

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

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18

19 **Abstract**

20 Animal African Trypanosomiasis (AAT) is a debilitating livestock disease prevalent across
21 sub-Saharan Africa, a main cause of which is the protozoan parasite *Trypanosoma*
22 *congolense*. In comparison to the well-studied *T. brucei*, there is a major paucity of
23 knowledge regarding the biology of *T. congolense*. Here, we use a combination of omics
24 technologies and novel genetic tools to characterise core metabolism in *T. congolense*
25 mammalian-infective bloodstream-form parasites, and test whether metabolic differences
26 compared to *T. brucei* impact upon drug sensitivity. Like *T. brucei*, glycolysis plays a major
27 part in *T. congolense* energy metabolism. However, the rate of glucose uptake is
28 significantly reduced in *T. congolense*, with cells remaining viable when cultured in
29 concentrations as low as 2 mM. Instead of pyruvate, the primary glycolytic endpoints are
30 succinate, malate and acetate. Comparative transcriptomics analysis showed higher levels
31 of activity associated with the mitochondrial pyruvate dehydrogenase complex, acetate
32 generation and the succinate shunt in *T. congolense*. However, based on omics analysis
33 and chemical inhibition, there does not appear to be significant levels of oxidative
34 phosphorylation. Stable-isotope labelling of glucose enabled the comparison of carbon
35 usage between *T. brucei* and *T. congolense*, highlighting differences in nucleotide and fatty
36 acid metabolism. To validate the metabolic similarities and differences, both species were
37 treated with pharmacological inhibitors, confirming a lack of essential electron transport
38 chain activity in *T. congolense*, but increased sensitivity to inhibition of mitochondrial
39 pyruvate import. Strikingly, *T. congolense* exhibited significant resistance to inhibitors of fatty
40 acid synthesis, including a 780-fold greater EC₅₀ against the lipase and fatty acid synthase
41 inhibitor Orlistat, compared to *T. brucei*. These data highlight that bloodstream form *T.*
42 *congolense* diverges from *T. brucei* in key areas of metabolism, with several features that
43 are intermediate between bloodstream- and insect-stage *T. brucei*. These results have
44 implications for drug development, mechanisms of drug resistance and host-pathogen
45 interactions.

46 **Introduction**

47 The hemoflagellate protozoan parasite *Trypanosoma congolense* is a primary causative
48 agent of animal African trypanosomiasis (AAT), which can also be caused by *T. vivax* and *T.*
49 *brucei* [1]. AAT accounts for livestock deaths in excess of 3 million annually with up to 120
50 million cattle at risk [2-4]. Thus, AAT is one of the most important livestock diseases across
51 sub-Saharan Africa.

52 Current methods of AAT control centre around chemotherapy and prophylaxis (reviewed in
53 [3]), but the very few available veterinary trypanocidal drugs have been used extensively for
54 decades, resulting in resistance and inadequate protection against AAT [5-7]. As such, there
55 is a dire need for the development of new and improved chemotherapeutics to manage AAT
56 [3, 8].

57 Most of our biological understanding of African trypanosomes derives from studies on *T.*
58 *brucei*, subspecies of which, *T. b. gambiense* and *T. b. rhodesiense*, cause Human African
59 Trypanosomiasis (HAT) [9]. The ability to culture both procyclic (PCF; tsetse fly) and
60 bloodstream (BSF; mammalian) forms of *T. brucei in vitro*, combined with its tractability with
61 respect to genetic manipulation, have enabled extensive study of this species on a molecular
62 level [10, 11]. In stark contrast, very few *T. congolense* strains are amenable to continuous
63 bloodstream form (BSF) culture, with a single strain (IL3000) used in most studies [12].
64 Whilst genetic modification is possible in *T. congolense* PCF stage, routine BSF transfection
65 has only recently become possible [13-15]. Additionally, although *T. congolense* exhibits a
66 superficially similar morphology and life cycle to *T. brucei* [16, 17] emerging evidence
67 increasingly suggests that *T. brucei*, *T. congolense* and *T. vivax* exhibit some profound
68 differences at the genomic level [18-22], including in genes and phenotypes of direct
69 relevance to infection biology and disease epidemiology. However, there is a lack of
70 understanding to what extent these genetic differences translate into biological differences,
71 including with respect to metabolism.

