sensitive (P = 0.0091,

655 unpaired *t*-test) to UK5099 compared to *T. brucei* (130.0 µM; Table 1).

656 Whilst acetate generation appears to be important in *T. congolense*, our data suggest that the

657 acetate does not appear to be utilised for the biosynthesis of fatty acids, in contrast to what

658 has been shown for *T. brucei*. To probe this further, we compared drug sensitivity of the two

659 species with compounds targeting fatty acid synthesis (Fig 8). Indeed, *T. congolense* was

660 significantly more resistant than *T. brucei* to an acetyl-CoA synthetase inhibitor (ACS inhibitor;

661 1-(2,3-di(thiophen-2-yl)quinoxalin-6-yl)-3-(2-methoxyethyl)urea, [94]; Fig 8A; Table 1),

662 indicating that acetyl-CoA synthetase is far less essential to this species. ACS is essential to

663 both BSF and PCF *T. brucei* [30, 95], thus indicating a key metabolic difference between the

664 species.

665 We next compared drug sensitivity to Orlistat, an inhibitor of fatty acid synthase and

666 phospholipase [32]. Here, a striking difference was found, with *T. congolense* exhibiting

667 significantly less sensitivity (780-fold increase in EC$_{50}$) to the compound compared to *T. brucei*

668 (Fig 8B; Table 1), providing further evidence that *T. congolense* primarily relies on fatty acid

669 scavenging, instead of synthesis, as predicted by the combination of metabolomics and

670 transcriptomics.

Discussion

672 The protozoan parasite *T. congolense* is a principal cause of AAT, but crucially, *T. brucei*

673 remains the dominant model for laboratory-led studies of African trypanosomes, even in the

674 face of mounting evidence that *T. brucei* and *T. congolense* differ profoundly in many facets

675 of their biology. In order to facilitate the identification and development of potential drug targets

676 for *T. congolense*, a detailed understanding of the fundamental cellular metabolism, leading

677 to an understanding of both the differences and commonalities between *T. congolense* and *T.*

678 *brucei*, would be a significant step forward. Thus, this study aimed to generate a detailed

679 comparison of metabolism in *T. congolense* and *T. brucei*, through a combination of

680 metabolomics, transcriptomics and gene knockdown approaches. Based on these

681 comparisons, areas of metabolism were further probed with chemical inhibition, in order to

682 validate findings.

683 Transcriptomic data was generated from *T. congolense* and *T. brucei* with parasite samples

684 isolated from both *in vitro* culture and purified from *in vivo* murine infections (*ex vivo*). Crucially,

685 there were high levels of correlation between *ex vivo* and *in vitro T. congolense* samples,

686 indicating that the cultured form of the parasite closely resembles the *in vivo* situation, at a

687 transcriptomic level. In contrast, there was lower inter-species correlation between *T. brucei*

688 and *T. congolense.*

689 To complement the transcriptomic data, several metabolomic analyses were carried out to

690 gain an understanding of specific areas of metabolism. These data demonstrate that BSF *T.*

691 *congolense*, while possessing some metabolic similarities with BSF *T. brucei* (as expected),

692 differs substantially in several core components, including in having a reduced reliance on

693 glucose, excretion of distinct glycolytic end products (acetate, malate and succinate in *T.*

694 *congolense* compared to pyruvate in *T. brucei*), and increased gene expression and metabolic

695 signatures of specific mitochondrial pathways, in particular pyruvate to acetate conversion.

696 Additionally, we show increased reliance on exogenous substrates such as ribose for

697 nucleotide synthesis as demonstrated by reduced glucose-derived carbon labelling in

698 nucleoside species in addition to upregulation of hydrolases and phosphoribosyltransferases.

699 Furthermore, while there is overlap in amino acid utilisation (e.g. glutamine), *T. congolense*

700 relies on more exogenous amino acids than *T. brucei*. Surprisingly, this included serine which,

701 in the case of *T. congolense*, appears to be important in the transsulfuration pathway that is

geared towards trypanothione biosynthesis. This may also explain the observed decreased reliance on exogenous cysteine. Unlike *T. brucei*, *T. congolense* also requires asparagine and proline for viable *in vitro* culture, although carbon usage from these amino acids is minimal. Finally, *T. congolense* exhibits increased acetate/acetyl-CoA metabolism compared to *T. brucei*, despite a reduction in fatty acid biosynthesis through the classical trypanosomatid pathways involving acetyl-CoA synthase, acetyl-CoA carboxylase, β-ketoacyl-CoA synthase and β-ketoacyl-CoA reductase, the expression of which are reduced in *T. congolense* (both in *ex vivo* and *in vitro* conditions). This is further underlined by lack of glucose-derived 2-carbon labelling of fatty acids, most notably myristic acid, a key GPI anchor component of variant surface glycoproteins of *T. brucei* and *T. congolense* [96]. However, fatty acid elongase 4, previously shown to extend exogenously scavenged arachidonic acid (C22:4) to docosatetraenoic acid (C22:4) [97], is upregulated under *in vitro* conditions, compared to *T. brucei*, which may indicate a reliance on long-chain polyunsaturated fatty acids. These findings are shown in a summary figure of *in vitro* transcriptomics data (Fig 9)

Analyses of culture supernatants showed that 10 mM glucose was not substantially depleted after *T. congolense* cultures reached high cell density, as would be expected from an equivalently dense *T. brucei* culture [56]. *T. brucei* requires at least 5 mM glucose in culture [67], whereas BSF *T. congolense* were viable and maintained doubling times in levels as low as 2 mM. Furthermore, confirming conclusions from one previous study on BSF *T. congolense* [43], the primary metabolic outputs *in vitro* were (S)-malate, succinate and acetate, in contrast to *T. brucei*, in which the main output is pyruvate, which is excreted in large amounts [42, 56]. Interestingly, we observed a reproducible reduction in pyruvate levels in *T. congolense* supernatants over time, before abundance of this metabolite returned to levels similar to those observed in negative controls. A recent study in PCF *T. brucei* demonstrated that these parasites can re-metabolize glycolytic end products such as pyruvate and succinate [37]. Stable isotope labelling patterns in catabolic products derived from glucose do not support cyclical TCA activity, nor re-uptake of excreted metabolites in BSF *T. congolense*. However,

729   it would be of interest to determine whether this species can recycle the aforementioned

730   metabolites.

731   *T. congolense* exhibits high levels of expression in genes involved in the glycosomal succinate

732   shunt (PEPCK, glycosomal malate dehydrogenase and fumarate hydratase; Fig 9). In *T.*

733   *brucei* these phenotypes are associated with PCF rather than BSF; thus, to further dissect

734   glycolytic metabolism, RNAi was employed to investigate the essentiality of PPDK and PEPCK

735   in *T. congolense*. In *T. brucei*, PPDK is only expressed in the PCF stage, and is absent in the

736   BSF stage. In contrast, BSF *T. congolense* expresses PPDK at both transcript (Fig 3) and

737   protein [44] levels, although our initial analyses suggest that the protein is not essential for

738   growth *in vitro*. PEPCK was previously found to be essential in BSF *T. brucei* [42]; though in

739   BSF *T. congolense*, PEPCK knock-down only led to a mild reduction in growth rate. Previous

740   studies in PCF *T. brucei* demonstrated that individual null mutants of PEPCK and PPDK

741   showed no change in growth rate, with moderate reductions in glycolytic flux [40]. However, a

742   PEPCK/PPDK null mutant did exhibit reduced growth rates, with further data showing that

743   PPDK functions in a glycolytic direction and contributes to glycosomal ATP/ADP balance [40].

744   Further work is required to establish the roles of PEPCK and PPDK in BSF *T. congolense*.

745   Gene knock-out has not been previously attempted for *T. congolense*, and consistent with

746   other studies RNAi penetrance does not appear as efficient as in *T. brucei* [69]. Techniques

747   such as CRISPR/Cas9 and conditional knock-out would greatly enhance our capabilities to

748   study this parasite.

749   Whilst the major PGK isoform in BSF *T. brucei* is expressed in the glycosome, a previous

750   study suggested that the major isoform of phosphoglycerate kinase in BSF *T. congolense*

751   lacks the glycosomal targeting signal present in *T. brucei*, and is thus expressed in the cytosol,

752   akin to PCF *T. brucei* [46]. This has significant implications for glycosomal ADP/ATP balance,

753   as the expression of cytosolic PGK in BSF *T. brucei* is lethal [39]. Taken together, these data

754   suggest that *T. congolense* appears to carry out glycolytic metabolism in the same fashion as

755   PCF, not BSF *T. brucei*, including in *ex vivo* cells.

756 Whilst 2-deoxy-D-glucose does cause *T. congolense* death *in vitro* and supplementation of

757 cultures with GlcNAc also has a detrimental impact on viability, knock-down of the glucose

758 transporter array did not affect growth, even though glucose uptake appeared to be reduced

759 by 37% subsequent to 72 h of RNAi induction. These experiments highlight a crucial difference

760 between BSF *T. congolense* and *T. brucei* in a pathway that has become a metabolic

761 paradigm in the latter species. Whilst *T. brucei* requires high levels of glucose to sustain a

762 significant glycolytic flux, *T. congolense* remains viable in significantly lower glucose

763 concentrations, with a reduced flux, more similar to PCF *T. brucei*. However, glucose remains

764 an essential carbon source in this species, as growth is abolished in the absence of glucose.

765 Of particular interest is whether the parasite generates the majority of ATP from this reduced

766 glucose intake, or if it can thrive on other carbon sources such as amino acids or even fatty

767 acids. If the latter, this adaptation could be due to the reduced bioavailability of glucose in the

768 ruminant host bloodstream. Blood concentrations of glucose in humans are approximately 5.5

769 mM [98]. Glucose concentrations in ruminants are typically lower (2–4 mM [99-101]), and

770 primary sources of energy are typically volatile fatty acids in the form of acetic, propionic and

771 butyric acid [102, 103]. To date, products of volatile fatty acid metabolism, such as 2-

772 methylcitrate and 2-methyl-cis-aconitase have not been reported in *T. congolense*. However,

773 it is thought *T. brucei* can metabolise ketone bodies such as β-hydroxybutyrate through the

774 action of a β-hydroxybutyrate dehydrogenase (Tb927.10.11930) to generate acetoacetate . *T.*

775 *congolense* possesses an orthologue of this gene (TcIL3000.A.H_000824100), and therefore,

776 the ability of *T. congolense* to utilise other products available in adult ruminant blood merits

777 further investigation.

778 RNAseq analyses of *T. congolense* indicate high levels of expression of mitochondrial

779 pathways associated with glucose catabolism, specifically acetate and acetyl-CoA metabolism

780 involving PDH, ASCT and succinyl-CoA synthetase (SCS; Fig 9). Given that the large amounts

781 of acetate generated by the parasite appear not to be required for fatty acid synthesis, these

782 findings could suggest significant reliance on mitochondrial substrate level phosphorylation for

783   growth, similar to PCF *T. brucei* cultured in glucose-rich medium [104, 105]. Interestingly, *T.*

784   *congolense* does not appear to encode a homologue of MPC1, and therefore likely relies on

785   MPC2 for pyruvate transport into the mitochondrion. The lack of multiple pyruvate transporters

786   combined with the importance of mitochondrial pyruvate catabolism likely explains the

787   increased sensitivity of *T. congolense* to UK5099, a mitochondrial pyruvate transport inhibitor,

788   compared to *T. brucei*.

789   Our data are consistent with the absence of oxidative phosphorylation, based on

790   transcriptomics and lack of sensitivity to chemical inhibition, compared to *T. brucei*.

791   Interestingly, a previous study reported NADH dehydrogenase (complex I) activity in *T.*

792   *congolense*, offering the possibility of ATP generation via complex V [48]. However, there was

793   no change in rotenone sensitivity in *T. congolense*, suggesting that the NADH dehydrogenase

794   activity may originate from a rotenone-insensitive NADH dehydrogenase such as NDH2,

795   known to be important for acetate production in BSF *T. brucei* [92, 106, 107]. Furthermore,

796   sensitivity to the TAO inhibitor, SHAM suggests that TAO is the terminal oxidase, with no

797   significant complex III or IV activity. These conclusions support one previous study of BSF *T.*

798   *congolense* [48].

799   Rather than oxidative phosphorylation, we propose it is more likely that considerable ATP

800   production occurs in the ASCT – SCS cycle, which would explain the high levels of acetate

801   generated by *T. congolense*, in addition to increased sensitivity to inhibition of mitochondrial

802   uptake of pyruvate, the key metabolic precursor. Given that 2-oxoglutarate dehydrogenase

803   complex expression appears to be less than, or equal to, that in *T. brucei* (under *in vitro*

804   culturing conditions; Fig 9), it is likely that SCS activity occurs in the acetate-generating

805   pathway rather than in the TCA cycle, which is not thought to be fully functional in BSF African

806   trypanosomes [35], although recent data have challenged this paradigm in PCF *T. brucei* [37].

807   The mechanisms proposed here bear some similarities to the scheme proposed by Dewar

808   and colleagues for stumpy-form *T. brucei* metabolism, which also exhibit increased

809   mitochondrial metabolism compared to BSF *T. brucei* [108].

810   In *T. brucei*, carbon atoms from glucose disseminate through multiple pathways in the cell [42]

811   and, using stable isotope-labelled glucose, our data demonstrate that this pattern is also seen

812   in *T. congolense*, in particular through the glycolytic pathway, suggesting some of the key

813   metabolic differences observed are quantitative, rather than qualitative. However, there were

814   key differences in glucose-derived carbon usage. In particular, a reduction in labelling was

815   observed in purine nucleotides in *T. congolense*. In both species, carbon labelling is likely due

816   to generation of ribose phosphate sugars via the PPP and these data suggest that *T.*

817   *congolense* does not obtain its ribose through the PPP (from glucose), to the same extent that

818   *T. brucei* does. Interestingly, *T. congolense* appears to express higher levels of APRT1

819   (cytosolic) compared to APRT2 (glycosomal) to synthesise adenosine (Fig 5). This

820   discrepancy could underpin the reduced fraction of glucose-derived purine labelling, with a

821   reliance on ribose from alternative sources (for example, exogenously).

822   Whilst the majority of pyrimidine labelling is 5-carbons in *T. brucei*, indicating labelled ribose,

823   there is decreased 5-carbon labelling and higher abundance of 2-carbon labelling in *T.*

824   *congolense*, likely through uridine generated from aspartate through orotate, again

825   highlighting a reduction in glucose-derived ribose, but conversely, an increase in glucose-

826   derived UMP and its derivatives.

827   There was also a reduced abundance of glucose-derived fatty acid labelling in *T. congolense*

828   relative to *T. brucei*. Coupled with a decreased abundance of acetyl-CoA synthetase mRNA,

829   these results suggest that *T. congolense* may scavenge exogenous lipids in favour of carrying

830   out fatty acid biosynthesis, for which it must break down extracellular lipids to their constituent

831   parts. Indeed, supernatant metabolomics showed accumulation of both choline and choline

832   phosphate, with a corresponding decrease in the LysoPC lipids, which appears to indicate

833   activity of the phospholipases which *T. congolense* is known to secrete [58, 109]. It is unknown

834   whether *T. congolense* is able to generate cytosolic acetyl-CoA for fatty acid biosynthesis

835   through the action of citrate lyase, although transcript abundance of this gene was reduced

836   compared to *T. brucei*. Analysis of drug sensitivity supports these conclusions, as *T.*

837   *congolense* is significantly less sensitive to acetyl-CoA synthetase inhibition, as well as

838   Orlistat, and inhibitor of fatty acid synthase, suggesting that fatty acid scavenging (e.g. lipid or

839   fatty acid transporters) could be a viable therapeutic target for this species. However, the

840   efficacy of orlistat *in vivo* has not been reported to our knowledge.

841   BSF *T. brucei* growth in CMM required only cysteine and glutamine when supplemented with

842   FBS gold, although a further 6 amino acids (Tyr, Phe, Trp, Leu, Met and Arg) were required

843   when supplemented with standard FBS [56]. As part of this study, 14 amino acids essential

844   for *T. congolense* growth were identified. Tryptophan and arginine, essential to *T. brucei*, were

845   not required to sustain *T. congolense* growth in 10% goat serum. Conversely, several amino

846   acids considered not essential to *T. brucei* were crucial for *T. congolense* growth *in vitro* (Asp,

847   His, Ile, Pro, Ser and Val). Proline is a well-established carbon source for PCF *T. brucei* [87].

848   However, based on stable isotope labelling experiments, this amino acid is solely used for

849   protein synthesis in BSF *T. congolense*, as there was no evidence of carbon dissemination

850   from proline into the metabolome (likewise for asparagine). Unlike BSF *T. congolense*, BSF

851   *T. brucei* must be able to synthesise sufficient amounts of these amino acids from alternative

852   sources, or obtain them from the serum supplement.

853   One metabolic area of interest in trypanosomatids is trypanothione biosynthesis, a crucial

854   pathway for parasite response to oxidative stress. Indeed, trypanothione synthase, as well as

855   proteins involved in the trypanothione biosynthesis pathway, such as ornithine decarboxylase

856   (targeted by Eflornithine), have long been considered prime chemotherapeutic targets due to

857   their absence from other organisms [110]. Whilst cysteine was previously known to be a main

858   carbon contributor to trypanothione synthesis in *T. brucei* along with glutamine and methionine

859   [85], we show here that serine, an amino acid essential to *T. congolense*, also contributes to

860   the generation of this metabolite in addition to the aforementioned amino acids. These data

861   indicate that *T. congolense* can both synthesise and transport cysteine. Interestingly, cysteine

862   was not significantly depleted from *T. congolense* culture supernatants and future work should

ascertain whether the presence of L-serine in medium can compensate for reduced cysteine levels in *T. congolense* culture.

The data presented here have led to the generation of a novel semi-defined medium for culturing the strain IL3000, which must be further optimized for the culture of multiple strains of *T. congolense.* Of interest is the peculiar requirement of adult bovine or goat serum for *in vitro* culture of *T. congolense*, rather than foetal bovine serum (FBS) which is typically used to culture *T. brucei* [15, 59]. Whilst this study made no attempts to adapt *T. congolense* to FBS-supplemented medium (indeed, even in SCM-7, growth rate is drastically reduced in the presence of FBS after 2-3 passages), this is of crucial importance, as it would allow the study of multiple species of African trypanosome under the same *in vitro* conditions. Analysis of metabolism presented here indicates that this phenomenon is likely to centre on the lipid requirements of *T. congolense*, although it remains to be seen if this requirement is for energy generation or synthesis of lipids in general. Furthermore, adult ruminant serum composition drastically differs from that of non-ruminants and of foetal ruminants [102, 103], and this likely has significant implications on the extracellular environment faced by livestock trypanosomes.

The information presented here is a significant step in laying the foundation for fundamental understanding of metabolism for an important livestock parasite. Understanding essential areas of metabolism in both *T. brucei* and *T. congolense* enables the development of drugs effectively targeting both species. Conversely, understanding the key differences between the two species aids in dissecting drug mechanisms of action and resistance, as well as enabling a greater understanding of host-pathogen dynamics.

Materials and Methods

Compounds and reagents

All compounds were obtained from Sigma/Merck with the exception of: Orlistat (Cambridge Bioscience), oligomycin A (VWR International), diminazene aceturate (Cambridge BioScience) and FCCP (Abcam).

Cell lines and *in vitro* culture

In all cases, *T. congolense* strain IL3000 [48] was used (originally received from Theo Baltz, University of Bordeaux). For RNAi experiments a *T. congolense* IL3000 single marker line, TcoSM, was used [69]. For *in vitro* experiments, cells were grown at 34°C, 5% CO_2 and routinely cultured in either TcBSF3 [14] or HMI-93 [15], in both cases without a serum plus supplement, with 20% goat serum (Gibco). For global metabolite analysis of culture supernatant, an experimental medium (SCM-3) was used with the following components: 77 mM NaCl, 1.5 mM $CaCl_2$, 4.5 mM KCl, 0.8 mM $MgSO_4$, 36 mM $NaHCO_3$, 25 mM HEPES, 0.05 mM bathocuproinedisulfonic acid, 0.22 mM 2-mercaptoethanol, 50 U/mL penicillin/streptomycin, 2.5 mM glucose, 1 mM pyruvate, 10 % goat serum, 10% TcBSF3 [14], 1 mM each of L-cysteine and L-glutamine, and 100 µM L-tyrosine, L-phenylalanine, L-tryptophan, L-leucine, L-methionine and L-arginine. BSF *T. congolense* in exponential growth phase were centrifuged at 1,500 × *g* for 10 minutes, washed with PBS and inoculated into this medium (0 h time point).