72 Understanding metabolism is critical to identifying how pathogens survive and thrive in the
73 varying host environments they encounter, as well as being a means of elucidating drug
74 targets, modes of drug action and mechanisms of drug resistance [23-25]. *T. brucei*
75 metabolism has been extensively studied, aided by the application of technologies such as
76 liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR)
77 spectroscopy (reviewed in detail by [26, 27]), which enable global profiling of the cellular
78 metabolome.

79 The BSF stage of *T. brucei* utilizes the high levels of glucose available in the mammalian
80 bloodstream, and depends almost exclusively on the glycolytic pathway to generate ATP
81 [28]. The first seven steps of glycolysis are encompassed by a specialized organelle, the
82 glycosome, which maintains its own ATP/ADP and NAD/NADH balance, allowing glycolysis
83 to proceed at an extraordinarily high rate in comparison to other eukaryotic cells [29]. The
84 endpoint of glycolysis, pyruvate, is a primary waste product of *T. brucei*, and excreted from
85 the cell in large quantities. As a result, only small amounts of pyruvate are further
86 metabolized in the mitochondrion to acetyl-CoA by pyruvate dehydrogenase (PDH), with

87 acetate the main excretory metabolite in this secondary, yet essential pathway [30]. The
88 acetyl-CoA generated from this pathway is utilized, at least partially, for the *de novo*
89 synthesis of fatty acids [31]. Indeed, both BSF and PCF *T. brucei* are highly sensitive to the
90 lipase and fatty acid synthase inhibitor Orlistat [32].

91 Conversely, in the absence of blood meals, glucose is scarce in the tsetse fly midgut [33],
92 and the main energy source of PCF *T. brucei* is L-proline, the catabolism of which leads to
93 production of acetate, succinate and L-alanine through a more developed and active
94 mitochondrion (including an active respiratory chain capable of generating ATP, as opposed
95 to the inactive respiratory chain in BSF *T. brucei* [34]). Until recently, it was thought that PCF
96 *T. brucei* did not exhibit active TCA metabolism, although recent data have shown that TCA
97 intermediates such as succinate and 2-oxoglutarate can stimulate PCF *T. brucei* growth. [35-
98 37].

99 Among the glycolytic enzymes, *T. brucei* expresses three isoforms of phosphoglycerate
100 kinase, which catalyze the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate
101 [38]. These are developmentally regulated, with the major isoform in BSF parasites present
102 in the glycosome (PGK-C), whilst the primary PCF isoform is found in the cytosol (PGK-B)
103 [39]. The localization of PGK-B in the PCF cytosol is thought to result in an ATP/ADP
104 imbalance in the glycosome, which is rectified by upregulating the glycosomal “succinate
105 shunt”, a pathway that includes the ATP-generating phospho*enol*pyruvate carboxykinase
106 (PEPCK)- and pyruvate phosphate dikinase (PPDK)-mediated conversion of
107 phospho*enol*pyruvate (PEP) to oxaloacetate and pyruvate respectively [39, 40]. The
108 succinate shunt, combined with amino acid metabolism, results in the excretion of high
109 levels of succinate in PCF *T. brucei* [41].

110 Stable isotope labelling data has revealed that BSF *T. brucei* utilize D-glucose to a greater
111 extent than first realized, with heavy carbons disseminating into amino acid, lipid and
112 nucleotide metabolism [42]. This study also showed that some of the succinate and malate
113 excreted from BSF parasites originates from glycolysis and, unexpectedly, inhibition of
114 PEPCK is lethal at this life-cycle stage [42]. It has also been shown that acetate production
115 is essential to BSF *T. brucei*, in particular for the synthesis of fatty acids (FAs) [30].
116 However, acetate excretion, as well as that of succinate and malate, is negligible in BSF *T.*
117 *brucei* compared to that of pyruvate and L-alanine.