For stable isotope labelling experiments, as well as experiments involving the removal or addition of specific medium components, a custom medium, Steketee's Congolense Medium-6 (SCM-6) was used (S6 Table). The final medium formulation based on this study's findings, SCM-7, is provided in S6 Table. This medium is essentially HMI-93, although i) vitamins (with the exception of folate) were removed, ii) D-glucose concentrations were modified depending on experimental procedure, but was routinely kept at 10 mM, iii) goat serum levels were reduced to 10% and, iv) of the 20 amino acids, 14 were added. Increasing the temperature to 37°C led to a detrimental effect on cell viability after several passages, as previously reported [15].

For experiments involving *T. brucei*, either the monomorphic Lister 427 (*in vitro* experiments and growth curves) or pleomorphic STIB 247 (RNAseq experiments, both *in vitro* and *ex vivo* sample groups) strains were used. Lister 427 cells were grown in HMI-11 [111], whilst STIB

915   247 were grown in modified HMI-9 containing 1.1% methylcellulose and 20% serum plus

916   (Sigma) [112, 113]. In both cases, cells were incubated at 37°C, 5% CO_2.

917   For both species, cell counts were carried out using a haemocytometer, and in the case of *T.*

918   *congolense*, cells were mechanically detached from the culturing plasticware by pipetting prior

919   to counting. Growth curves were routinely carried out in 2 mL samples incubated in 24-well

920   plates, resuspended using a P1000. For detachment of cells from flasks, 10 mL plastic pipettes

921   were used. In cases where cells were harvested for experiments other than those involving

922   metabolomics, cells could also be detached by replacing the medium with PBS for incubating

923   at room temperature for several minutes, prior to vigorously tapping the flask to detach

924   parasites.

925   RNAi experiments using TcoSM were carried out in HMI-93 in 20 mL cultures. Cells were

926   seeded at 7×10^5 cells/mL and RNAi induction was initiated with the addition of 1 μg/mL

927   tetracycline (Sigma) and 1×10^7 cells were isolated every 24 hours for RNA analysis (outlined

928   below) before cells were passaged

929 **Ethics statement**

930 All animal experiments were performed in accordance with the Animals (Scientific Procedures)

931 Act 1986 and the University of Glasgow care and maintenance guidelines. All animal protocols

932 and procedures were approved by The Home Office of the UK government and the University

933 of Glasgow Ethics Committee.

934 **Animal experiments**

935 Adult female CD-1 mice (20–30 g body weight; Charles River Laboratories) were infected with

936 5×10^4 wild-type *T. brucei* STIB 247 or 1×10^5 wild-type *T. congolense* IL3000 by

937 intraperitoneal injection. Parasitaemia was monitored daily by venesection of the lateral tail

938 vein [114]. At first peak of parasitaemia ($>10^7$ cells/mL) mice were euthanised and blood

939 isolated. Parasites of both species were purified from blood by anion exchange using DEAE

940 cellulose [115]. Purified cells were counted, and a total of 1×10^8 cells were centrifuged for 10

941 minutes at $1,500 \times g$ prior to RNA extraction.

RNA extraction

For RNAseq experiments, 10^8 cells were isolated either from *in vitro* culture or from mouse infections. RNA was extracted using the QIAgen RNeasy kit (Qiagen) with an on-column DNase treatment step. Sample concentrations were analysed by Nanodrop and QuBit, and concentrations adjusted to 37 ng/µL of which 80 µL (2.96 µg) was submitted for RNAseq.

For RNAi time course experiments, cell pellets (10^7 cells) were resuspended in 1 mL TRIzol (Invitrogen) and stored at -80°C. Samples were thawed, 200 µL chloroform was added, samples were shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes, prior to centrifugation at 12,000 × *g* for 15 minutes, 4°C. The aqueous layer was transferred to a fresh tube and 500 µL isopropanol and 1 µL Glycoblue (Invitrogen) were added. Samples were mixed by inverting, incubated at room temperature for 10 minutes and centrifuged at 12,000 × *g* for 10 minutes at 4°C. RNA pellet was washed in ice-cold 75% ethanol and centrifuged at 12,000 × *g* for 10 minutes at 4°C. After air-drying, RNA was resuspended in 20 µL RNase-free water and concentration adjusted to 100 ng/µL. DNase treatment was carried out using the Ambion TURBO DNase kit (Applied Biosystems) as per manufacturer's instructions.

Metabolomics sample preparation

For metabolomics analysis of supernatants, 10 mL *T. congolense* cultures were incubated in T25 flasks in relevant media. Cells were centrifuged at 1,500 × *g* for 10 minutes, washed with PBS, resuspended in relevant media and density adjusted to 1×10^5 cells/mL. At each time-point, 500 µL medium was transferred to a 1.5 mL Eppendorf tube and briefly quenched in a dry ice/ethanol bath, before centrifuging at 1,500 × *g* for 10 minutes at 4°C. A 5 µL aliquot was then transferred to a new Eppendorf containing 200 µL metabolite extraction solvent (chloroform:methanol:water in a 1:3:1 ratio) and samples vortexed at 4°C for one hour. Samples were centrifuged for 5 minutes at 13,000 × *g* (4°C) and supernatants transferred to new Eppendorf tubes. Samples were stored at -80°C prior to analysis.

968 For analysis of intracellular metabolites, cells were grown to a final density of 2×10^6 cells/mL

969 and a total of 10^8 cells isolated. Cells were quenched in 50 mL falcon tubes to 4°C using a dry

970 ice/ethanol bath (stirred and measured by thermometer) and all subsequent steps were carried

971 out at 4°C. Cells were centrifuged at $1,500 \times g$ for 10 minutes and if supernatant samples were

972 required in addition to cell pellets, 5 µL was transferred to an Eppendorf containing 200 µL

973 extraction solvent. Cells were resuspended in residual medium before transfer to Eppendorf

974 tubes. Cells were then centrifuged ($1,500 \times g$, 5 minutes) and washed twice with ice-cold

975 phosphate buffered saline (PBS) before resuspension in 200 µL extraction solvent

976 (chloroform:methanol:water in a 1:3:1 ratio). Samples were vortexed at 4°C for 1 hour, and

977 then centrifuged for 5 minutes at $13,000 \times g$. Supernatants were transferred to clean

978 Eppendorf tubes. For all experiments, a quality control sample was generated by pooling 10

979 µL from each sample and samples were stored under argon gas at -80°C.

Primers and plasmids

981 RNAi experiments were carried out using a *T. congolense* single marker line, TcoSM [69] that

982 expresses Tet repressor and T7 polymerase, maintained in 0.5 µg/mL puromycin, and gene

983 specific RNAi constructs were introduced with a *T. congolense* specific plasmid, p3T7-TcoV

984 [69]. Primers carrying a HindIII (5'-AAGCTT-forward) or an FseI (5'-GGCCGGCC-reverse)

985 restriction site were used to amplify *TcoPEPCK*, *TcoPPDK* and *TcoHT* (S7 Table). Gene

986 fragments were amplified using a HiFi polymerase master mix (NEB) and cloned into pGEM-

987 T easy (Promega) and sequenced to confirm correct sequence identity of each fragment. The

988 constructs were then digested with HindIII and FseI and ligated into the p3T7-TcoV vector

989 using T4 DNA ligase (Promega). The final plasmid was linearised with NotI before purification

990 by ethanol precipitation prior to electroporation into TcoSM cells.

Transfections/electroporations

992 *T. congolense* IL3000 electroporation experiments and selection experiments were performed

993 as developed by [69]. A total of 4×10^7 cells were used per transfection, including a negative

994 (buffer only) control. A transfection buffer previously published for use with *T. brucei* was used

995   for *T. congolense* transfections [116]. Cells were centrifuged at 1,500 × *g* for 10 minutes,

996   pellets resuspended in residual medium and transferred to Eppendorf tubes for a further

997   centrifugation step. Cells were subsequently washed in transfection buffer prior to final

998   resuspension in 100 µL buffer per transfection. Up to 12 µg linearised plasmid DNA was added

999   to an electroporation cuvette (Sigma), and 100 µL cells were subsequently added.

1000   Electroporation was carried out using a Nucleofector II (Lonza) programme Z-001.

1001   Transfected cells were then incubated overnight in 25 mL warm medium in the absence of

1002   selective antibiotics, prior to their addition and plating out at dilutions of 1:50, 1:100 and 1:200

1003   in 96-well plates. Antibiotics were added at the following concentrations: Puromycin: 0.5

1004   µg/mL; Neomycin (G418): 0.4 µg/mL. Clones were retrieved after 7-10 days, and these were

1005   maintained in 0.25 µg/mL puromycin and 0.2 µg/mL G418.

Drug sensitivity assays

1007 Drug sensitivity assays were carried out using the alamar blue method developed by Raz and

1008 colleagues [117]. Briefly, Compounds were diluted to 2× starting concentration in SCM-6 (with

1009 10% goat serum for *T. congolense* IL3000 or 10% FBS for *T. brucei* Lister 427) and 200 µL

1010 was transferred to the first well of a solid white flat-bottomed 96-well plate. 100 µL medium

1011 was then added to 23 further wells and compounds were diluted 1:2 over this series of wells,

1012 with the exception of the last well, for a negative control. Subsequently, 100 µL cells were

1013 added at 2× starting density (4×10^4 cells/mL for *T. brucei* and 5×10^5 cells/mL for *T.*

1014 *congolense*). Plates were incubated for 48 hours (37°C or 34°c for *T. brucei* and *T.*

1015 *congolense*, respectively, 5% CO_2 in both cases), prior to addition of 20 µL resazurin sodium

1016 salt (0.49 mM in 1× PBS, pH 7.4) to each well. Plates were then incubated for a further 24

1017 hours before measurements of cell viability.

1018 Reduction of the resazurin salt was measured as a function of cell viability. Fluorescence of

1019 each plate was read using a Cytation 5 imaging reader (BioTek) and GEN5 software.

1020 Parameters were as follows: $\lambda_{excitation}$ = 540 nm and $\lambda_{emission}$ = 590 nm. Raw values were plotted

1021 against concentrations (converted to Log_{10} values) and normalised (0% defined as smallest

1022   mean in the dataset; 100% defined as largest mean in the dataset) using Graphpad Prism

1023   version 8.4.0. EC_{50} values for each compound were calculated using a non-linear sigmoidal

1024   dose-response curve. Each assay was performed in duplicate and each EC_{50} value represents

1025   a mean of three independent experiments.

Real-time quantitative PCR (RT-qPCR)

1027   RNA was extracted as described above, and reverse transcription was carried out in 20 μL

1028   using 1 μg RNA, using a high capacity cDNA kit (Applied Biosystems). Primers for RT-qPCR

1029   analysis were designed using Primer 3 [118], and primer efficiency was tested using serial

1030   dilutions of *T. congolense* IL3000 genomic DNA by plotting Ct value against Log_{10}(DNA

1031   concentration). Real-time PCR was carried out using the SensiFAST SYBR Hi-ROX kit

1032   (Bioline, BIO92005). Briefly, a 20 μL reaction was set up using 10 μL SYBR mix, RT template

1033   and 400 nM of each primer. Cycling conditions were: 96°C, 120 seconds, followed by 40 cycles

1034   of 95°C for 5 seconds, 62°C for 10 seconds and 72°C for 20 seconds. Previously published

1035   endogenous control primers for TcoTERT were used for within sample normalisation [119],

1036   and normalised transcript level was calculated using the delta delta Ct method [120].

Glucose uptake assays

1038   For analysis of wild-type *T. congolense* and *T. brucei* glucose uptake, cells were seeded in 10

1039   mL cultures of SCM-6 at an initial density of 2×10^5 cells/mL (four cultures per species), with

1040   10 mM glucose added separately at the start of the experiment. Upon the addition of glucose,

1041   1 mL supernatant was immediately centrifuged ($1,500 \times g$, 10 minutes) and supernatant stored

1042   at -80°C. This process was repeated at 12, 15, 18, 21 and 24 h, and cell density measured by

1043   haemocytometer. A medium-only control (4 replicates) was also incubated alongside *in vitro*

1044   cultures. Glucose concentration of each supernatant sample was analysed using the Glucose

1045   (GO) assay kit (GAGO-20; Sigma) in a 96-well format. Briefly, 40 μL supernatant sample

1046   (diluted if necessary) was incubated with 80 μL assay reagent for 30 minutes at 37°C, after

1047   which 80 μL 12 N sulphuric acid was added and absorbance measured at 540 nm using a

1048 spectrophotometer. A standard curve was also run to calculate glucose concentration. Rate

1049 of glucose consumption was calculated using a custom script [57].

1050 For glucose consumption of the TcoHT RNAi line, the Glucose Uptake-Glo kit (Promega) was

1051 used. RNAi was induced for 72 hours prior to carrying out the assay. Cells were centrifuged,

1052 washed in PBS and resuspended in assay buffer (77 mM NaCl, 1.5 mM $CaCl_2$-$2H_2O$, 4.5 mM

1053 KCl, 0.8 mM $MgSO_4$-$7H_2O$, 36 mM $NaHCO_3$, 25 mM HEPES and 0.02 mM

1054 bathocuproinedisulfonic acid), as it was determined *T. congolense* viability is reduced in PBS

1055 alone. Density was adjusted to 10^8 cells/mL, and three 100 μL replicates of each sample were

1056 added to wells of a black flat-bottomed 96-well plate. The uptake reaction was started by the

1057 addition of 50 μL 1 mM 2-deoxy-D-glucose. Plate was shaken for 15 minutes at 34°C prior to

1058 addition of 25 μL stop buffer, 25 μL neutralisation buffer and 100 μL pre-prepared 2DG6P

1059 detection reagent. Plates were shaken in between addition of the buffers. Finally, the plate

1060 was read with 0.3–1 second integration on a luminometer (Cytation 5 Imaging reader, BioTek).

1061 Wild-type *T. congolense*, and *T. congolense* supplemented with glucose were used as

1062 controls, in addition to cells without 2-deoxy-D-glucose and assays in the absence of cells.

1063 **Acetate concentration assay**

1064 Acetate was not detectable by mass spectrometry and therefore, a commercial colorimetric

1065 acetate assay kit (MAK086, Merck) was used to analyse changes in supernatant acetate

1066 concentrations in trypanosome cultures over time. *T. congolense* IL3000 were seeded in 10

1067 mL SCM-6 at a density of 1×10^5 cells/mL as outlined in the supernatant metabolomics

1068 experiment. At each time-point, 500 μL supernatant was taken from each flask and transferred

1069 to an Eppendorf tube. Samples were centrifuged at 1,500 × *g*, the supernatant was transferred

1070 to a fresh Eppendorf tube, and samples were stored at -80°C until samples from all time-points

1071 had been collected. Acetate concentration assays were carried out according to the

1072 manufacturer's instructions. Briefly, for each sample, 5 μL was added to the wells of a 96-well

1073 plate in duplicate and 45 μL assay buffer was added to each sample. Subsequently, 50 μL

1074 reaction mix was added to each well, and the plate was mixed and incubated for 40 minutes

at room temperature before absorbance was read at 450 nm. Acetate concentrations were

1076 calculated using the standard curve comprised of six concentrations run alongside the

1077 experimental samples.

Metabolomics – Liquid chromatography mass spectrometry

1079 Hydrophilic interaction liquid chromatography (HILIC) was carried out by Glasgow Polyomics

1080 (Glasgow, UK), using a Dionex UltiMate 3000 RSLC system (Thermo Fischer Scientific)

1081 coupled to a ZIC-pHILIC column (150 mm × 4.6 mm, 5 µm column, Merch Sequant). The

1082 column was maintained at 30°C and samples were eluted with a linear gradient (20 mM

1083 ammonium carbonate in water and acetonitrile) over 26 minutes with a flow rate of 0.3

1084 mL/minute.

1085 Sample injection volume was 10 µL and samples were maintained at 4°C before injection. A

1086 Thermo Orbitrap Exactive (Thermo Fischer Scientific) was used to generate mass spectra,

1087 and was operated in polarity switching mode with the following settings: Resolution: 50,000;

1088 AGC: 106; m/z range: 70–1,400; sheath gas: 40; auxiliary gas: 5; sweep gas: 1; probe

1089 temperature: 150°C; capillary temperature: 275°C. Samples were run in both positive and

1090 negative polarity with the following ionisation: source voltage +4.5 kV, capillary voltage +50 V,

1091 tube voltage +70 kV and skimmer voltage +20 V for positive mode; source voltage -3.5 kV,

1092 capillary voltage -50 V, tube voltage -70 V and skimmer voltage -20 V for negative mode. Mass

1093 calibration was performed for each polarity immediately prior to each analysis batch. The

1094 calibration mass range was extended to cover small metabolites by inclusion of low-mass

1095 contaminants with the standard Thermo calmix masses (below m/z 1400), $C_2H_6NO_2$ for

1096 positive ion electrospray ionisation (PIESI) mode (m/z 76.0393) and $C_3H_5O_3$ for negative ion

1097 electrospray ionisation (NIESI) mode (m/z 89.0244). To enhance calibration stability, lock-

1098 mass correction was also applied to each analytical run using these ubiquitous low-mass

1099 contaminants. A set of authentic standards was run prior to the sample set for each

1100 experiment.

Metabolomics data analysis

RAW spectra were converted to mzXML files (mzML files for fragmentation data) using XCMS for untargeted peak detection [121]. The resultant files were further processed using mzMatch [122] for peak matching and annotation, resulting in a tabular output that was analysed using IDEOM with default settings [123]. For stable-isotope assisted metabolomics experiments, mzMatch output (in .peakml format) was analysed using mzMatch-ISO to extract all carbon isotopologue abundances from putative metabolites [124]. Data analysis of stable isotope-labelled metabolomics was based on a 48 hour time-point in all experiments. Data was further analysed using Microsoft Excel or Metaboanalyst v4.0 [125]. The raw data from all metabolomics analyses are available in Metabolights (accession number: MTBLS2372; URL: www.ebi.ac.uk/metabolights/MTBLS2372).

RNA sequencing and data processing

RNA sequencing was carried out by Edinburgh Genomics (Edinburgh, UK). Libraries were prepared from 8 samples (4× *T. brucei*, 4× *T. congolense*) using the TruSeq Stranded mRNA kit (Illumina) and 2 × 75 bp paired-end sequencing was carried out using a HiSeq 4000 system (Illumina). Sequencing reads were aligned to the corresponding genome sequence using HiSat2 (--no-spliced-alignment) [126]. For *T. brucei*, the TREU 927 reference genome sequence was used (v34.0 from TriTrypDB [127]), whilst a PacBio assembly of *T. congolense* IL3000 was used for *T. congolense* [65]. The resulting SAM files were converted to BAM files using samtools [128], and subsequently filtered for quality and primary alignment (-q 1 -F 0x100), the latter to reduce the effects of multimapping. Read counts were extracted from the filtered BAM files using HTSeq-count (-s reverse -f bam -t CDS -i ID -m union -a 0 --nonunique all).

For all samples, transcripts per million (TPM) values for each gene were calculated manually using Microsoft Excel as follows: 1) Reads per kilobase (RPK) were calculated by dividing the read counts by the length of gene in kilobases; 2) All RPK values in a sample were summed and divided by 1 million as a scaling factor; 3) Each RPK value was divided by the scaling

factor to yield TPM values [53]. To compare transcript abundances between the two species, Orthofinder [54] was used to infer orthologue genes or gene groups. Default parameters were used to compare the TriTrypDB v34.0 TREU 927 annotated proteins and the PacBio *T. congolense* IL3000 annotated proteins (S2 Table). A custom MATLAB (version R2019a) was used to combine the Orthofinder dataset and the TPM values for 1-to-1 orthologues, as well as "sum of TPM" values for groups containing multiple genes, where TPM value for each gene was summed, resulting in a final dataset (S1 Table). Raw RNA-seq data is deposited at GEO (accession number: GSE165290). Transcriptomics data were cross-referenced with the TrypanoCyc database (vm-trypanocyc.toulouse.inra.fr/; [60]) to enable pathway analysis of the data.

Computation

Figures were generated using Graphpad Prism version 8.4.0 (www.graphpad.com) with the exception of scatter plots and heatmaps, which were generated using R [129]. Heatmaps were generated using the R packages pheatmap and ComplexHeatmap [130]; scatter plots were generated using GGplot2 and GGally; and pathway maps were generated with Inkscape v1.0.

Acknowledgements

Figure legends

Figure 1: Overview of comparative transcriptomics analysis of *T. brucei* and *T. congolense*, isolated from *ex vivo* and *in vitro* conditions. RNAseq data from *T. congolense* (IL3000) and *T. brucei* (STIB247) in both *in vitro* and *ex vivo* (from mouse infections) conditions was aligned to the species' respective genome sequence and read counts were normalised by the transcripts per million (TPM) method. To directly compare the species, a pseudogenome was generated using the Orthofinder tool [54]. TPM values from the 4 sample groups were plotted against each other to analyse correlation between conditions (A and B) and between species in the same conditions (C and D). Correlation was assessed using both Spearman's rank correlation (ρ) and Pearson correlation (ρ; Pearson's *r*) coefficients.

Figure 2: Analysis of supernatant metabolites after *T. congolense* culture. A heatmap covering the 80 putative medium components judged to be significantly altered after 56 hours of *in vitro* cell culture containing *T. congolense* strain IL3000, as calculated by a one-way repeated measures ANOVA ($P < 0.05$). Peak abundances were log transformed and mean centred and metabolites were clustered based on Pearson correlation. Two clusters of interest were identified, which are shown in a larger format on the right. Metabolites in the top cluster were observed to increase significantly over time, whilst those in the bottom cluster decreased. Metabolite names follow by [*] were matched to an authentic standard. B) Comparison of metabolite changes in medium supernatants after 56 hours between *T. brucei* [56] and *T. congolense* (S4 Table). Relative changes in metabolite abundance were calculated as Log_2 fold change of 56 h vs 0 h. Key differences are highlighted numerically: 1, guanine; 2, *N*6-acetyl-L-lysine; 3, succinate; 4, 4-hydroxy-4-methylglutamate; 5, *N*6,*N*6,*N*6-trimethyl-L-lysine; 6, choline; 7, 2-oxoglutarate; 8, L-1-pyrroline-3-hydroxy-5-carboxylate; 9, D-glycerate; 10,

45

1182   pyruvate; 11, 12-hydroxydodecanoic acid; 12, L-cystine; 13, diacetyl; 14, [PC (18:0)] 1-

1183   octadecanoyl-sn-glycero-3-phosphocholine; 15, LysoPC(17:0); 16, [PC (16:0)] 1-

1184   hexadecanoyl-sn-glycero-3-phosphocholine; 17, inosine; 18, [PC (16:1)] 1-(9Z-

1185   hexadecenoyl)-sn-glycero-3-phosphocholine; 19, [FA trihydroxy(18:1)] 9S,12S,13S-

1186   trihydroxy-10E-octadecenoic acid; 20, inosine.

1187   **Figure 3: Energy metabolism in *T. congolense*.** A-E) Supernatant metabolomics analysis

1188   of metabolites involved in glycolytic metabolism in *T. congolense*. Grey bars indicate a

1189   negative medium control incubated for 56 hours. F) A commercial kit was used to measure

1190   acetate concentration during *T. congolense* culture, with supernatant samples analysed at the

1191   same time points as the supernatant metabolomics experiment. G) A simplified overview of

1192   the glycolytic pathway. Typically, the succinate shunt is only active in PCF *T. brucei*, with low

1193   levels of activity in BSF *T. brucei*. Numbers refer to the following proteins: 1, glucose

1194   transporters; 2, hexokinase; 3, glucose 6-phosphate isomerase; 4, phosphofructokinase; 5,

1195   aldolase; 6, triosephosphate isomerase; 7, glycerol 3-phosphate dehydrogenase; 8, glycerol

1196   kinase; 9, glyceraldehyde 3-phosphate dehydrogenase; 10, phosphoglycerate kinase; 11,

1197   phosphoglycerate mutase and enolase; 12, phosphenolpyruvate carboxykinase; 13, malate

1198   dehydrogenase; 14, fumarate hydratase; 15, NADH-dependent fumarate reductase; 16,

1199   pyruvate kinase; 17, alanine aminotransferase; 18, pyruvate dehydrogenase complex; 19,

1200   acetate:succinate CoA-transferase and acetyl-CoA thioesterase. H) Tracing glucose derived

1201   carbon usage through glycolytic metabolism. *T. congolense* were incubated with a 50:50 mix

1202   of ^{12}C-D-glucose:^{13}C-U-D-glucose before cell pellets were isolated for metabolomics analysis.

1203   Results were compared to those generated in *T. brucei* by Creek and colleagues [42]. Colours

1204   indicate the number of ^{13}C atoms in each metabolite. I) Comparative analysis of transcript level

1205   activity of glycolysis in *T. brucei* and *T. congolense* from both *in vitro* and *ex vivo* conditions.

1206   Gene IDs: HK1 & 2, hexokinase, TbTc_0341; GPI, glucose 6-phosphate isomerase,

1207   TbTc_1840; PFK, phosphofructokinase, TbTc_1399; ALDA, aldolase, TbTc_0358; TPI,

1208   Triosephosphate isomerase, TbTc_1075; GPDH, glycerol 3-phosphate dehydrogenase,

1209 TbTc_2722; GK, glycerol kinase, TbTc_0392; GAPDH, glyceraldehyde 3-phosphate

1210 dehydrogenase, TbTc_0377; PGK, phosphoglycerate kinase, TbTc_6030; PGKA,

1211 phosphoglycerate kinase A, TbTc_0241; PGKB/C, phosphoglycerate kinase B & C,

1212 TbTc_0240, ENO1, enolase, TbTc_0465; ENO2, enolase, putative, TbTc_3614, PK1,

1213 pyruvate kinase 1, TbTc_0372; FBPase, fructose-1,6-bisphosphatase, TbTc_1967; PEPCK,

1214 phosphoenolpyrvuate carboxykinase, TbTc_0348; gMDH, glycosomal malate

1215 dehydrogenase, TbTc_0642, FH, fumarate hydratase, TbTc_0242; Frd, NADH-dependent

1216 fumarate reductase, TbTc_0141; PPDK, pyruvate phosphate dikinase, TbTc_1304; AAT,

1217 alanine aminotransferase, TbTc_0675; PDH E1α, pyruvate dehydrogenase E1 alpha subunit,

1218 TbTc_4169; PDH E1β, pyruvate dehydrogenase E1 beta subunit, TbTc_5437.

1219 **Figure 4: *In vitro* analysis of glycolytic metabolism.** To further probe glycolytic metabolism

1220 in *T. congolense*, novel RNAi technology was employed to knock-down key glycolytic and

1221 gluconeogenic steps. A) *T. congolense* remains viable in reduced glucose concentrations. A

1222 growth defect was only observed when glucose concentrations were reduced to <2 mM. B)

1223 Supplementation with increased concentrations of 2-deoxy-D-glucose leads to *T. congolense*

1224 cell death (red dotted line indicates detection limit by haemocytometer). C) Growth analysis of

1225 RNAi-mediated knock-down of PEPCK in *T. congolense* IL3000 single marker induced with 1

1226 µg/mL tetracycline. D) Growth analysis of RNAi-mediated knock-down of PPDK in *T.*

1227 *congolense* IL3000 single marker induced with 1 µg/mL tetracycline. E-F) Transcript

1228 abundance over time, following tetracycline-mediated RNAi induction of PEPCK and PPDK.

1229 G) Knock-down of the entire glucose transporter (HT) array does not affect *in vitro* cell viability.

1230 H) Normalised HT mRNA abundance over time after RNAi induction. I) Changes in glucose

1231 uptake in RNAi-induced cells were detected via an enzyme-linked luminescence assay

1232 coupled to 2-deoxy-D-glucose uptake over a period of 30 minutes. The assay was carried out

1233 72-hours post-induction. Of the three RNAi lines, 2 showed a significant reduction in glucose

1234 uptake capability (*$P < 0.05$; ***$P < 0.001$)

Figure 5: Nucleotide metabolism in *T. congolense*. Supernatant analysis of *T. congolense*

in vitro cultures showing changes in abundance of D-ribose (A), guanine (B), xanthine (C) and

inosine (D) over 56 hours. Grey bar indicates a negative medium control group E) Simplified

overview of purine salvage and synthesis in trypanosomatids adapted from [131]. Numbers

indicate the following enzymes: 1, APRT; 2, AD; 3, HGPRT; 4, IMPD; 5, HGXPRT; 6, GMPR;

7, GMPS; 8, HGPRT. Red cross indicates guanine deaminase, which is not

encoded/annotated in the *T. congolense* genome. F) Comparison of glucose-derived purine

carbon labelling in *T. congolense* and *T. brucei* [42]. Colours indicate the number of ^{13}C atoms

in each metabolite. D) Comparative RNAseq analysis of *T. congolense* and *T. brucei* under

both *in vitro* and *ex vivo* conditions. Gene IDs from top to bottom: P121-PWY

(adenine/adenosine salvage): IMPDH1, inosine-5'-monophosphate dehydrogenase,

TbTc_1648; ADSS, adenylosuccinate synthetase, TbTc_1142; APRT-1, cytosolic adenine

phosphoribosyltransferase, TbTc_3522; HGPRT, hypoxanthine-guanine

phosphoribosyltransferase, TbTc_0726; GMPR, GMP reductase, TbTc_4627; HGXPRT,

hypoxanthine-guanine-xanthine phosphoribosyltransferase, TbTc_3696; APRT-2, glycosomal

adenine phosphoribosyltransferase, TbTc_5918; ADSL, adenylosuccinate lyase, TbTc_1986.

PWY0-162 (pyrimidine biosynthesis): DHODH, dihydroorotate dehydrogenase (fumarate),

TbTc_0620; PYR1A-B, glutamine hydrolysing carbomoyl phosphate synthase, TbTc_1631;

PYR2, aspartate carbamoyltransferase, TbTc_1630; PYR3, dihydroorotase, TbTc_3801;

CTPS, cytidine triphosphate synthase, TbTc_0920; OMPDC/OPRT, orotidine-5-

monophosphate decarboxylase/orotate phosphoribosyltransferase, TbTc_0735; CMF40a,

nucleoside diphosphate kinase, TbTc_5784. PWY0-163 (pyrimidine salvage): UP, uridine

phosphorylase, TbTc_5794; CDA, cytidine deaminase, TbTc_3318; UPRT, uracil

phosphoribosyltransferase, TbTc_4220; NDPK, nucleoside diphosphate kinase, TbTc_0593;

CMF40a, nucleoside diphosphate kinase, TbTc_5784; NDPK3, nucleoside diphosphate

kinase 3, TbTc_2560.

Figure 6: Amino acid metabolism in *T. congolense* IL3000. A-C) Analysis of indicated amino acids in *T. congolense* IL3000 culture supernatants over a 56 h time course. Grey bars indicate a negative medium control group. D-F) Growth curves in SCM-6 excluding one amino acid at a time, to determine those essential to *T. congolense* viability. In each experiment, full SCM-6 was used as a positive control. Legends indicate which amino acid was removed in each experiment. G) Growth analysis of SCM-6 and SCM-7, the latter containing only amino acids deemed essential, compared to HMI-93 [111]. H) Simplified map of intracellular glutamine metabolism. Numbers refer to the following enzymes: 1, glutaminase; 2, glutamate decarboxylase; 3, 4-aminobutyrate aminotransferase; 4, succinate semialdehyde dehydrogenase; 5, glutamate dehydrogenase; 6, 2-oxoglutarate dehydrogenase; 7, Succinyl-CoA synthetase; 8, isocitrate dehydrogenase; 9 & 10, aconitase. I) Carbon utilisation from L-glutamine was analysed in *T. congolense* (100% ^{13}C-U-L-glutamine) and compared to that in *T. brucei* (50:50 ratio of L-glutamine and ^{13}C-U-L-glutamine) [85].

Figure 7: Fatty acid metabolism in *T. congolense*. A) Glucose-derived ^{13}C carbon labelling of saturated fatty acids in *T. congolense* and *T. brucei* [42]. Colours correspond to the number of ^{13}C labels detected in each metabolite. B) L-threonine-derived saturated fatty acid 13C labelling in T. congolense. Fatty acid systematic names and numbers: lauric acid: dodecanoic acid, C12:0; myristic acid: tetradecanoic acid, C14:0; palmitic acid: hexadecanoic acid, C16:0; nonadecyclic acid: nonadecanoic acid, C19:0. C) Transcriptomics analysis of acetate and lipid metabolism. Gene names and IDs: ACH, acetyl-CoA hydrolase, TbTc_5515; ACS, acetyl-CoA synthetase, TbTc_0318; AKCT, 2-amino-3-ketobutyrate-CoA ligase, TbTc_6236; TDH, L-threonine 3-dehydrogenase, TbTc_5991; PDHe1α, pyruvate dehydrogenase E1 α subunit, TbTc_4169; PDHe1β, pyruvate dehydrogenase E1 β subunit; SCSα, succinyl-CoA synthetase α subunit, TbTc_0813; PPDK, pyruvate phosphate dikinase, TbTc_1304; PDHe2, dihydrolipoamide acetyltransferase, TbTc_1015; PDHe3, pyruvate dehydrogenase E3, TbTc_4765; PYK1, pyruvate kinase, TbTc_0372; BKR, β-ketoacyl-ACP reductase, TbTc_1241; BKS, β-ketoacyl synthase, TbTc_3372; ACC, acetyl-CoA carboxylase,

1288   TbTc_0754; HMGCL, hydroxymethylglutaryl-CoA lyase, TbTc_6160; FPPS, farnesyl

1289   pyrophosphate synthase, TbTc_5375; LSS, lanosteral synthase, TbTc_4540; MVK,

1290   mevalonate kinase, TbTc_3761; SM, squalene monooxygenase, TbTc_3357; MDD,

1291   mevalonate diphosphate decarboxylase, TbTc_0546; SMT, sterol 24-c methyltransferase,

1292   TbTc_0387; CYP51A1, lanosterol 14α demethylase, TbTc_4837; SQase, squalene synthase,

1293   TbTc_2577; SPPS, solanesyl-diphosphate synthase, TbTc_3025; IDI, isopentenyl-

1294   diphosphate delta-isomerase, TbTc_1099; PTase, prenyltransferase, TbTc_1352; GGTase-

1295   IIβ, geranylgeranyl transferase type II β subunit, TbTc_0680; SCP2, 3-ketoacyl-CoA thiolase,

1296   TbTc_4024; PMVK, phosphomevalonate kinase, TbTc_3039; HMGR, 3-hydroxy-3-

1297   methylglutaryl-CoA reductase, TbTc_3189; LACS5, fatty acyl-CoA synthetase, TbTc_0099;

1298   ACSL_0688, long-chain-fatty-acid-CoA ligase, TbTc_0688; ECHD, enoyl-CoA hydratase,

1299   TbTc_3283; ACS3/ACS4, fatty acyl-CoA synthetase 3 & 4, TbTc_0101; ACS1, fatty acyl-CoA

1300   synthetase 1, TbTc_0100; ACS2, fatty acyl-CoA synthetase 2, TbTc_0102; ECI_4184, 3,2-

1301   trans-enoyl-CoA isomerase, TbTc_4184; ACSL_2381, long-chain-fatty-acid-CoA ligase,

1302   TbTc_2381; TFEα1, enoyl-CoA hydratase/enoyl-CoA isomerase, TbTc_3362; SCP2, 3-

1303   ketoacyl-CoA thiolase, TbTc_4024; ECI_0360, 3,2-trans-enoyl-CoA isomerase, TbTc_0360;

1304   ACAD, acyl-CoA dehydrogenase, TbTc_4954.

1305   **Figure 8: Pharmacological inhibition of fatty acid synthesis in *T. brucei* and *T.**

1306   ***congolense*.** Dose-response curves to determine differential sensitivity of the two species of

1307   parasite to inhibition of an ACS inhibitor (panel A) and Orlistat (B).

1308   **Figure 9: Summary of *T. congolense* and *T. brucei in vitro* transcriptome.** Log_2 fold

1309   change *T. congolense*/*T.brucei*) was calculated for each gene (for ratio changes, see the key

1310   on the bottom-left). Dashed lines represent transport processes. Genes: 1, hexose

1311   transporters, TbTc_0095; 2, hexokinase, TbTc_0341; 3, glucose-6-phosphate isomerase,

1312   TbTc_1840; 4, phosphofructokinase, TbTc_1399; 5, fructose-1,6-bisphosphatase,

1313   TbTc_1967; 6, aldolase, TbTc_0358; 7, triosephosphate isomerase, TbTc_1075; 8, glycerol-

1314   3-phosphate dehydrogenase, TbTc_2722; 9, glycerol kinase, TbTc_0392; 10, glyceraldehyde

3-phosphate dehydrogenase, TbTc_0377; 11, phosphoglycerate kinase, TbTc_0240; 12, phosphoglycerate mutase, TbTc_5039; 13, enolase, TbTc_0465; 14, pyruvate kinase 1, TbTc_0372; 15, alanine aminotransferase, TbTc_0675; 16, pyruvate phosphate dikinase, TbTc_1304; 17, Phosphoenolpyruvate carboxykinase, TbTc_0348; 18, glycosomal malate dehydrogenase, TbTc_0642; 19, glycosomal fumarate hydratase, TbTc_0242; 20, glycosomal NADH-dependent fumarate reductase, TbTc_0140; 21, glucose-6-phosphate dehydrogenase, TbTc_0931; 22, 6-phosphogluconolactonase, TbTc_4165; 23, 6-phosphogluconate dehydrogenase, TbTc_2025; 24, ribulose-5-phosphate epimerase, TbTc_4356; 25, ribose 5-phosphate isomerase, TbTc_3090; 26, transketolase, TbTc_1701; 27, transaldolase, TbTc_1823; 28, ribokinase, TbTc_5212; 29, malic enzyme, TbTc_0296; 30, Mitochondrial pyruvate carrier 2, TbTc_2668; 31, FAD-dependent glycerol-3-phosphate dehydrogenase, TbTc_2282; 32, NADH dehydrogenase (NDH2), TbTc_5033; 33, Alternative oxidase, TbTc_6589; 34, mitochondrial fumarate hydratase, TbTc_0243; 35, mitochondrial NADH-dependent fumarate reductase, TbTc_0141; 36, mitochondrial malate dehydrogenase, TbTc_0256; 37, citrate synthase, TbTc_0486; 38, aconitase, TbTc_5765; 39, isocitrate dehydrogenase, TbTc_0510; 40, 2-oxoglutarate dehydrogenase E1 component, TbTc_2864; 41, 2-oxoglutarate dehydrogenase E1 component, TbTc_3111; 42, 2-oxoglutarate dehydrogenase E2 component, TbTc_3057; 43, succinyl-CoA synthetase α, TbTc_0813; 44, succinyl-CoA ligase β, TbTc_3392; 45, glutamine synthetase, TbTc_2226; 46, glutamate dehydrogenase, TbTc_0872; 47, pyruvate dehydrogenase E1 α subunit, TbTc_4169; 48, pyruvate dehydrogenase E1 β subunit, TbTc_5437; 49, dihydrolipoamide acetyltransferase, TbTc_1015; 50, pyruvate dehydrogenase complex E3, TbTc_4765; 51, L-threonine 3-dehydrogenase, TbTc_5991; 52, 2-amino-3-ketobutyrate coenzyme A ligase, TbTc_6236; 53, Acetyl-CoA hydrolase (ACH), TbTc_5515; 54, Succinyl-CoA:3-ketoacid coenzyme A transferase (ASCT), TbTc_0236; 55, Acyl carrier protein, TbTc_5262; 56, beta-ketoacyl-ACP synthase, TbTc_3372; 57, beta-ketoacyl-ACP reductase, TbTc_1241; 58, Trans-2-enoyl-ACP reductase 1, TbTc_5269; 59, acetyl-CoA synthetase, TbTc_0318; 60, acetyl-CoA carboxylase, TbTc_0754; 61, Fatty acid elongase (ELO1), TbTc_0159; 62, Fatty acid

1343 elongase (ELO2), TbTc_1882; 63, Fatty acid elongase (ELO3), TbTc_0235; 64, elongation of

1344 very long chain fatty acids protein (ELO4), TbTc_0737; 65, aspartate aminotransferase,

1345 TbTc_0799; 66, aspartate carbamoyltransferase, TbTc_1630; 67, dihydroorotase,

1346 TbTc_3801; 68, dihydroorotate dehydrogenase, TbTc_0620; 69, orotidine-5-phosphate

1347 decarboxylase/orotate phosphoribosyltransferase, TbTc_0735; 70, uracil

1348 phosphoribosyltransferase, TbTc_4220; 71, Adenine phosphoribosyltransferase (APRT-2),

1349 TbTc_3522; 72, inosine-adenosine-guanosine-nucleoside hydrolase, TbTc_4998; 73,

1350 adenosine kinase, TbTc_1024; 74, AMP deaminase, TbTc_5808; 75, hypoxanthine-guanine

1351 phosphoribosyltransferase (HGPRT), TbTc_0726; 76, inosine-guanine nucleoside hydrolase,

1352 TbTc_0808; 77, inosine-5'-monophosphate dehydrogenase, TbTc_1648; 78, Hypoxanthine-

1353 guanine-xanthine phosphoribosyltransferase (HGXPRT), TbTc_3696; 79, GMP reductase,

1354 TbTc_4627; 80, GMP synthase, TbTc_1452. Abbreviations: PUFA, polyunsaturated fatty acid.

1355 **S1 figure: comparative analysis of published *T. congolense* RNAseq data and data**

1356 **generated in this study.** Scatter matrix of *T. congolense* datasets from this study compared

1357 to ascending and peak parasitaemia *in vivo* transcriptomics data generated by Silvester and

1358 colleagues [55]. TPM values were calculated for each gene in the *T. congolense* genome and

1359 Log_2 TPM was plotted. Lower panels: Scatter plots of individual comparisons of the 4 datasets.

1360 Red dots correspond to genes associated with glycolysis; Diagonal panels: sample names;

1361 Upper panels: Pearson correlation coefficients for comparisons of entire datasets (black),

1362 glycolytic pathway ("Glyc", green) and proteins with predicted transmembrane domains

1363 ("Trans", red).