118 In contrast to *T. brucei*, the literature on metabolism in *T. congolense* is scarce. More than
119 half a century ago it was suggested that BSF *T. congolense* has a significantly lower rate of
120 glucose consumption compared to BSF *T. brucei* [43]. Furthermore, pyruvate is not the main
121 glycolytic end product and instead, acetate and succinate are excreted at high levels,

122 indicative of metabolism more akin to PCF *T. brucei* [43]. Further work has revealed
123 additional differences that support this hypothesis [44-46]. For example, BSF *T. congolense*
124 primarily expresses cytosolic PGK-C, rather than glycosomal PGK-B [46]. Microscopy has
125 also revealed a more developed mitochondrion in BSF *T. congolense*, with visible cristae,
126 suggesting that mitochondrial energy metabolism could play a more prominent role in BSF *T.*
127 *congolense* [47]. The high levels of acetate excretion first shown by Agosin & Von Brand [43]
128 are consistent with this hypothesis. However, other studies have shown that BSF *T.*
129 *congolense* is sensitive to inhibitors of Trypanosome Alternative Oxidase (TAO), including
130 salicylhydroxamide (SHAM); and is insensitive to cyanide, suggesting that, as for BSF *T.*
131 *brucei*, TAO is the sole terminal oxidase, responsible for reoxidising glycerol 3-phosphate, in
132 BSF *T. congolense* [48-51]. Notably, nitroblue tetrazolium staining of BSF *T. congolense*
133 does indicate the presence of NADH dehydrogenase (complex I) activity [48]. However, to
134 date, no studies have assessed BSF *T. congolense* sensitivity to chemical inhibition of the
135 electron transport chain, or the F₁F₀-ATPase.

136 Post-genomic technologies allow for the generation of large datasets that enable analysis of
137 cellular processes on a systems scale, including metabolomics and transcriptomics.
138 Integration of these data can provide a detailed snapshot of cell metabolism at the transcript
139 and metabolite levels and help to dissect differences between species or conditions in
140 unprecedented detail [52]. Furthermore, this knowledge can aid in predication and
141 understanding of drug efficacy and mode of action

142 This study aimed to generate the first comprehensive overview of the metabolome of
143 bloodstream-form *T. congolense* IL3000 parasites, allowing a global metabolic comparison
144 of differences between *T. congolense* and *T. brucei*. Glycolytic metabolism in BSF *T.*
145 *congolense* appears to be similar to PCF *T. brucei*, particularly in terms of metabolic outputs
146 and gene expression. However, there are pronounced differences in parasite reliance on
147 exogenous amino acids as well as carbon dissemination into pathways involved in
148 nucleotide and lipid metabolism, as shown by stable isotope-labelled metabolomics. Using
149 these data, we further validated these metabolic differences in *T. congolense* by
150 pharmacological inhibition, which highlighted increased sensitivity to inhibition of
151 mitochondrial pyruvate uptake, as well as significant resistance to inhibition of fatty acid
152 synthesis, tested using inhibitors of fatty acid synthase and acetyl-coA synthetase. Taken
153 together, these results suggest that *T. congolense* and *T. brucei* differ in some fundamental
154 aspects of their core metabolism, which has important implications in terms drug sensitivity,
155 and therefore, development of novel chemotherapeutics.