1364 **S2 figure: Growth of *T. congolense* IL3000 in absence or presence of N-acetyl-D-**

1365 **glucosamine.** Parasites were cultured in SCM-6 supplemented with 10 mM or 2 mM glucose

1366 in the presence or absence of 60 mM GlcNAc and density monitored by haemocytometer

1367 every 24 hours.

1368 **S3 figure: Comparative transcriptomics analysis of the electron transport chain in *T.***

1369 ***congolense* and *T. brucei*.** A heatmap of all ETC complexes based on a table generated by

1370   Zikova and colleagues [73]. Heatmaps are divided into the alternative oxidases (AOX), NADH

1371   dehydrogenase 2 (NDH2), complex I, II, III, IV and ATPase (complex V).

1372   **S4 figure: Stable isotope labelled (^{13}C)-glucose derived pyrimidine labelling.**

1373   Comparative analysis of glucose-derived pyrimidine labelling in *T. congolense* and *T. brucei*

1374   (taken from [42]).

1375   **S5 figure: Effect of cysteine exclusion on *T. congolense* growth.** Parasites were grown in

1376   SCM-6 supplemented with 1.5 mM, 1.0 mM or absence of L-cysteine. Cell density was

1377   monitored every 24 hours.

1378   **S6 figure: Comparison of amino acid metabolism in *T. congolense* and *T. brucei.*** A)

1379   glucose-derived carbon labelling of amino acids B) Transcriptomics pathway analysis. Gene

1380   IDs: A) ARG+POLYAMINE-SYN: AdoMetDC_3193, AdoMet decarboxylase, TbTc_3193;

1381   ODC, ornithine decarboxylase, TbTc_5903; AdoMetDC_0696, AdoMet decarboxylase,

1382   TbTc_0696; SpSyn, spermidine synthase, TbTc_1034. B) ASPASN-PWY: cASAT, cytosolic

1383   aspartate aminotransferase, TbTc_0799; ASNS, asparagine synthetase, TbTc_4894;

1384   mASAT, mitochondrial aspartate aminotransferase, TbTc_5877. C) GLUCAT-PWY: OGDH-

1385   E1, 2-oxoglutarate dehydrogenase E1, TbTc_2864; GDH, glutamate dehydrogenase,

1386   TbTc_0872; SCSα, succinyl-CoA synthetase, TbTc_0813; SUCLG2, succinyl-CoA ligase,

1387   TbTc_3392; OGDH-E2, 2-oxoglutarate dehydrogenase E2, TbTc_3057. D) ILEUDEG-PWY:

1388   ECH, enoyl-CoA hydratase, TbTc_3283; BCAAT, branched-chain amino acid

1389   aminotransferase, TbTc_0559; SCP2, 3-ketoacyl-CoA thiolase, TbTc_4024. E) LEUDEG-

1390   PWY: ECH, enoyl-CoA hydratase, TbTc_3283; BCKDHα, 2-oxoisovalerate dehydrogenase α,

1391   TbTc_1182; BCKDHβ, 2-oxoisovalerate dehydrogenase β, TbTc_0682; AUH,

1392   methylglutaconyl-CoA hydratase, TbTc_5348; HMGCL, hydroxymethylglutaryl-CoA lyase,

1393   TbTc_6160; BCAAT, branched-chain amino acid aminotransferase, TbTc_0559; MCCβ, 3-

1394   methylcrotonyl-CoA carboxylase β, TbTc_5385; MCCα, 3-methylcrotonyl-CoA carboxylase α,

1395   TbTc_1670; SCP2, 3-ketoacyl-CoA thiolase, TbTc_4024; IVDH, isovaleryl-CoA

1396   dehydrogenase, TbTc_3112. F) PWY0-781: cASAT, cytosolic aspartate aminotransferase,

TbTc_0799; MTR - 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase, TbTc_5805; NMNAT, nicotinamide/nicotinic acid mononucleotide adenylyltransferase, TbTc_4133; NADSYN, NAD+ synthase, TbTc_2404; mASAT, mitochondrial aspartate aminotransferase, TbTc_5877; METK1, AdoMet synthase, TbTc_0178. G) PWY1V8-11: AKCT, 2-amino-3-ketobutyrate-CoA ligase, TbTc_6236; TDH, L-threonine dehydrogenase, TbTc_5991. H) VALDEG-PWY: ECH, enoyl-CoA hydratase, TbTc_3283; HOPR, 2-hydroxy-3-oxopropionate reductase, TbTc_2903; BCAAT, branched-chain amino acid aminotransferase, TbTc_0559. I) PROLINE-DEG2-PWY: P5CDH, delta-1-pyrroline-5-carboxylate dehydrogenase, TbTc1695; GDH, glutamate dehydrogenase, TbTc_0872; ProDH, proline dehydrogenase, TbTc_1591.

S7 figure: Carbon utilisation for trypanothione biosynthesis in *T. congolense*. Metabolomics and transcriptomics analyses were carried out to analyse trypanothione biosynthesis. A) A simplified map of trypanothione biosynthesis as known in *T. brucei*. Numbers refer to the following enzymes: 1, S-adenosyl-L-methionine synthase, METK1; 2, S-adenosyl-L-methionine decarboxylase, AdoMetDC; 3, spermidine synthase, SpSyn; 4, methyltransferase reaction, MTase; 5, S-adenosyl-L-homocysteine dehydrolase, AdoHycase; 6, cystathionine beta synthase, CBS; 7, cystathione gamma lyase, CTH; 8, glutaminase/amidase, AM; 9, gamma-glutamylcysteine synthetase, GCS; 10, glutathione synthetase, GSS; 11, ornithine decarboxylase, ODC; 12, spermidine synthase, SpSyn; 13, glutathionylspermidine synthase, GSP; 14, trypanothione synthetase, TRYS; 15, tryparedoxin peroxidase, TXN1b; 16, trypanothione reductase, TRYR. B) Isotopologue labelling experiments using 100% ^{13}C-L-serine, ^{13}C-L-glutamine, ^{13}C-L-methionine or ^{13}C-L-cysteine, showing the abundance of carbon labelling derived from these amino acids in components of the trypanothione biosynthesis pathway. C) Transcriptomics analysis using the following TrypanoCyc pathways: PWY1V8-6 (trypanothione biosynthesis), HOMOCYSDESGR-PWY1 (homocysteine degradation/cysteine biosynthesis) & METHIONINE-DEG1-PWY (methionine degradation I). GeneIDs: TNX1b, tryparedoxin 1b, TbTc_0324; TRYS, trypanothione

synthetase, TbTc_1359; SpSyn, Spermidine synthase, TbTc_1034; TRYR, trypanothione reductase, TbTc_4239; AdoMetDC_0696, S-adenosylmethionine decarboxylase, TbTc0696; GCS, gamma-glutamylcysteine synthetase, TbTc_3424; METK1, S-adenosylmethionine synthetase, TbTc_0178; GSS, glutathione synthetase, TbTc_3678; AdoMetDC_3193, S-adenosylmethionine decarboxylase, TbTc_3193; AM, amidase, TbTc_5549; ODC, ornithine decarboxylase, TbTc_5903; CTH, cystathione gamma lyase, TbTc_1051; CBS, cystathionine beta synthase, TbTc_0413; AdoHcyase, S-adenosylhomocysteine hydrolase, TbTc_0685; METK1, S-adenosylmethionine synthase, TbTc_0178.

S8 Figure: Analysis of LC-MS utilising stable isotope labelled amino acids. Percentage total labelling of metabolites identified in data from 6 stable isotope labelling experiments using ^{13}C-L-asparagine, ^{13}C-L-cysteine, ^{13}C-L-glutamine, ^{13}C-L-methionine, ^{13}C-L-proline and ^{13}C-L-serine. Colour intensity correlates to the total fraction of the metabolite that was ^{13}C-labeled.

Supplementary Data

S1 Table: RNAseq dataset – *T. congolense ex vivo*, *T. congolense in vitro*, *T. brucei ex vivo*, *T. brucei in vitro*, Silvester *et al* dataset

S2 Table: Orthofinder output comparing *T. congolense* TriTrypDB (v34.0), *T. congolense* Liverpool pacbio, *T. brucei* TriTrypDB (v34.0) and other trypanosomatids

S3 Table: RNAseq dataset – *T. congolense* only, Pacbio assembly, single genes.

S4 Table: Supernatant metabolomics dataset for *in vitro* cultured *T. congolense* over a period of 56 hours. Metabolites highlighted in yellow were confidently predicted using a set of metabolite standards run alongside the experimental samples. Results of statistical analysis by means of a one-way repeated measures ANOVA (false discovery rate-adjusted P value, FDR) is also shown for metabolites that were taken forward for downstream analysis

S5 Table: TrypanoCyc pathways and linked Orthogroup gene IDs

S6 Table: Formulation of Steketee's congolense medium (SCM)-6 & -7

1449 **S7 table:** List of primers used in this study

1450

1451

References

1453 1.   Morrison LJ, Vezza L, Rowan T, Hope JC. Animal African Trypanosomiasis: Time to Increase Focus

1454   on Clinically Relevant Parasite and Host Species. Trends Parasitol. 2016;32(8): 599-607. doi:

1455   10.1016/j.pt.2016.04.012.

1456 2.   Auty H, Torr SJ, Michoel T, Jayaraman S, Morrison LJ. Cattle trypanosomosis: the diversity of

1457   trypanosomes and implications for disease epidemiology and control. Rev Sci Tech. 2015;34(2):

1458   587-98.

1459 3.   Giordani F, Morrison LJ, Rowan TG, HP DEK, Barrett MP. The animal trypanosomiases and their

1460   chemotherapy: a   review.   Parasitology.   2016;143(14):   1862-89.   doi:

1461   10.1017/S0031182016001268.

1462 4.   Shaw AP, Cecchi G, Wint GR, Mattioli RC, Robinson TP. Mapping the economic benefits to

1463   livestock keepers from intervening against bovine trypanosomosis in Eastern Africa. Prev Vet

1464   Med. 2014;113(2): 197-210. doi: 10.1016/j.prevetmed.2013.10.024.

1465 5.   Chitanga S, Marcotty T, Namangala B, Van den Bossche P, Van Den Abbeele J, Delespaux V. High

1466   prevalence of drug resistance in animal trypanosomes without a history of drug exposure. PLoS

1467   Negl Trop Dis. 2011;5(12): e1454. doi: 10.1371/journal.pntd.0001454.

1468 6.   Delespaux V, Dinka H, Masumu J, Van den Bossche P, Geerts S. Five-fold increase in

1469   Trypanosoma congolense isolates resistant to diminazene aceturate over a seven-year period

1470   in Eastern Zambia. Drug Resist Updat. 2008;11(6): 205-9. doi: 10.1016/j.drup.2008.10.002.

1471 7.   Geerts S, Holmes PH, Eisler MC, Diall O. African bovine trypanosomiasis: the problem of drug

1472   resistance. Trends Parasitol. 2001;17(1): 25-8.

56

1473   8.   Diall O, Cecchi G, Wanda G, Argiles-Herrero R, Vreysen MJB, Cattoli G, et al. Developing a

1474   Progressive Control Pathway for African Animal Trypanosomosis. Trends Parasitol. 2017;33(7):

1475   499-509. doi: 10.1016/j.pt.2017.02.005.

1476   9.   Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, et al. The trypanosomiases.

1477   Lancet. 2003;362(9394): 1469-80. doi: 10.1016/S0140-6736(03)14694-6.

1478   10. Alsford S, Kawahara T, Glover L, Horn D. Tagging a T. brucei RRNA locus improves stable

1479   transfection efficiency and circumvents inducible expression position effects. Mol Biochem

1480   Parasitol. 2005;144(2): 142-8. doi: 10.1016/j.molbiopara.2005.08.009.

1481   11. Rico E, Jeacock L, Kovarova J, Horn D. Inducible high-efficiency CRISPR-Cas9-targeted gene

1482   editing and precision base editing in African trypanosomes. Sci Rep. 2018;8(1): 7960. doi:

1483   10.1038/s41598-018-26303-w.

1484   12. Gibson W. The origins of the trypanosome genome strains Trypanosoma brucei brucei TREU

1485   927, T. b. gambiense DAL 972, T. vivax Y486 and T. congolense IL3000. Parasit Vectors. 2012;5:

1486   71. doi: 10.1186/1756-3305-5-71.

1487   13. Kay C, Peacock L, Gibson W. Trypanosoma congolense: In Vitro Culture and Transfection. Curr

1488   Protoc Microbiol. 2019;53(1): e77. doi: 10.1002/cpmc.77.

1489   14. Coustou V, Guegan F, Plazolles N, Baltz T. Complete in vitro life cycle of Trypanosoma

1490   congolense: development of genetic tools. PLoS Negl Trop Dis. 2010;4(3): e618. doi:

1491   10.1371/journal.pntd.0000618.

1492   15. Hirumi H, Hirumi K. In vitro cultivation of Trypanosoma congolense bloodstream forms in the

1493   absence of feeder cell layers. Parasitology. 1991;102 Pt 2: 225-36.

1494   16. Rotureau B, Van Den Abbeele J. Through the dark continent: African trypanosome development

1495   in the tsetse fly. Front Cell Infect Microbiol. 2013;3: 53. doi: 10.3389/fcimb.2013.00053.

1496   17. Silvester E, Young J, Ivens A, Matthews KR. Interspecies quorum sensing in co-infections can

1497   manipulate trypanosome transmission potential. Nat Microbiol. 2017;2(11): 1471-9. doi:

1498   10.1038/s41564-017-0014-5.

1499   18.   Jackson AP, Allison HC, Barry JD, Field MC, Hertz-Fowler C, Berriman M. A cell-surface phylome

1500   for African trypanosomes. PLoS Negl Trop Dis. 2013;7(3): e2121. doi:

1501   10.1371/journal.pntd.0002121.

1502   19.   Jackson AP, Berry A, Aslett M, Allison HC, Burton P, Vavrova-Anderson J, et al. Antigenic diversity

1503   is generated by distinct evolutionary mechanisms in African trypanosome species. Proc Natl

1504   Acad Sci U S A. 2012;109(9): 3416-21. doi: 10.1073/pnas.1117313109.

1505   20.   Silva Pereira S, Casas-Sanchez A, Haines LR, Ogugo M, Absolomon K, Sanders M, et al. Variant

1506   antigen repertoires in Trypanosoma congolense populations and experimental infections can

1507   be profiled from deep sequence data using universal protein motifs. Genome Res. 2018;28(9):

1508   1383-94. doi: 10.1101/gr.234146.118.

1509   21.   Silva Pereira S, de Almeida Castilho Neto KJG, Duffy CW, Richards P, Noyes H, Ogugo M, et al.

1510   Variant antigen diversity in Trypanosoma vivax is not driven by recombination. Nat Commun.

1511   2020;11(1): 844. doi: 10.1038/s41467-020-14575-8.

1512   22.   Tihon E, Imamura H, Dujardin JC, Van Den Abbeele J, Van den Broeck F. Discovery and genomic

1513   analyses of hybridization between divergent lineages of Trypanosoma congolense, causative

1514   agent of Animal African Trypanosomiasis. Mol Ecol. 2017;26(23): 6524-38. doi:

1515   10.1111/mec.14271.

1516   23.   Creek DJ, Barrett MP. Determination of antiprotozoal drug mechanisms by metabolomics

1517   approaches. Parasitology. 2014;141(1): 83-92. doi: 10.1017/S0031182013000814.

1518   24.   Haanstra JR, Gerding A, Dolga AM, Sorgdrager FJH, Buist-Homan M, du Toit F, et al. Targeting

1519   pathogen metabolism without collateral damage to the host. Sci Rep. 2017;7: 40406. doi:

1520   10.1038/srep40406.

1521   25.   Vincent IM, Ehmann DE, Mills SD, Perros M, Barrett MP. Untargeted Metabolomics To Ascertain

1522   Antibiotic Modes of Action. Antimicrob Agents Chemother. 2016;60(4): 2281-91. doi:

1523   10.1128/AAC.02109-15.

1524   26.   Bringaud F, Biran M, Millerioux Y, Wargnies M, Allmann S, Mazet M. Combining reverse genetics

1525   and nuclear magnetic resonance-based metabolomics unravels trypanosome-specific metabolic

1526   pathways. Mol Microbiol. 2015;96(5): 917-26. doi: 10.1111/mmi.12990.

1527   27.   Creek DJ, Anderson J, McConville MJ, Barrett MP. Metabolomic analysis of trypanosomatid

1528   protozoa. Mol Biochem Parasitol. 2012;181(2): 73-84. doi: 10.1016/j.molbiopara.2011.10.003.

1529   28.   Allmann S, Bringaud F. Glycosomes: A comprehensive view of their metabolic roles in T. brucei.

1530   Int J Biochem Cell Biol. 2017;85: 85-90. doi: 10.1016/j.biocel.2017.01.015.

1531   29.   Haanstra JR, van Tuijl A, Kessler P, Reijnders W, Michels PA, Westerhoff HV, et al.

1532   Compartmentation prevents a lethal turbo-explosion of glycolysis in trypanosomes. Proc Natl

1533   Acad Sci U S A. 2008;105(46): 17718-23. doi: 10.1073/pnas.0806664105.

1534   30.   Mazet M, Morand P, Biran M, Bouyssou G, Courtois P, Daulouede S, et al. Revisiting the central

1535   metabolism of the bloodstream forms of Trypanosoma brucei: production of acetate in the

1536   mitochondrion is essential for parasite viability. PLoS Negl Trop Dis. 2013;7(12): e2587. doi:

1537   10.1371/journal.pntd.0002587.

1538   31.   Millerioux Y, Mazet M, Bouyssou G, Allmann S, Kiema TR, Bertiaux E, et al. De novo biosynthesis

1539   of sterols and fatty acids in the Trypanosoma brucei procyclic form: Carbon source preferences

1540   and   metabolic   flux   redistributions.   PLoS   Pathog.   2018;14(5):   e1007116.   doi:

1541   10.1371/journal.ppat.1007116.

1542   32.   Yang PY, Wang M, Liu K, Ngai MH, Sheriff O, Lear MJ, et al. Parasite-based screening and

1543   proteome profiling reveal orlistat, an FDA-approved drug, as a potential anti Trypanosoma

1544   brucei agent. Chemistry. 2012;18(27): 8403-13. doi: 10.1002/chem.201200482.

1545   33.   Lamour N, Riviere L, Coustou V, Coombs GH, Barrett MP, Bringaud F. Proline metabolism in

1546   procyclic Trypanosoma brucei is down-regulated in the presence of glucose. J Biol Chem.

1547   2005;280(12): 11902-10. doi: 10.1074/jbc.M414274200.

1548   34.   Tielens AG, Van Hellemond JJ. Differences in energy metabolism between trypanosomatidae.

1549   Parasitol Today. 1998;14(7): 265-72.

1550   35.   van Weelden SW, Fast B, Vogt A, van der Meer P, Saas J, van Hellemond JJ, et al. Procyclic

1551   Trypanosoma brucei do not use Krebs cycle activity for energy generation. J Biol Chem.

1552   2003;278(15): 12854-63. doi: 10.1074/jbc.M213190200.

1553   36.   van Hellemond JJ, Opperdoes FR, Tielens AG. The extraordinary mitochondrion and unusual

1554   citric acid cycle in Trypanosoma brucei. Biochem Soc Trans. 2005;33(Pt 5): 967-71. doi:

1555   10.1042/BST20050967.

1556   37.   Villafraz O, Biran M, Pineda E, Plazolles N, Cahoreau E, Ornitz R, et al. Fly stage trypanosomes

1557   recycle glucose catabolites and TCA cycle intermediates to stimulate growth in near

1558   physiological conditions. 2020: 2020.12.17.423221. doi: 10.1101/2020.12.17.423221 %J

1559   bioRxiv.

1560   38.   Colasante C, Robles A, Li CH, Schwede A, Benz C, Voncken F, et al. Regulated expression of

1561   glycosomal phosphoglycerate kinase in Trypanosoma brucei. Mol Biochem Parasitol.

1562   2007;151(2): 193-204. doi: 10.1016/j.molbiopara.2006.11.003.

1563   39.   Blattner J, Helfert S, Michels P, Clayton C. Compartmentation of phosphoglycerate kinase in

1564   Trypanosoma brucei plays a critical role in parasite energy metabolism. Proc Natl Acad Sci U S

1565   A. 1998;95(20): 11596-600.

1566   40.   Deramchia K, Morand P, Biran M, Millerioux Y, Mazet M, Wargnies M, et al. Contribution of

1567   pyruvate phosphate dikinase in the maintenance of the glycosomal ATP/ADP balance in the

1568   Trypanosoma brucei procyclic form. J Biol Chem. 2014;289(25): 17365-78. doi:

1569   10.1074/jbc.M114.567230.

1570   41.   van Grinsven KW, Van Den Abbeele J, Van den Bossche P, van Hellemond JJ, Tielens AG.

1571   Adaptations in the glucose metabolism of procyclic Trypanosoma brucei isolates from tsetse

1572   flies and during differentiation of bloodstream forms. Eukaryot Cell. 2009;8(8): 1307-11. doi:

1573   10.1128/EC.00091-09.

1574   42.   Creek DJ, Mazet M, Achcar F, Anderson J, Kim DH, Kamour R, et al. Probing the metabolic

1575   network in bloodstream-form Trypanosoma brucei using untargeted metabolomics with stable

1576   isotope   labelled   glucose.   PLoS   Pathog.   2015;11(3):   e1004689.   doi:
1577   10.1371/journal.ppat.1004689.

1578   43.   Agosin M, Von Brand T. Studies on the carbohydrate metabolism of Trypanosoma congolense.
1579   Exp Parasitol. 1954;3(6): 517-24.

1580   44.   Bringaud F, Baltz D, Baltz T. Functional and molecular characterization of a glycosomal PPi-
1581   dependent enzyme in trypanosomatids: pyruvate, phosphate dikinase. Proc Natl Acad Sci U S A.
1582   1998;95(14): 7963-8.

1583   45.   Ohashi-Suzuki M, Yabu Y, Ohshima S, Nakamura K, Kido Y, Sakamoto K, et al. Differential kinetic
1584   activities of glycerol kinase among African trypanosome species: phylogenetic and therapeutic
1585   implications. J Vet Med Sci. 2011;73(5): 615-21.

1586   46.   Parker HL, Hill T, Alexander K, Murphy NB, Fish WR, Parsons M. Three genes and two isozymes:
1587   gene conversion and the compartmentalization and expression of the phosphoglycerate kinases
1588   of Trypanosoma (Nannomonas) congolense. Mol Biochem Parasitol. 1995;69(2): 269-79.

1589   47.   Vickerman K. The fine structure of Trypanosoma congolense in its bloodstream phase. J
1590   Protozool. 1969;16(1): 54-69.

1591   48.   Bienen EJ, Webster P, Fish WR. Trypanosoma (Nannomonas) congolense: changes in respiratory
1592   metabolism during the life cycle. Exp Parasitol. 1991;73(4): 403-12.

1593   49.   Ebiloma GU, Ayuga TD, Balogun EO, Gil LA, Donachie A, Kaiser M, et al. Inhibition of
1594   trypanosome alternative oxidase without its N-terminal mitochondrial targeting signal
1595   (DeltaMTS-TAO) by cationic and non-cationic 4-hydroxybenzoate and 4-alkoxybenzaldehyde
1596   derivatives active against T. brucei and T. congolense. Eur J Med Chem. 2018;150: 385-402. doi:
1597   10.1016/j.ejmech.2018.02.075.

1598   50.   Fueyo Gonzalez FJ, Ebiloma GU, Izquierdo Garcia C, Bruggeman V, Sanchez Villamanan JM,
1599   Donachie A, et al. Conjugates of 2,4-Dihydroxybenzoate and Salicylhydroxamate and
1600   Lipocations Display Potent Antiparasite Effects by Efficiently Targeting the Trypanosoma brucei

1601   and Trypanosoma congolense Mitochondrion. J Med Chem. 2017;60(4): 1509-22. doi:

1602   10.1021/acs.jmedchem.6b01740.

1603   51.   Steiger RF, Steiger E, Trager W, Schneider I. Trypanosoma congolense: partial cyclic

1604   development in a Glossina cell system and oxygen consumption. J Parasitol. 1977;63(5): 861-7.

1605   52.   Hsu HH, Araki M, Mochizuki M, Hori Y, Murata M, Kahar P, et al. A Systematic Approach to Time-

1606   series Metabolite Profiling and RNA-seq Analysis of Chinese Hamster Ovary Cell Culture. Sci Rep.

1607   2017;7: 43518. doi: 10.1038/srep43518.

1608   53.   Wagner GP, Kin K, Lynch VJ. Measurement of mRNA abundance using RNA-seq data: RPKM

1609   measure is inconsistent among samples. Theory Biosci. 2012;131(4): 281-5. doi:

1610   10.1007/s12064-012-0162-3.

1611   54.   Emms DM, Kelly S. OrthoFinder: solving fundamental biases in whole genome comparisons

1612   dramatically improves orthogroup inference accuracy. Genome Biol. 2015;16: 157. doi:

1613   10.1186/s13059-015-0721-2.

1614   55.   Silvester E, Ivens A, Matthews KR. A gene expression comparison of Trypanosoma brucei and

1615   Trypanosoma congolense in the bloodstream of the mammalian host reveals species-specific

1616   adaptations to density-dependent development. PLoS Negl Trop Dis. 2018;12(10): e0006863.

1617   doi: 10.1371/journal.pntd.0006863.

1618   56.   Creek DJ, Nijagal B, Kim DH, Rojas F, Matthews KR, Barrett MP. Metabolomics guides rational

1619   development of a simplified cell culture medium for drug screening against Trypanosoma

1620   brucei. Antimicrob Agents Chemother. 2013;57(6): 2768-79. doi: 10.1128/AAC.00044-13.

1621   57.   Kim DH, Achcar F, Breitling R, Burgess KE, Barrett MP. LC-MS-based absolute metabolite

1622   quantification: application to metabolic flux measurement in trypanosomes. Metabolomics.

1623   2015;11(6): 1721-32. doi: 10.1007/s11306-015-0827-2.

1624   58.   Nok AJ, Esievo KA, Ibrahim S, Ukoha AI, Ikediobi CO. Phospholipase A2 from Trypanosoma

1625   congolense: characterization and haematological properties. Cell Biochem Funct. 1993;11(2):

1626   125-30. doi: 10.1002/cbf.290110208.

1627   59.   Duszenko M, Ferguson MA, Lamont GS, Rifkin MR, Cross GA. Cysteine eliminates the feeder cell

1628   requirement for cultivation of Trypanosoma brucei bloodstream forms in vitro. J Exp Med.

1629   1985;162(4): 1256-63.

1630   60.   Shameer S, Logan-Klumpler FJ, Vinson F, Cottret L, Merlet B, Achcar F, et al. TrypanoCyc: a

1631   community-led biochemical pathways database for Trypanosoma brucei. Nucleic Acids Res.

1632   2015;43(Database issue): D637-44. doi: 10.1093/nar/gku944.

1633   61.   Szoor B, Ruberto I, Burchmore R, Matthews KR. A novel phosphatase cascade regulates

1634   differentiation in Trypanosoma brucei via a glycosomal signaling pathway. Genes Dev.

1635   2010;24(12): 1306-16. doi: 10.1101/gad.570310.

1636   62.   Mugo E, Clayton C. Expression of the RNA-binding protein RBP10 promotes the bloodstream-

1637   form differentiation state in Trypanosoma brucei. PLoS Pathog. 2017;13(8): e1006560. doi:

1638   10.1371/journal.ppat.1006560.

1639   63.   Dean S, Marchetti R, Kirk K, Matthews KR. A surface transporter family conveys the

1640   trypanosome differentiation signal. Nature. 2009;459(7244): 213-7. doi: 10.1038/nature07997.

1641   64.   Aranda A, Maugeri D, Uttaro AD, Opperdoes F, Cazzulo JJ, Nowicki C. The malate dehydrogenase

1642   isoforms from Trypanosoma brucei: subcellular localization and differential expression in

1643   bloodstream and procyclic forms. Int J Parasitol. 2006;36(3): 295-307. doi:

1644   10.1016/j.ijpara.2005.09.013.

1645   65.   Abbas AH, Silva Pereira S, D'Archivio S, Wickstead B, Morrison LJ, Hall N, et al. The Structure of

1646   a Conserved Telomeric Region Associated with Variant Antigen Loci in the Blood Parasite

1647   Trypanosoma congolense. Genome Biol Evol. 2018;10(9): 2458-73. doi: 10.1093/gbe/evy186.

1648   66.   Spitznagel D, Ebikeme C, Biran M, Nic a' Bhaird N, Bringaud F, Henehan GT, et al. Alanine

1649   aminotransferase of Trypanosoma brucei--a key role in proline metabolism in procyclic life

1650   forms. FEBS J. 2009;276(23): 7187-99. doi: 10.1111/j.1742-4658.2009.07432.x.

1651   67.   Natesan SK, Peacock L, Leung KF, Gibson W, Field MC. Evidence that low endocytic activity is

1652   not directly responsible for human serum resistance in the insect form of African trypanosomes.

1653   BMC Res Notes. 2010;3: 63. doi: 10.1186/1756-0500-3-63.

1654   68.   Ebikeme CE, Peacock L, Coustou V, Riviere L, Bringaud F, Gibson WC, et al. N-acetyl D-

1655   glucosamine stimulates growth in procyclic forms of Trypanosoma brucei by inducing a

1656   metabolic shift. Parasitology. 2008;135(5): 585-94. doi: 10.1017/S0031182008004241.

1657   69.   Awuah-Mensah G, McDonald J, Steketee PC, Autheman D, Whipple S, D'Archivio S, et al.

1658   Reliable, scalable functional genetics in bloodstream-form Trypanosoma congolense in vitro

1659   and in vivo. PLoS Pathog. 2021;17(1): e1009224. doi: 10.1371/journal.ppat.1009224.

1660   70.   Kovarova J, Nagar R, Faria J, Ferguson MAJ, Barrett MP, Horn D. Gluconeogenesis using glycerol

1661   as a substrate in bloodstream-form Trypanosoma brucei. PLoS Pathog. 2018;14(12): e1007475.

1662   doi: 10.1371/journal.ppat.1007475.

1663   71.   Tielens AG, van Hellemond JJ. Surprising variety in energy metabolism within

1664   Trypanosomatidae. Trends Parasitol. 2009;25(10): 482-90. doi: 10.1016/j.pt.2009.07.007.

1665   72.   Trindade S, Rijo-Ferreira F, Carvalho T, Pinto-Neves D, Guegan F, Aresta-Branco F, et al.

1666   Trypanosoma brucei Parasites Occupy and Functionally Adapt to the Adipose Tissue in Mice.

1667   Cell Host Microbe. 2016;19(6): 837-48. doi: 10.1016/j.chom.2016.05.002.

1668   73.   Zikova A, Verner Z, Nenarokova A, Michels PAM, Lukes J. A paradigm shift: The mitoproteomes

1669   of procyclic and bloodstream Trypanosoma brucei are comparably complex. PLoS Pathog.

1670   2017;13(12): e1006679. doi: 10.1371/journal.ppat.1006679.

1671   74.   Geiger A, Hirtz C, Becue T, Bellard E, Centeno D, Gargani D, et al. Exocytosis and protein

1672   secretion in Trypanosoma. BMC Microbiol. 2010;10: 20. doi: 10.1186/1471-2180-10-20.

1673   75.   Manivel G, Meyyazhagan A, Durairaj DR, Piramanayagam S. Genome-wide analysis of

1674   Excretory/Secretory proteins in Trypanosoma brucei brucei: Insights into functional

1675   characteristics and identification of potential targets by immunoinformatics approach.

1676   Genomics. 2019;111(5): 1124-33. doi: 10.1016/j.ygeno.2018.07.007.

1677   76.   Henriques C, Sanchez MA, Tryon R, Landfear SM. Molecular and functional characterization of

1678   the first nucleobase transporter gene from African trypanosomes. Mol Biochem Parasitol.

1679   2003;130(2): 101-10. doi: 10.1016/s0166-6851(03)00167-1.

1680   77.   Burchmore RJ, Wallace LJ, Candlish D, Al-Salabi MI, Beal PR, Barrett MP, et al. Cloning,

1681   heterologous expression, and in situ characterization of the first high affinity nucleobase

1682   transporter from a protozoan. J Biol Chem. 2003;278(26): 23502-7. doi:

1683   10.1074/jbc.M301252200.

1684   78.   Munday JC, Rojas Lopez KE, Eze AA, Delespaux V, Van Den Abbeele J, Rowan T, et al. Functional

1685   expression of TcoAT1 reveals it to be a P1-type nucleoside transporter with no capacity for

1686   diminazene uptake. Int J Parasitol Drugs Drug Resist. 2013;3: 69-76. doi:

1687   10.1016/j.ijpddr.2013.01.004.

1688   79.   Al-Salabi MI, Wallace LJ, Luscher A, Maser P, Candlish D, Rodenko B, et al. Molecular interactions

1689   underlying the unusually high adenosine affinity of a novel Trypanosoma brucei nucleoside

1690   transporter. Mol Pharmacol. 2007;71(3): 921-9. doi: 10.1124/mol.106.031559.

1691   80.   de Koning HP, Bridges DJ, Burchmore RJ. Purine and pyrimidine transport in pathogenic

1692   protozoa: from biology to therapy. FEMS Microbiol Rev. 2005;29(5): 987-1020. doi:

1693   10.1016/j.femsre.2005.03.004.

1694   81.   Papageorgiou IG, Yakob L, Al Salabi MI, Diallinas G, Soteriadou KP, De Koning HP. Identification

1695   of the first pyrimidine nucleobase transporter in Leishmania: similarities with the Trypanosoma

1696   brucei U1 transporter and antileishmanial activity of uracil analogues. Parasitology. 2005;130(Pt

1697   3): 275-83. doi: 10.1017/s0031182004006626.

1698   82.   Gudin S, Quashie NB, Candlish D, Al-Salabi MI, Jarvis SM, Ranford-Cartwright LC, et al.

1699   Trypanosoma brucei: a survey of pyrimidine transport activities. Exp Parasitol. 2006;114(2):

1700   118-25. doi: 10.1016/j.exppara.2006.02.018.

1701   83.   de Koning HP, Jarvis SM. A highly selective, high-affinity transporter for uracil in Trypanosoma

1702   brucei brucei: evidence for proton-dependent transport. Biochem Cell Biol. 1998;76(5): 853-8.

1703   doi: 10.1139/bcb-76-5-853.

1704   84.   Ali JA, Tagoe DN, Munday JC, Donachie A, Morrison LJ, de Koning HP. Pyrimidine biosynthesis is

1705   not an essential function for Trypanosoma brucei bloodstream forms. PLoS One. 2013;8(3):

1706   e58034. doi: 10.1371/journal.pone.0058034.

1707   85.   Johnston K, Kim DH, Kerkhoven EJ, Burchmore R, Barrett MP, Achcar F. Mapping the metabolism

1708   of five amino acids in bloodstream form Trypanosoma brucei using U-(13)C-labelled substrates

1709   and LC-MS. Biosci Rep. 2019;39(5). doi: 10.1042/BSR20181601.

1710   86.   Marchese L, Nascimento JF, Damasceno FS, Bringaud F, Michels PAM, Silber AM. The Uptake

1711   and Metabolism of Amino Acids, and Their Unique Role in the Biology of Pathogenic

1712   Trypanosomatids. Pathogens. 2018;7(2). doi: 10.3390/pathogens7020036.

1713   87.   Mantilla BS, Marchese L, Casas-Sanchez A, Dyer NA, Ejeh N, Biran M, et al. Proline Metabolism

1714   is Essential for Trypanosoma brucei brucei Survival in the Tsetse Vector. PLoS Pathog.

1715   2017;13(1): e1006158. doi: 10.1371/journal.ppat.1006158.

1716   88.   Paul KS, Jiang D, Morita YS, Englund PT. Fatty acid synthesis in African trypanosomes: a solution

1717   to the myristate mystery. Trends Parasitol. 2001;17(8): 381-7. doi: 10.1016/s1471-

1718   4922(01)01984-5.

1719   89.   Stephens JL, Lee SH, Paul KS, Englund PT. Mitochondrial fatty acid synthesis in Trypanosoma

1720   brucei. J Biol Chem. 2007;282(7): 4427-36. doi: 10.1074/jbc.M609037200.

1721   90.   Millerioux Y, Ebikeme C, Biran M, Morand P, Bouyssou G, Vincent IM, et al. The threonine

1722   degradation pathway of the Trypanosoma brucei procyclic form: the main carbon source for

1723   lipid biosynthesis is under metabolic control. Mol Microbiol. 2013;90(1): 114-29. doi:

1724   10.1111/mmi.12351.

1725   91.   Millerioux Y, Morand P, Biran M, Mazet M, Moreau P, Wargnies M, et al. ATP synthesis-coupled

1726   and -uncoupled acetate production from acetyl-CoA by mitochondrial acetate:succinate CoA-

1727   transferase and acetyl-CoA thioesterase in Trypanosoma. J Biol Chem. 2012;287(21): 17186-97.

1728   doi: 10.1074/jbc.M112.355404.

1729 92.   Surve SV, Jensen BC, Heestand M, Mazet M, Smith TK, Bringaud F, et al. NADH dehydrogenase

1730   of Trypanosoma brucei is important for efficient acetate production in bloodstream forms. Mol

1731   Biochem Parasitol. 2017;211: 57-61. doi: 10.1016/j.molbiopara.2016.10.001.

1732 93.   Stafkova J, Mach J, Biran M, Verner Z, Bringaud F, Tachezy J. Mitochondrial pyruvate carrier in

1733   Trypanosoma brucei. Mol Microbiol. 2016;100(3): 442-56. doi: 10.1111/mmi.13325.

1734 94.   Comerford SA, Huang Z, Du X, Wang Y, Cai L, Witkiewicz AK, et al. Acetate dependence of

1735   tumors. Cell. 2014;159(7): 1591-602. doi: 10.1016/j.cell.2014.11.020.

1736 95.   Riviere L, Moreau P, Allmann S, Hahn M, Biran M, Plazolles N, et al. Acetate produced in the

1737   mitochondrion is the essential precursor for lipid biosynthesis in procyclic trypanosomes. Proc

1738   Natl Acad Sci U S A. 2009;106(31): 12694-9. doi: 10.1073/pnas.0903355106.

1739 96.   Lamont GS, Fox JA, Cross GA. Glycosyl-sn-1,2-dimyristylphosphatidylinositol is the membrane

1740   anchor for Trypanosoma equiperdum and T. (Nannomonas) congolense variant surface

1741   glycoproteins. Mol Biochem Parasitol. 1987;24(2): 131-6. doi: 10.1016/0166-6851(87)90099-5.