156 **Results**

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

158 To permit direct comparison of BSF *T. congolense* and *T. brucei* at the transcriptome level,
159 RNAseq analysis was carried out on parasites cultured *in vitro* and trypanosome samples
160 isolated from infected mice at first peak parasitaemia (*ex vivo*) (Fig 1). Samples were
161 prepared using *T. congolense* (strain IL3000, *in vitro* and *ex vivo*) and pleomorphic *T. brucei*
162 (strain STIB 247; *in vitro* and *ex vivo*), to assess similarities and differences between
163 trypanosomes grown in culture and those from an infection (Fig 1A and 1B), and to compare
164 and contrast the transcriptome across the species (Fig 1C and 1D). Sequencing data were
165 aligned to the respective genome sequence with a mean overall alignment rate of $88.0 \pm$
166 2.3% and $94.1 \pm 0.7\%$ for *T. brucei* and *T. congolense* reads, respectively. Resultant files
167 were sorted and filtered for quality, and to minimize artefacts from multigene families, only
168 uniquely aligned reads were used for downstream analyses. Read counts were normalised
169 using transcripts per million (TPM) [53]. Orthologues were inferred between the species
170 using Orthofinder [54], in order to compare directly TPM values for 1-to-1 orthologues, as
171 well as sum-of-TPM values for groups containing families of paralogues (e.g. hexose
172 transporters). These normalised read counts are henceforth referred to as orthoTPM values
173 (S1 Table). The Orthofinder dataset (S2 Table) consisted of 6,677 orthogroups (denoted with
174 the prefix “TbTc”), of which 5,398 (80.84%) were 1-to-1 orthologues. The Orthofinder tool
175 was also used to predict genes only present in one of the two species (S2 Table). There are
176 several metabolic genes that are not present in the *T. congolense* genome, including
177 putative delta-4 and delta-6 desaturases (Tb927.10.7100 & Tb11.v5.0580), a succinate
178 dehydrogenase subunit (SDH11; Tb927.8.6890) and guanine deaminase (Tb927.5.4560,
179 Tb05.5K5.200 & Tb11.v5.0409), in addition to mitochondrial pyruvate carrier 1 (MPC1;
180 Tb927.9.3780) (S2 Table).

181 Differences between four sample groups were assessed based on orthoTPM values (Fig 1;
182 full dataset in S1 Table). There was a strong intra-species correlation between the *in vitro*
183 and *ex vivo* conditions at the transcriptome level (*T. congolense* Pearson correlation
184 coefficient, $\rho = 0.765$, Fig 1A; *T. brucei* $\rho = 0.803$, Fig 1B), showing that *in vitro*-derived BSF
185 *T. congolense* and *T. brucei* closely resemble parasites isolated from infections at the
186 transcriptome level. However, correlations between species even in the same condition were
187 lower, implying transcriptional differences between the species (*ex vivo*: $\rho = 0.651$, Fig 1C; *in*
188 *vitro*: $\rho = 0.687$, Fig 1D).

189 To compare data from this study to BSF *T. congolense* transcriptomics data generated by
190 Silvester *et al.* (generated at ascending and peak parasitaemia [55]), TPM values for each

191 annotated *T. congolense* gene were compared directly (S1 Fig, S3 Table). There was good
192 correlation between both *in vitro* and *ex vivo* *T. congolense* BSF datasets and the data from
193 Silvester *et al.* ($p > 0.8$, S1 Fig), with the highest correlation being between the *ex vivo* and
194 ascending data as expected ($p = 0.897$, S1 Fig), albeit the correlation between the
195 'ascending' and 'peak parasitaemia' in Silvester *et al.* was higher ($p = 0.979$, S1 Fig).

196 ***T. congolense* metabolite consumption and output**

197 Global metabolite (metabolomics) analysis of *in vitro* culture supernatant samples provides a
198 detailed insight into the metabolic inputs and outputs of cultured cells [56]. However, high
199 levels of medium components can often mask subtle but significant changes in culture
200 medium composition over time. To counteract this, a modified culture medium was designed
201 for *T. congolense* strain IL3000, based on previous published medium formulations (SCM-3;
202 for details see Materials and Methods) [14, 15].