1742 97.   Lee SH, Stephens JL, Paul KS, Englund PT. Fatty acid synthesis by elongases in trypanosomes.

1743   Cell. 2006;126(4): 691-9. doi: 10.1016/j.cell.2006.06.045.

1744 98.   Danaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, Paciorek CJ, et al. National, regional, and

1745   global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis

1746   of health examination surveys and epidemiological studies with 370 country-years and 2.7

1747   million participants. Lancet. 2011;378(9785): 31-40. doi: 10.1016/S0140-6736(11)60679-X.

1748 99.   Cozzi G, Ravarotto L, Gottardo F, Stefani AL, Contiero B, Moro L, et al. Short communication:

1749   reference values for blood parameters in Holstein dairy cows: effects of parity, stage of

1750   lactation, and season of production. J Dairy Sci. 2011;94(8): 3895-901. doi: 10.3168/jds.2010-

1751   3687.

1752   100.   Jackson PGG, Cockcroft PD. Appendix 2: Laboratory Reference Values: Haematology. Clinical
1753   Examination of Farm Animals2002. p. 302-.

1754   101.   Mair B, Drillich M, Klein-Jobstl D, Kanz P, Borchardt S, Meyer L, et al. Glucose concentration in
1755   capillary blood of dairy cows obtained by a minimally invasive lancet technique and determined
1756   with three different hand-held devices. BMC Vet Res. 2016;12: 34. doi: 10.1186/s12917-016-
1757   0662-3.

1758   102.   Nafikov RA, Beitz DC. Carbohydrate and lipid metabolism in farm animals. J Nutr. 2007;137(3):
1759   702-5. doi: 10.1093/jn/137.3.702.

1760   103.   Puppel K, Kuczynska B. Metabolic profiles of cow's blood; a review. J Sci Food Agric. 2016;96(13):
1761   4321-8. doi: 10.1002/jsfa.7779.

1762   104.   Bochud-Allemann N, Schneider A. Mitochondrial substrate level phosphorylation is essential for
1763   growth of procyclic Trypanosoma brucei. J Biol Chem. 2002;277(36): 32849-54. doi:
1764   10.1074/jbc.M205776200.

1765   105.   Mochizuki K, Inaoka DK, Mazet M, Shiba T, Fukuda K, Kurasawa H, et al. The ASCT/SCS cycle
1766   fuels mitochondrial ATP and acetate production in Trypanosoma brucei. Biochim Biophys Acta
1767   Bioenerg. 2020;1861(11): 148283. doi: 10.1016/j.bbabio.2020.148283.

1768   106.   Verner Z, Skodova I, Polakova S, Durisova-Benkovicova V, Horvath A, Lukes J. Alternative NADH
1769   dehydrogenase (NDH2): intermembrane-space-facing counterpart of mitochondrial complex I
1770   in the procyclic Trypanosoma brucei. Parasitology. 2013;140(3): 328-37. doi:
1771   10.1017/S003118201200162X.

1772   107.   Fang J, Beattie DS. Novel FMN-containing rotenone-insensitive NADH dehydrogenase from
1773   Trypanosoma brucei mitochondria: isolation and characterization. Biochemistry. 2002;41(9):
1774   3065-72. doi: 10.1021/bi015989w.

1775   108.   Dewar CE, MacGregor P, Cooper S, Gould MK, Matthews KR, Savill NJ, et al. Mitochondrial DNA
1776   is critical for longevity and metabolism of transmission stage Trypanosoma brucei. PLoS Pathog.
1777   2018;14(7): e1007195. doi: 10.1371/journal.ppat.1007195.

1778   109.   Tizard IR, Mellors A, Holmes WL, Nielsen K. The generation of phospholipase A and hemolytic

1779   fatty acids by autolysing suspensions of Trypanosoma congolense. Tropenmed Parasitol.

1780   1978;29(1): 127-33.

1781   110.   Field MC, Horn D, Fairlamb AH, Ferguson MAJ, Gray DW, Read KD, et al. Anti-trypanosomatid

1782   drug discovery: an ongoing challenge and a continuing need. Nat Rev Microbiol. 2017;15(7):

1783   447. doi: 10.1038/nrmicro.2017.69.

1784   111.   Hirumi H, Hirumi K. Axenic culture of African trypanosome bloodstream forms. Parasitol Today.

1785   1994;10(2): 80-4.

1786   112.   Vassella E, Kramer R, Turner CM, Wankell M, Modes C, van den Bogaard M, et al. Deletion of a

1787   novel protein kinase with PX and FYVE-related domains increases the rate of differentiation of

1788   Trypanosoma brucei. Mol Microbiol. 2001;41(1): 33-46.

1789   113.   Hirumi H, Hirumi K. Continuous cultivation of Trypanosoma brucei blood stream forms in a

1790   medium containing a low concentration of serum protein without feeder cell layers. J Parasitol.

1791   1989;75(6): 985-9.

1792   114.   Herbert WJ, Lumsden WH. Trypanosoma brucei: a rapid "matching" method for estimating the

1793   host's parasitemia. Exp Parasitol. 1976;40(3): 427-31. doi: 10.1016/0014-4894(76)90110-7.

1794   115.   Lanham SM, Godfrey DG. Isolation of salivarian trypanosomes from man and other mammals

1795   using DEAE-cellulose. Exp Parasitol. 1970;28(3): 521-34. doi: 10.1016/0014-4894(70)90120-7.

1796   116.   Schumann Burkard G, Jutzi P, Roditi I. Genome-wide RNAi screens in bloodstream form

1797   trypanosomes identify drug transporters. Mol Biochem Parasitol. 2011;175(1): 91-4. doi:

1798   10.1016/j.molbiopara.2010.09.002.

1799   117.   Raz B, Iten M, Grether-Buhler Y, Kaminsky R, Brun R. The Alamar Blue assay to determine drug

1800   sensitivity of African trypanosomes (T.b. rhodesiense and T.b. gambiense) in vitro. Acta Trop.

1801   1997;68(2): 139-47. doi: 10.1016/s0001-706x(97)00079-x.

1802   118.   Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3--new

1803   capabilities and interfaces. Nucleic Acids Res. 2012;40(15): e115. doi: 10.1093/nar/gks596.

1804 119. Tihon E, Imamura H, Van den Broeck F, Vermeiren L, Dujardin JC, Van Den Abbeele J. Genomic

1805   analysis of Isometamidium Chloride resistance in Trypanosoma congolense. Int J Parasitol Drugs

1806   Drug Resist. 2017;7(3): 350-61. doi: 10.1016/j.ijpddr.2017.10.002.

1807 120. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative

1808   PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4): 402-8. doi:

1809   10.1006/meth.2001.1262.

1810 121. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data

1811   for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal

1812   Chem. 2006;78(3): 779-87. doi: 10.1021/ac051437y.

1813 122. Scheltema RA, Jankevics A, Jansen RC, Swertz MA, Breitling R. PeakML/mzMatch: a file format,

1814   Java library, R library, and tool-chain for mass spectrometry data analysis. Anal Chem.

1815   2011;83(7): 2786-93. doi: 10.1021/ac2000994.

1816 123. Creek DJ, Jankevics A, Burgess KE, Breitling R, Barrett MP. IDEOM: an Excel interface for analysis

1817   of LC-MS-based metabolomics data. Bioinformatics. 2012;28(7): 1048-9. doi:

1818   10.1093/bioinformatics/bts069.

1819 124. Chokkathukalam A, Jankevics A, Creek DJ, Achcar F, Barrett MP, Breitling R. mzMatch-ISO: an R

1820   tool for the annotation and relative quantification of isotope-labelled mass spectrometry data.

1821   Bioinformatics. 2013;29(2): 281-3. doi: 10.1093/bioinformatics/bts674.

1822 125. Chong J, Xia J. Using MetaboAnalyst 4.0 for Metabolomics Data Analysis, Interpretation, and

1823   Integration with Other Omics Data. Methods Mol Biol. 2020;2104: 337-60. doi: 10.1007/978-1-

1824   0716-0239-3_17.

1825 126. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements.

1826   Nat Methods. 2015;12(4): 357-60. doi: 10.1038/nmeth.3317.

1827 127. Aslett M, Aurrecoechea C, Berriman M, Brestelli J, Brunk BP, Carrington M, et al. TriTrypDB: a

1828   functional genomic resource for the Trypanosomatidae. Nucleic Acids Res. 2010;38(Database

1829   issue): D457-62. doi: 10.1093/nar/gkp851.
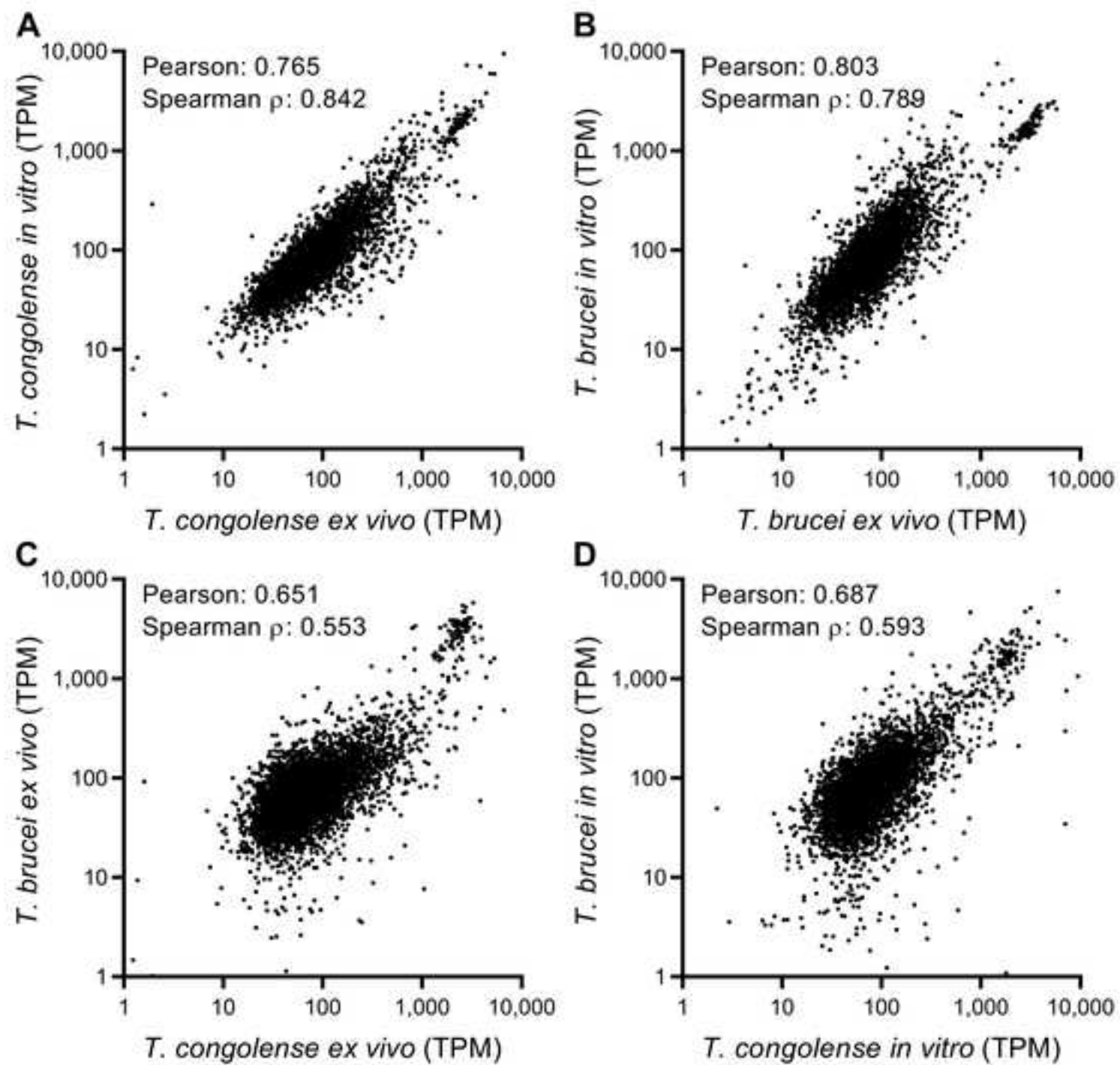
1830   128.   Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map

1831   format   and   SAMtools.   Bioinformatics.   2009;25(16):   2078-9.   doi:

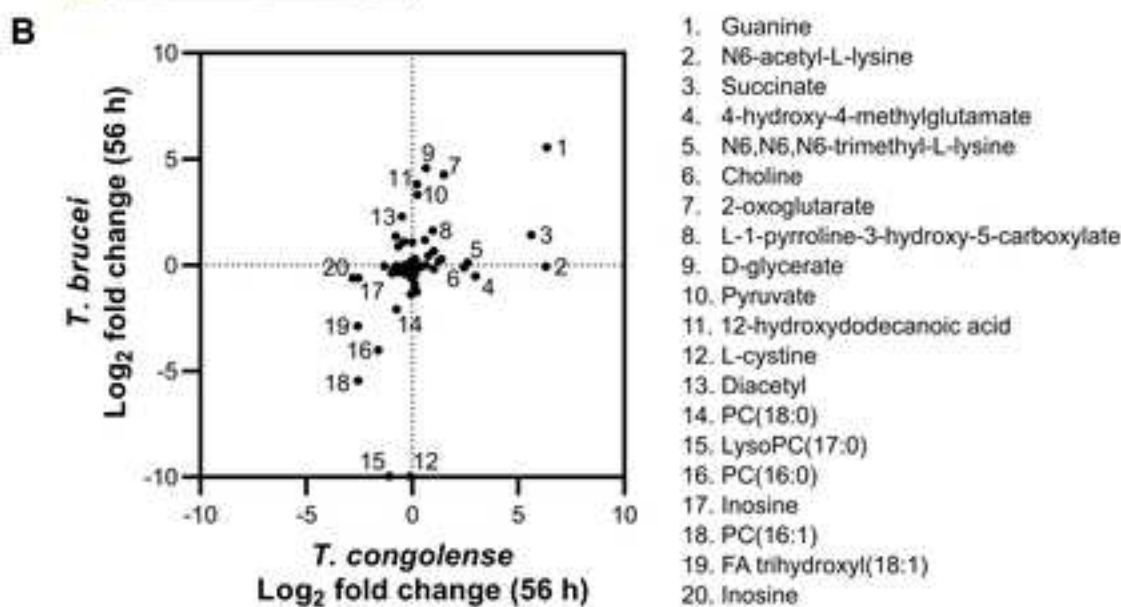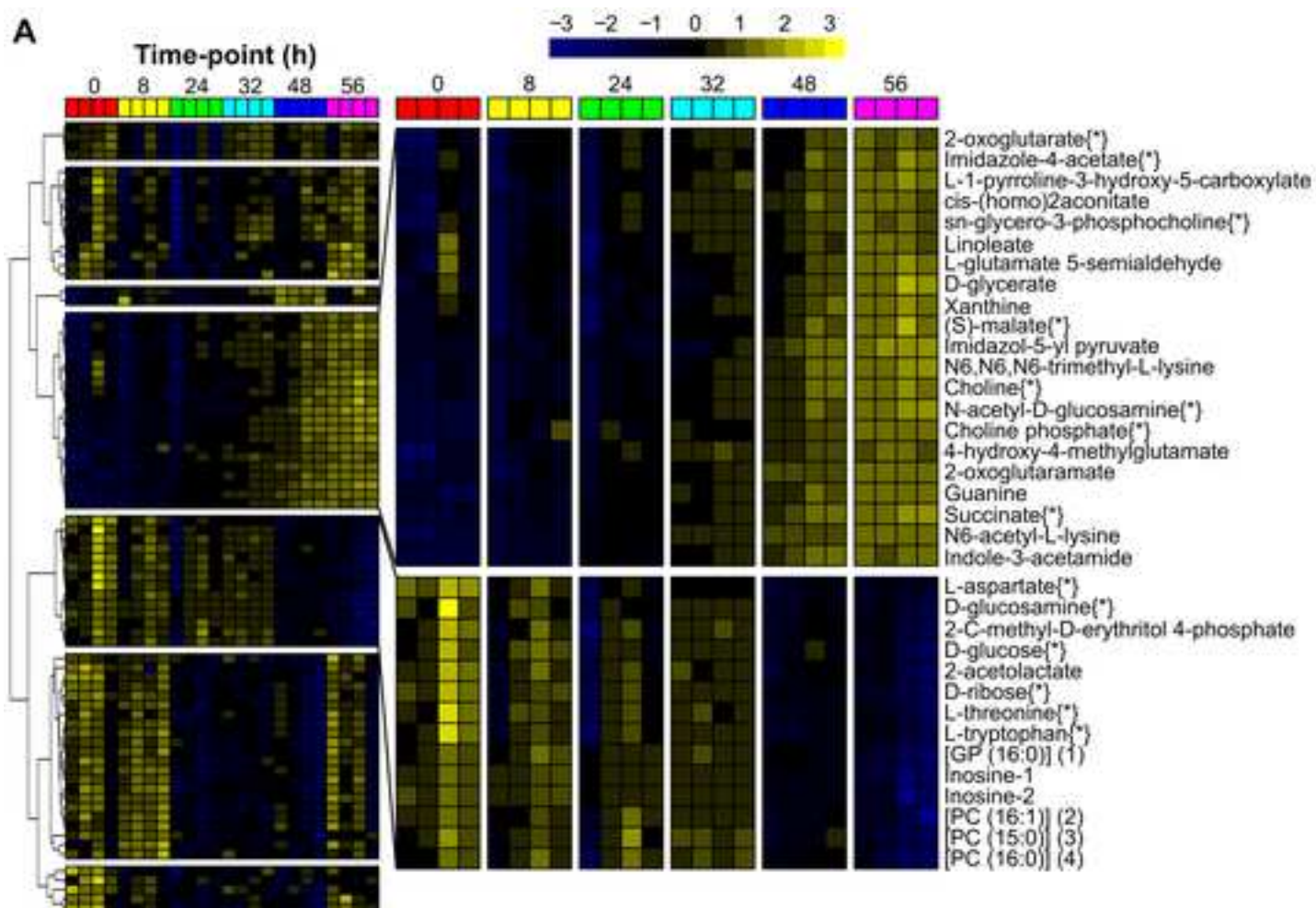1832   10.1093/bioinformatics/btp352.