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

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

211 80 of the 206 putative metabolites were significantly altered across the dataset (false
212 discovery rate-adjusted $P < 0.05$; one-way repeated measures ANOVA; Fig 2A and S4
213 Table). To analyse metabolites undergoing similar changes, K-means clustering with
214 Pearson correlation coefficient as the similarity metric was used, highlighting seven clusters
215 with two clusters of particular interest: one containing metabolites that accumulated over
216 time, and the other containing metabolites depleted over time (Fig. 2A). Log_2 fold change
217 (Log_2 FC) between the first and final time points (0 and 56 h, respectively) was also
218 calculated for each metabolite (S4 Table).

219 Glucose, the primary energy source for *T. brucei*, whilst clearly consumed, was not fully
220 depleted after 56 hours in *T. congolense* culture (Log_2 FC: -0.76; Fig 2A and 3A), in contrast
221 to *T. brucei*, where 10 mM glucose is consumed by the same time-point [56]. Ribose,
222 glucosamine, inosine and threonine were similarly depleted in *T. congolense* culture (Log_2
223 FC: -0.78, -0.97, -2.82 and -0.89, respectively).

224 In contrast, a number of metabolites accumulated in the medium (Fig 2A). The most
225 significant of these were guanine (Log₂ FC: 6.34; Fig 2A and 5A), succinate (Log₂ FC: 5.60;
226 Fig 2A & 3B) and (S)-malate (malate, Log₂ FC: 1.37; Fig 2A and 3B). Interestingly, pyruvate
227 (Log₂ FC: 0.24; Fig 3B) was not excreted at the high levels relative to starting concentration
228 consistently observed in BSF *T. brucei* culture, in both HMI-11 and in Creek's Minimal
229 medium (CMM) [56]. Succinate and malate appear to be the primary glycolytic outputs from
230 BSF *T. congolense*, similar to PCF *T. brucei*. Elevated levels of 2-oxoglutarate and a
231 metabolite putatively identified as 2-oxoglutamamate were observed, which potentially
232 originate from alanine aminotransferase activity using L-glutamate and L-glutamine,
233 respectively, as substrates [42, 57]. Moreover, a significant build-up of N6-Acetyl-L-lysine
234 (Log₂ FC: 6.30) was observed (Fig 2B). Whilst the low molecular weight of acetate means it
235 could not be detected by the LC-MS platform used here, concentrations of this molecule
236 were measured directly using an acetate assay in samples taken at the same time points
237 from four independent cultures, which confirmed high levels of acetate excretion by BSF *T.*
238 *congolense* (Fig 3F).

239 Other notable observations included the depletion of several putative
240 lysophosphatidylcholine species at 56 hours (Fig 2A; S4 Table), coincident with increased
241 medium levels of *sn*-glycero-3-phosphocholine, choline and choline phosphate, indicating
242 lyso-phospholipase activity where the charged headgroup moiety of a lyso-species is
243 cleaved from its bound fatty acid [58]. In addition, tryptophan (Log₂ FC: -0.74; Fig 6B; S4
244 Table) was significantly consumed ($P = 0.042$), in contrast with cysteine (Log₂ FC: -0.07; $P >$
245 0.05), despite the latter being essential to *T. brucei* [59] (S4 Table).

246 The Log₂ metabolite fold changes after 56 hours of culture of *T. congolense* were compared
247 to those of *T. brucei* grown in HMI-11 (Fig 2B) [56]. A total of 90 metabolites were identified
248 in both datasets, with some showing divergence between the two species (Fig 2B). Several
249 metabolites only accumulated in *T. brucei* supernatant, in particular pyruvate, D-glycerate, 2-
250 oxoglutarate and 12-hydroxydodecanoic acid (Fig 2B). Conversely, succinate, N6-acetyl-L-
251 lysine, 4-hydroxy-4-methylglutamate, N6,N6,N6-trimethyl-L-lysine and choline only
252 accumulated in *T. congolense* supernatant (Fig 2B). Whilst cystine (Fig 2B; 12) was depleted
253 in *T. brucei* samples, this metabolite remained unchanged in those from *T. congolense*.

254 In summary, whilst core elements of metabolism have been conserved between BSF *T.*
255 *congolense* and *T. brucei*, several pronounced differences in *T. congolense* metabolism
256 were identified based solely on metabolic input and output in *in vitro* culture. An integrated
257 analysis of the metabolomic and transcriptomic datasets was then undertaken in order to
258 further define the metabolic differences between the two species.

259 **Energy metabolism**

260 As described above, RNA sequencing and culture supernatant metabolomics provided initial
261 indications that *T. congolense* energy metabolism, specifically with respect to glucose
262 usage, diverges substantially from that characterized in *T. brucei* BSFs (simplified map of
263 glycolysis depicted in Fig 3G).

264 To dissect metabolic differences at the transcriptome level, pathway analysis was carried out
265 using the TrypanoCyc database [60], which contains 186 manually curated pathways
266 covering 422 genes or groups of multi-copy genes (S5 Table). These analyses showed
267 broadly similar levels of gene expression of glycolytic components between BSF *T. brucei*
268 and *T. congolense* (Fig 3G and 3I). However, the *T. brucei* *ex vivo* samples displayed a
269 more distinct expression profile, with low transcript abundances for most glycolytic
270 components compared to all sample groups. This is most likely the result of cells being
271 sampled near peak parasitaemia, and so having a higher proportion of tsetse-transmissible,
272 quiescent short stumpy forms – consistent with this there was elevated expression of stumpy
273 markers such as the PAD array (TbTc_0074), PIP39 (TbTc_0700) and reduced expression
274 of RBP10 (TbTc_0619) (S1 Table) [61-63].

275 Transcripts associated with gluconeogenesis, the succinate shunt, and the acetate
276 generation pathway were upregulated in BSF *T. congolense* under both *in vitro* and *ex vivo*
277 conditions compared to BSF *T. brucei*. Key examples of this are pyruvate phosphate
278 dikinase (PPDK), phosphoenolpyruvate carboxykinase (PEPCK), glycosomal malate
279 dehydrogenase (gMDH) and two subunits of pyruvate dehydrogenase (PDH) (Fig 3I). PPDK
280 was previously reported to be expressed in BSF *T. congolense*, but not BSF *T. brucei* [44],
281 and it may be assumed that the enzyme serves a similar function in BSF *T. congolense* as it
282 does in PCF *T. brucei*; in a mainly glycolytic role to maintain ATP/ADP balance in the
283 glycosome. The high levels of glycosomal MDH expressions in BSF *T. congolense* contrasts
284 with BSF *T. brucei*, where gMDH expression is reported to be mostly absent, and cytosolic
285 MDH (cMDH) is the major isoform [64]. The RNAseq analysis also supports a previous study
286 showing high levels of glycerol kinase expression in BSF *T. congolense* [45]. The most
287 recent PacBio assembly of the *T. congolense* genome indicates that the parasite encodes
288 five copies of PEPCK in tandem array (TcIL3000.A.H_000300300,
289 TcIL3000.A.H_000300400, TcIL3000.A.H_000300500, TcIL3000.A.H_000300600 &
290 TcIL3000.A.H_000300700; compared to one copy in *T. brucei* – Tb927.2.4210; [65]), whilst
291 there are only three copies of glycerol kinase in *T. congolense* (compared to five in *T.*
292 *brucei*).

293 To confirm that the elevated levels of succinate and malate seen in *T. congolense* spent
294 medium samples originated from glucose, LC-MS analysis using ^{13}C -U-D-glucose was
295 carried out on intracellular metabolites from cell pellet extracts. Stable isotope analysis has
296 provided valuable insights into *T. brucei* central carbon metabolism [42], and generating *T.*
297 *congolense* datasets enabled comparative analysis of glucose catabolism (albeit with an
298 unavoidable difference in medium supplementation of goat serum for *T. congolense*, rather
299 than foetal bovine serum for *T. brucei*).