1833   129.   Team. RC. R: A language and environment for statistical computing.: R Foundation for Statistical

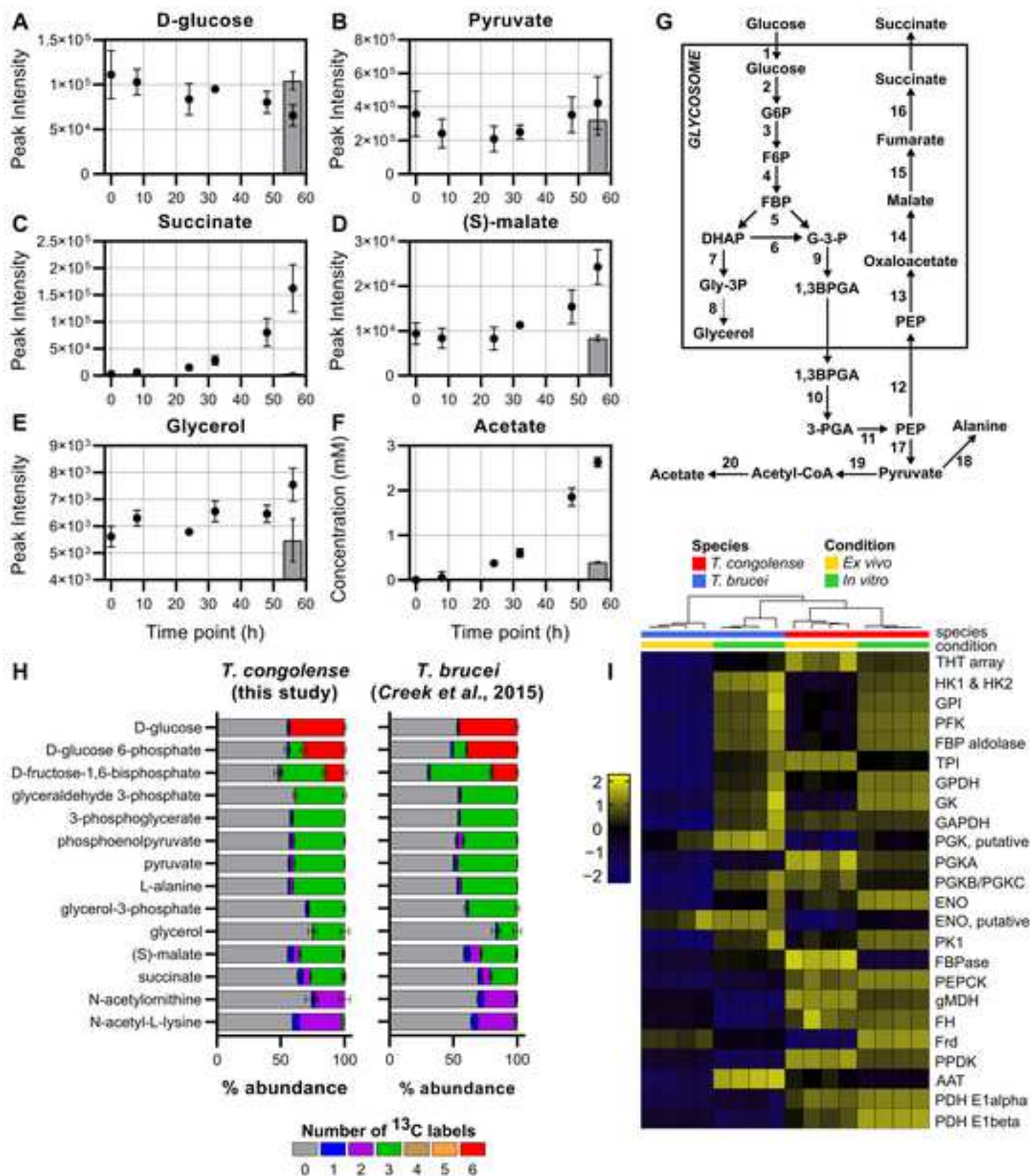1834   Computing, Vienna, Austria.; 2013.

1835   130.   Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in

1836   multidimensional   genomic   data.   Bioinformatics.   2016;32(18):   2847-9.   doi:

1837   10.1093/bioinformatics/btw313.

1838   131.   Dolezelova E, Teran D, Gahura O, Kotrbova Z, Prochazkova M, Keough D, et al. Evaluation of the

1839   Trypanosoma brucei 6-oxopurine salvage pathway as a potential target for drug discovery. PLoS

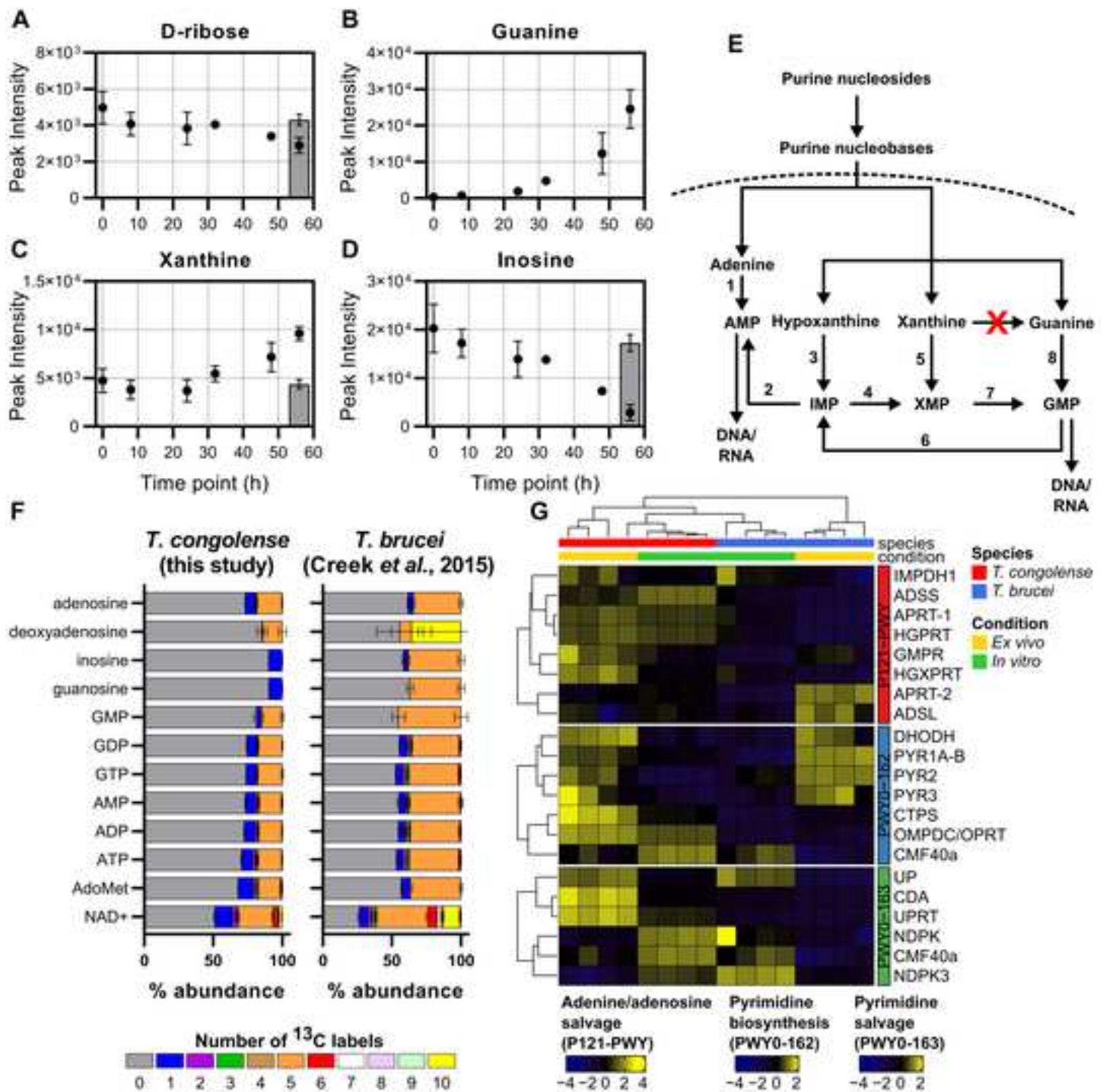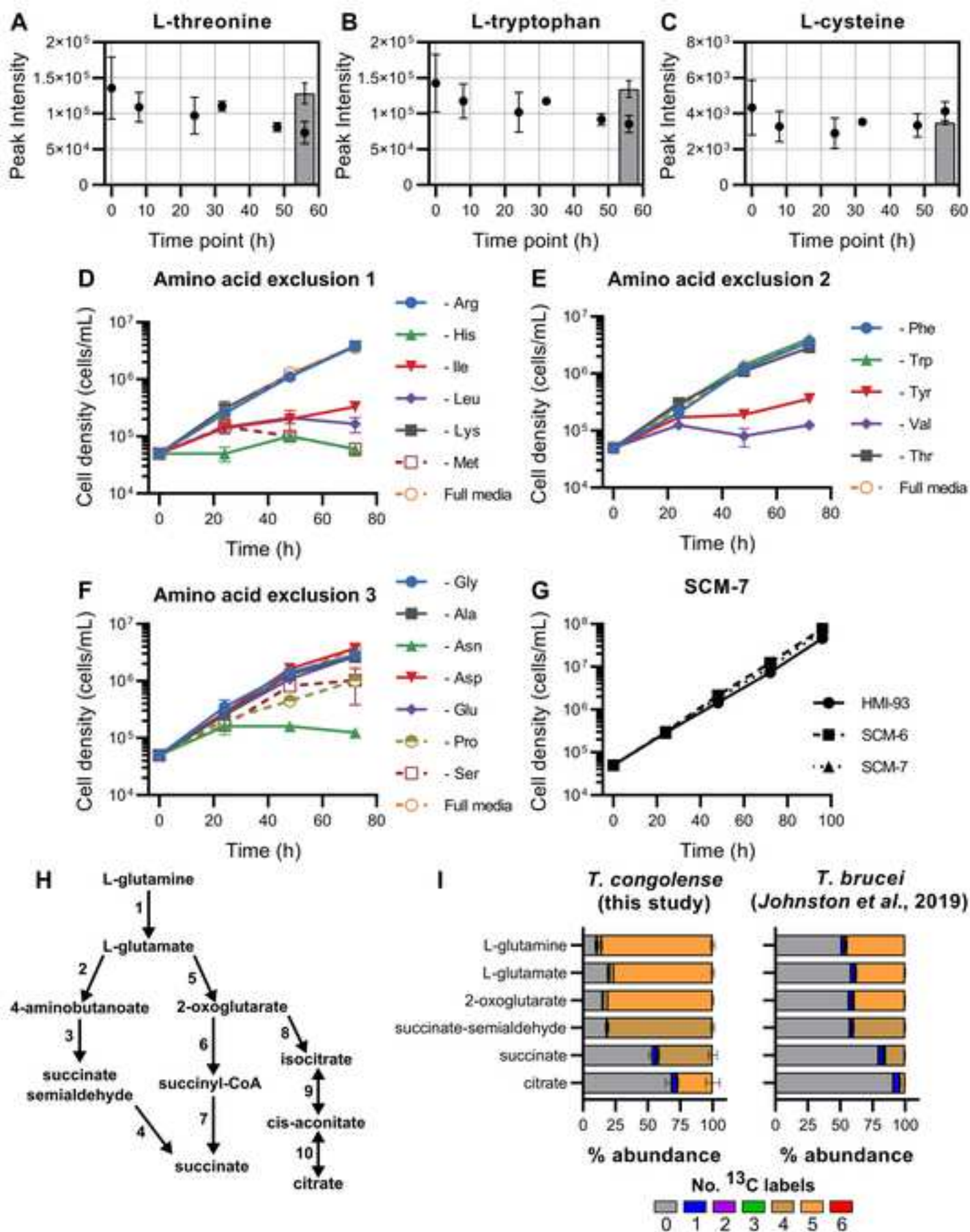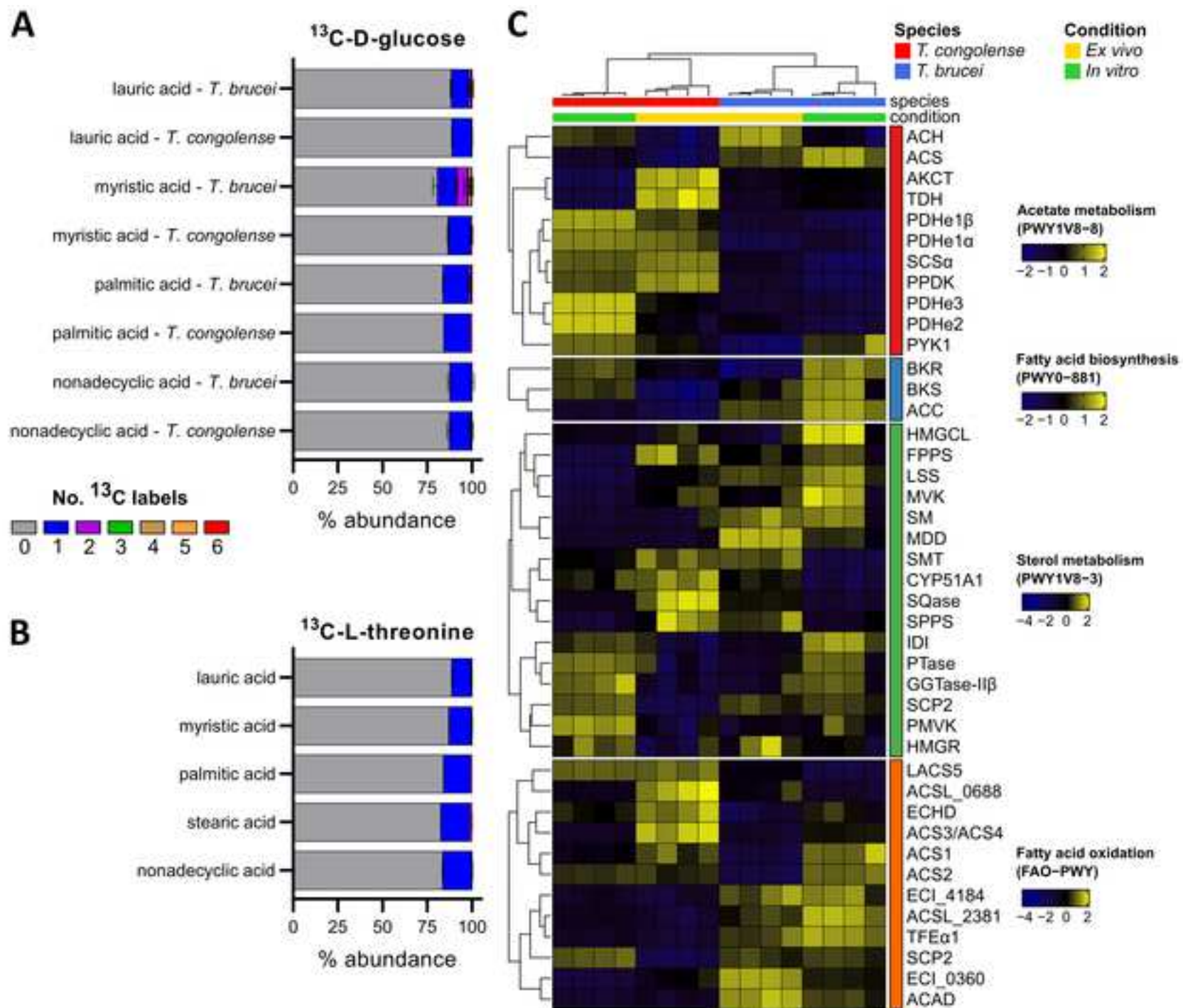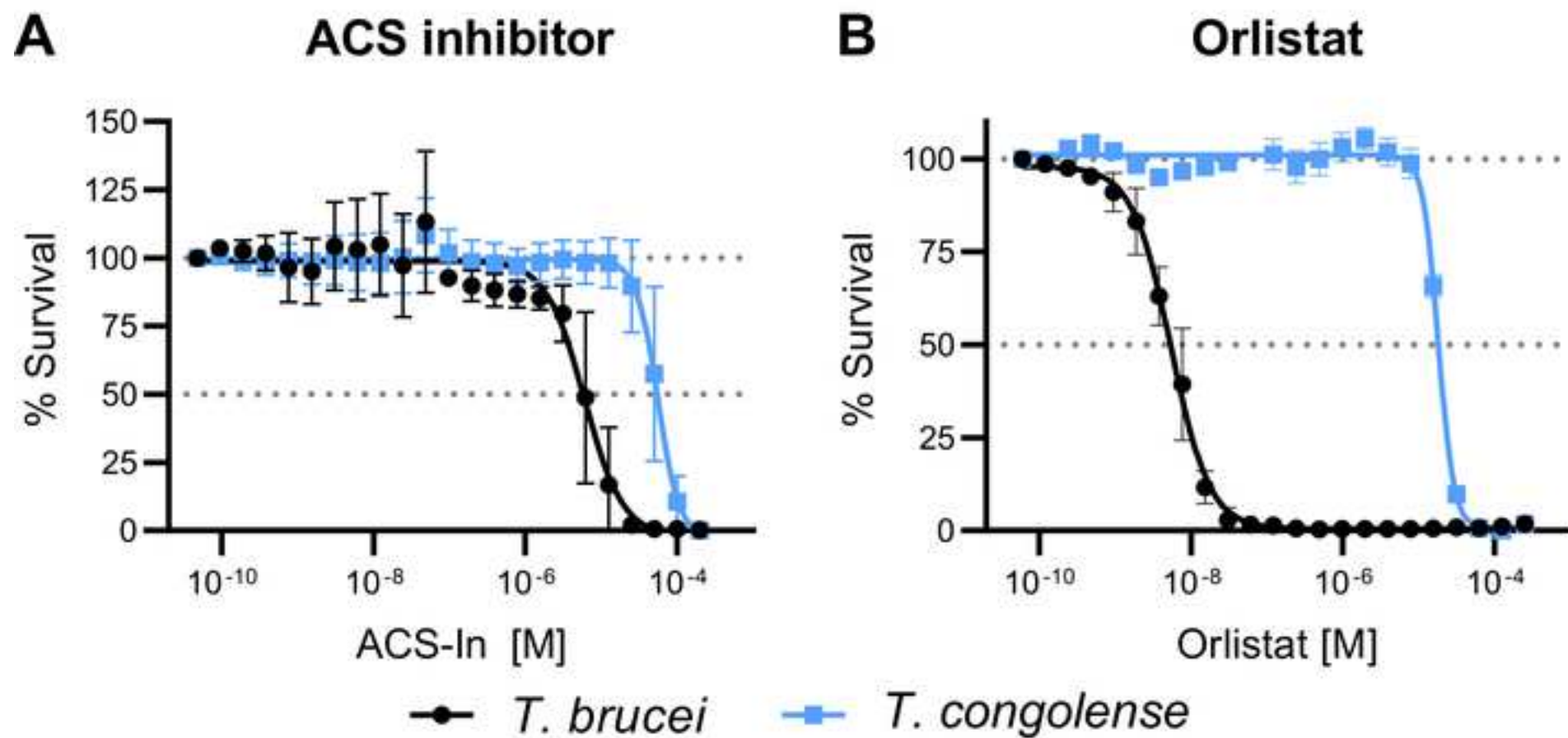1840   Negl Trop Dis. 2018;12(2): e0006301. doi: 10.1371/journal.pntd.0006301.

1841

Figure 1

Figure 2   Click here to access/download;Figure;Fig2.tiff ⬇

A

Time-point (h)

-3 -2 -1 0 1 2 3

2-oxoglutarate{*}
Imidazole-4-acetate(*)
L-1-pyrroline-3-hydroxy-5-carboxylate
cis-(homo)2aconitate
sn-glycero-3-phosphocholine(*)
Linoleate
L-glutamate 5-semialdehyde
D-glycerate
Xanthine
(S)-malate(*)
Imidazol-5-yl pyruvate
N6,N6,N6-trimethyl-L-lysine
Choline(*)
N-acetyl-D-glucosamine(*)
Choline phosphate(*)
4-hydroxy-4-methylglutamate
2-oxoglutaramate
Guanine
Succinate(*)
N6-acetyl-L-lysine
Indole-3-acetamide

L-aspartate(*)
D-glucosamine(*)
2-C-methyl-D-erythritol 4-phosphate
D-glucose(*)
2-acetolactate
D-ribose(*)
L-threonine(*)
L-tryptophan{*)
[GP (16:0)] (1)
Inosine-1
Inosine-2
[PC (16:1)] (2)
[PC (15:0)] (3)
[PC (16:0)] (4)

B

1. Guanine
2. N6-acetyl-L-lysine
3. Succinate
4. 4-hydroxy-4-methylglutamate
5. N6,N6,N6-trimethyl-L-lysine
6. Choline
7. 2-oxoglutarate
8. L-1-pyrroline-3-hydroxy-5-carboxylate
9. D-glycerate
10. Pyruvate
11. 12-hydroxydodecanoic acid
12. L-cystine
13. Diacetyl
14. PC(18:0)
15. LysoPC(17:0)
16. PC(16:0)
17. Inosine
18. PC(16:1)
19. FA trihydroxyl(18:1)
20. Inosine

Figure 4　　　　　　　　　　　　　　　　　　　　　　　　　　　　　Click here to access/download;Figure;Fig4.tiff

Figure 6

Figure 7

Click here to access/download;Figure;Fig7.tiff ±
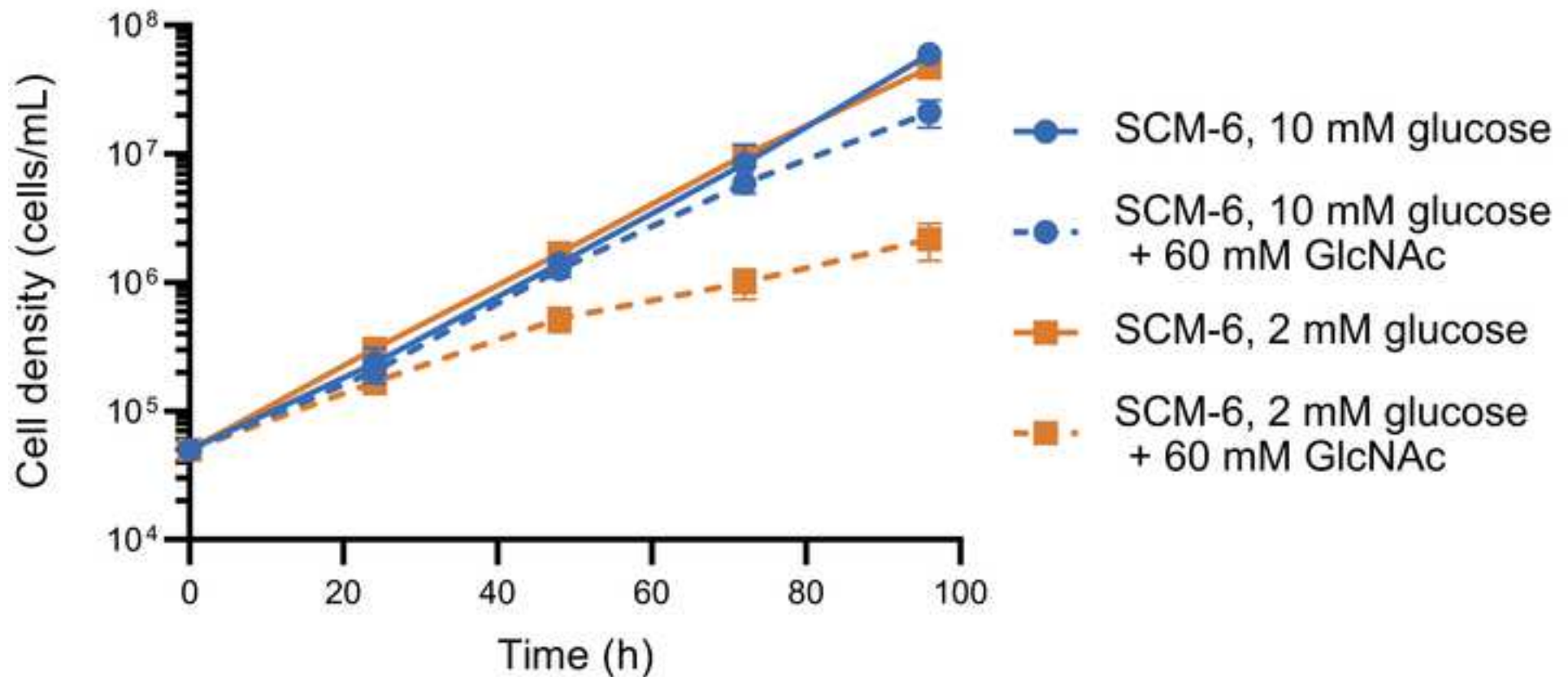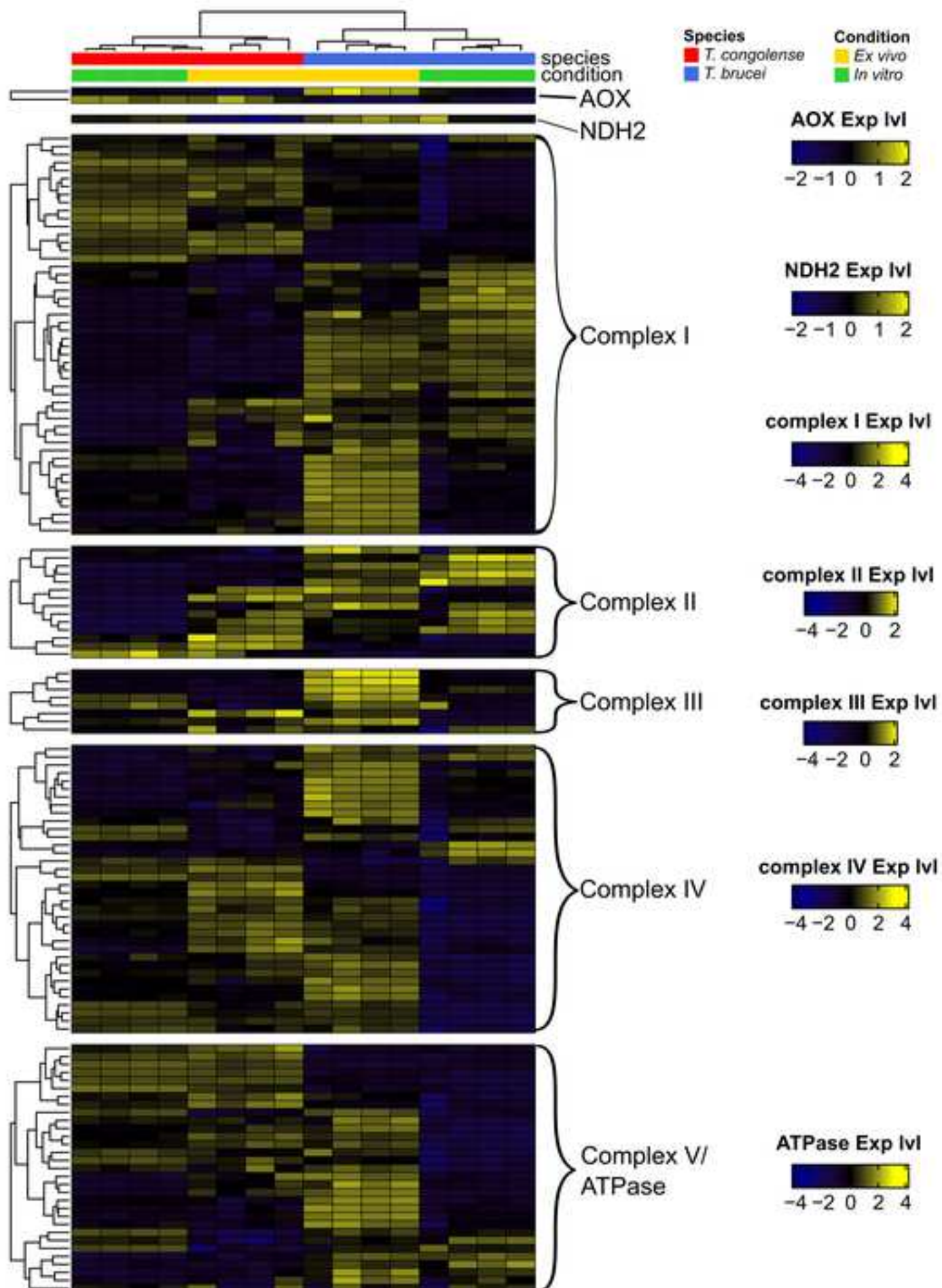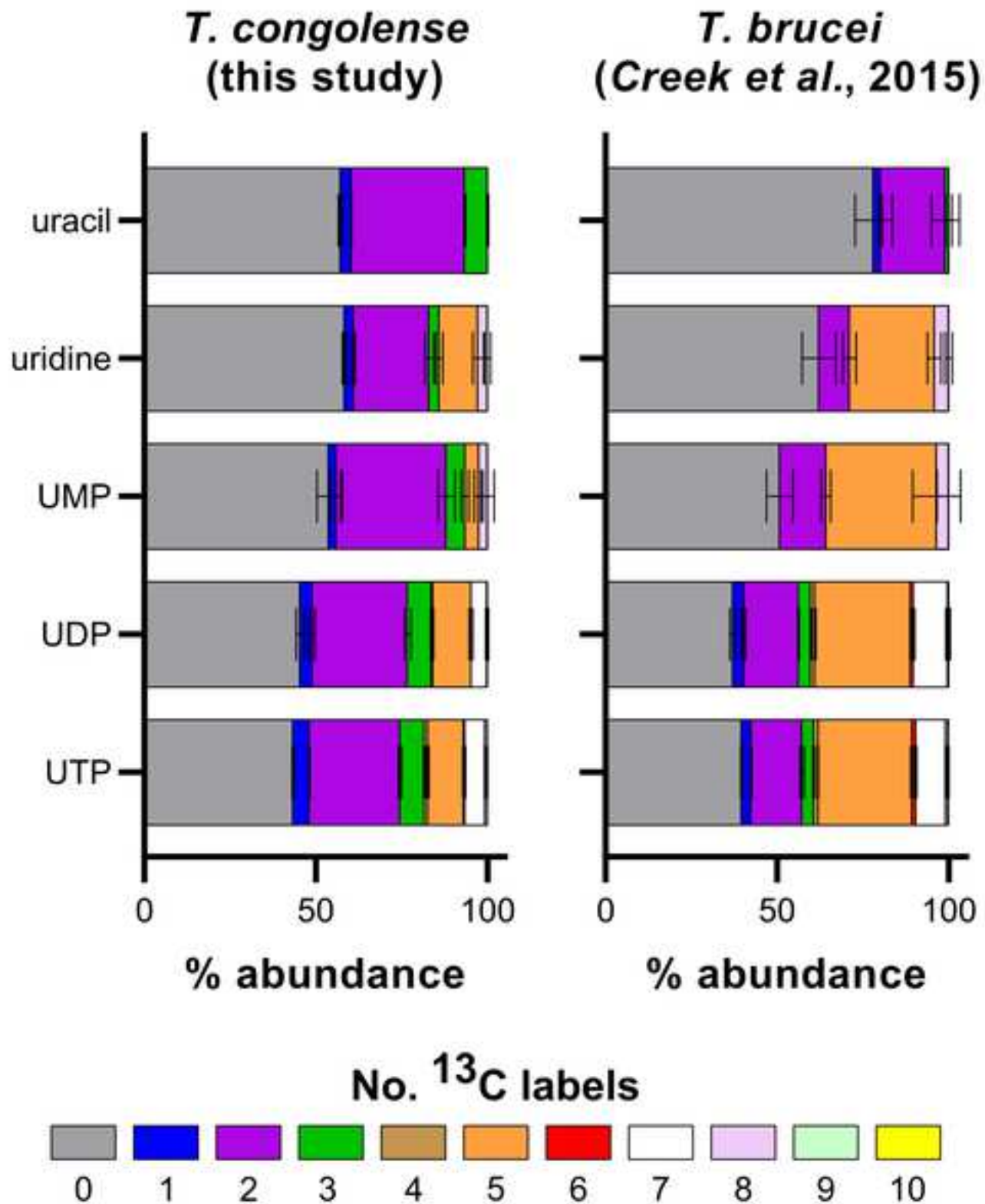
Figure 8

Figure 8

Figure 9

Figure 9

Supplementary Figure 1

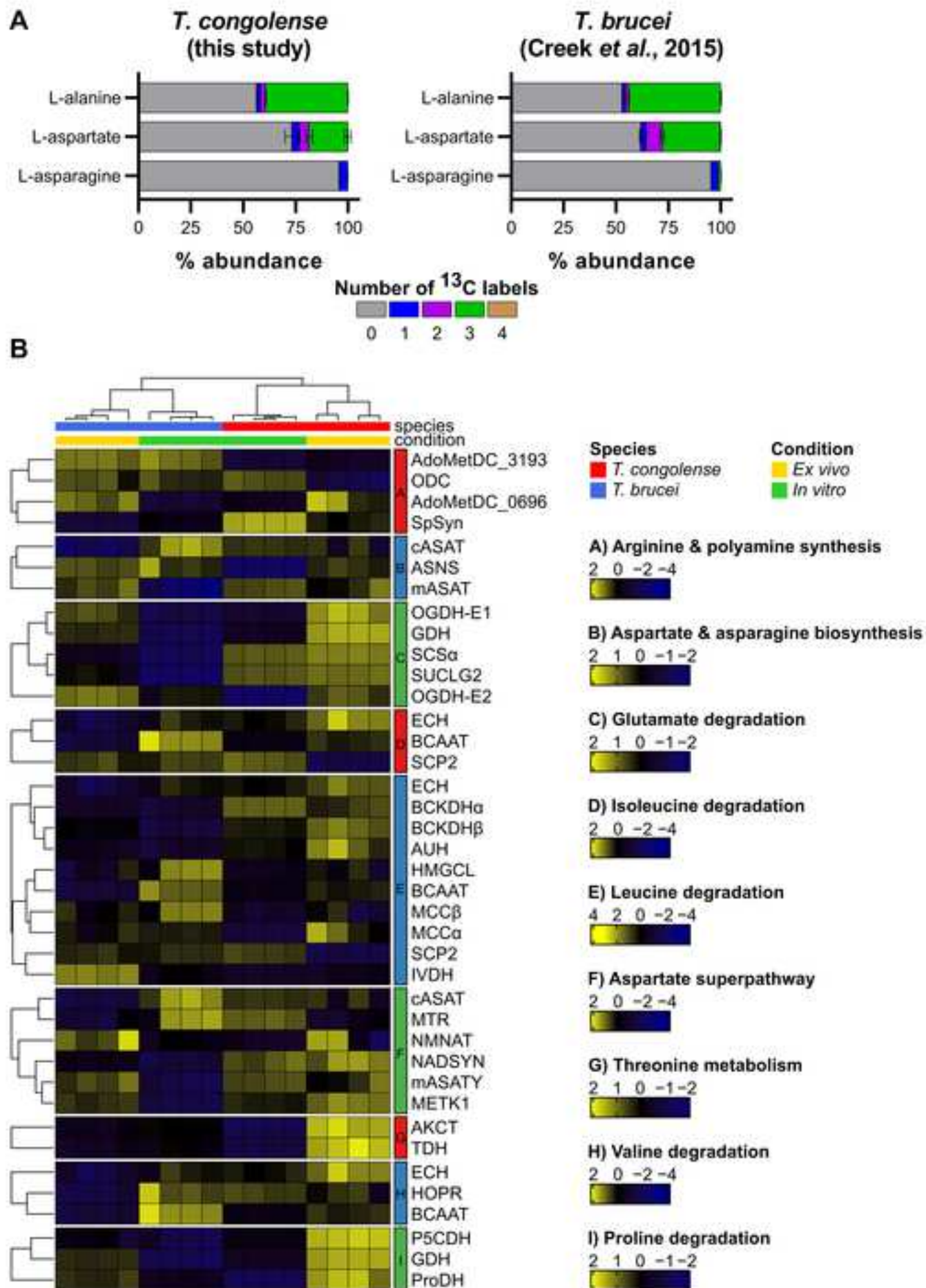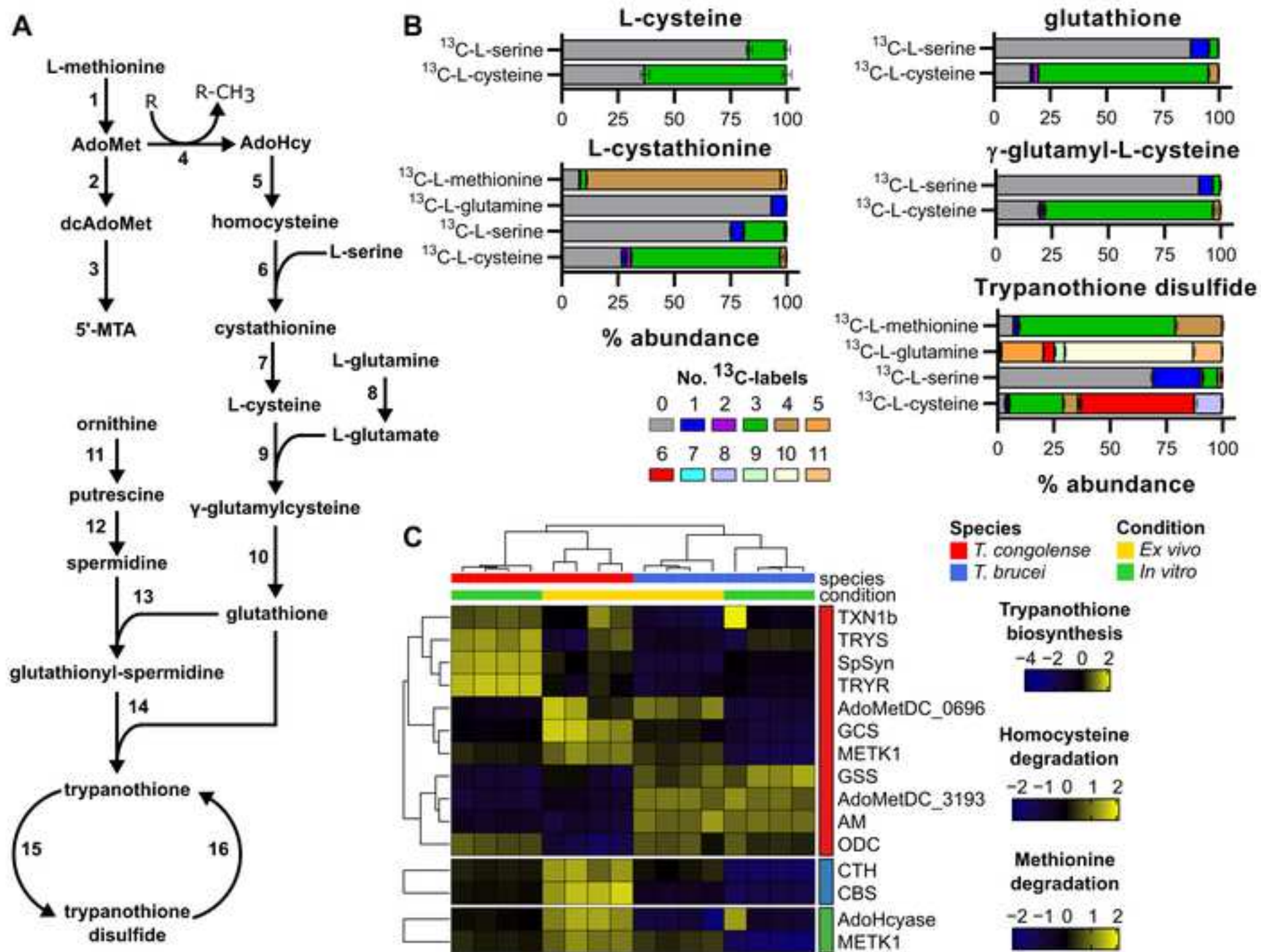*T. congolense* growth ± N-acetyl-D-glucosamine

Species
- T. congolense (red)
- T. brucei (blue)

Condition
- Ex vivo (yellow)
- In vitro (green)

species
condition
AOX
NDH2

Complex I

Complex II

Complex III

Complex IV

Complex V/ATPase

AOX Exp lvl
−2 −1 0 1 2

NDH2 Exp lvl
−2 −1 0 1 2

complex I Exp lvl
−4 −2 0 2 4

complex II Exp lvl
−4 −2 0 2

complex III Exp lvl
−4 −2 0 2

complex IV Exp lvl
−4 −2 0 2 4

ATPase Exp lvl
−4 −2 0 2 4

Supplementary Figure 4

A

T. congolense (this study)

T. brucei (Creek *et al.*, 2015)

Number of ^{13}C labels

0 1 2 3 4

B

Species
- *T. congolense*
- *T. brucei*

Condition
- *Ex vivo*
- *In vitro*

A) Arginine & polyamine synthesis

B) Aspartate & asparagine biosynthesis

C) Glutamate degradation

D) Isoleucine degradation

E) Leucine degradation

F) Aspartate superpathway

G) Threonine metabolism

H) Valine degradation

I) Proline degradation

Click here to access/download

**Supporting Information
S1_Table_RNAseq_Tcongolense_vs_Tbrucei.xlsx**

Click here to access/download
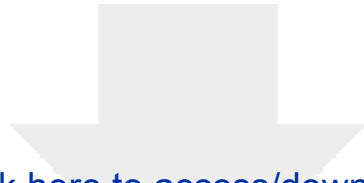**Supporting Information**
S2_Table_OrthoFinder_dataset.xlsx

Click here to access/download

Supporting Information

S3_Table_RNAseq_Tcongolense_only.xlsx
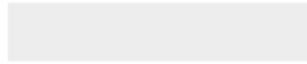
Click here to access/download

Supporting Information
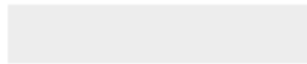
S4_Table_supernatant_metabolomics.xlsx

Click here to access/download
Supporting Information
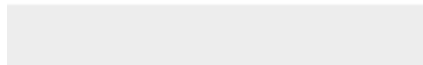S5_Table_Trypanocyc_pathways_pseudo_genome.xlsx

Click here to access/download

Supporting Information

S6_Table_SCM-6_SCM-7_formulations.xlsx

Click here to access/download

**Supporting Information
S7_Table_primers.xlsx**