300 BSF *T. congolense* was grown for 48 hours in a custom medium (Steketee's *congolense*
301 medium; SCM-6; S6 Table), containing a total D-glucose concentration of 10 mM in a 1:1
302 ratio of D-glucose: ^{13}C -U-D-glucose. Following metabolite extraction, LC-MS analysis was
303 undertaken and the majority of glycolytic intermediates were detected, including ^{13}C -labels
304 (Fig 3H). Moreover, labelling ratios of downstream metabolites were largely similar to that of
305 intracellular glucose, and the number of carbons found to be labelled in each metabolite
306 matched that which would be expected in the BSF *T. brucei* glycolytic pathway (i.e. three ^{13}C
307 atoms in all metabolites downstream of glyceraldehyde 3-phosphate and glycerol-3-
308 phosphate). Similar to *T. brucei*, a high percentage of 3-carbon labelled fructose-1,6-
309 bisphosphate (FBP) (34.8%) was observed in *T. congolense* (Fig 3H), probably a result of
310 the "reverse" aldolase reaction occurring in the glycosome [42]. Importantly, two-carbon
311 labelling was observed in several acetylated compounds (N-acetylmethionine & N-acetyl-L-
312 lysine; Fig 3H), confirming that acetyl groups used to generate these metabolites originate
313 from D-glucose. Although acetyl-CoA, the product of pyruvate oxidation, was not detected
314 for technical reasons, labelling of acetylated metabolites indicate that glucose-derived
315 pyruvate is used to generate acetyl-CoA and subsequently acetate in the mitochondrion,
316 similar to other trypanosomatids. Taken together, these data indicate that the flow of carbon
317 atoms for glycolytic components in *T. congolense* is very similar to that in *T. brucei*.
318 However, the metabolic outputs differ drastically from BSF *T. brucei* and appear to be more
319 similar to PCF *T. brucei*.

320 To determine whether the elevated succinate in supernatants originated from glucose
321 catabolism, metabolite labelling was corrected for the 1:1 (50%) ratio of natural glucose to
322 ^{13}C -U-D-glucose, which equated to a mean percentage labelling of 43.1% (the value is less
323 than 50% due to D-glucose in the serum). All glycolytic metabolites up to pyruvate showed
324 >90% labelling when corrected (for glucose 6-phosphate and fructose-1,6-bisphosphate,
325 both 3-carbon and 6-carbon labels were taken into account), although glycerol and glycerol
326 3-phosphate exhibited 57.2% and 64.4% labelling, respectively, as these metabolites can
327 also be obtained from catabolism of lipid precursors. Moreover, 40.1% (93.0% corrected)
328 labelling was detected in L-alanine, suggesting that the alanine aminotransferase reaction

329 that utilizes pyruvate to generate 2-oxoglutarate and L-alanine in both BSF and PCF *T.*
330 *brucei*, also occurs in BSF *T. congolense* [42, 66]. For both succinate and malate, 3 carbons
331 are derived from glucose and these metabolites showed 33.6% (78.1% corrected) and
332 26.0% (60.3% corrected) labelling, respectively. These results suggest that glucose is not
333 the only source of intracellular succinate and malate in *T. congolense*. However, these
334 values were higher than those reported in *T. brucei* (70% and 52% for malate and succinate,
335 respectively [42]).

336 Whilst PCF *T. brucei* exhibit citric acid (TCA) cycle activity, this pathway is not used to
337 catabolize glucose [35]. Similarly, no citric acid cycle intermediate isotopologues (e.g. citrate)
338 were found when BSF *T. congolense* were incubated with ¹³C-U-D-glucose, although small
339 amounts of 2-carbon labelled succinate and malate were observed (Fig 3H). This is similar
340 to BSF *T. brucei* [42], indicating that, like *T. brucei*, *T. congolense* does not appear to couple
341 glycolysis to TCA metabolism and instead directs high amounts of pyruvate through pyruvate
342 dehydrogenase (PDH) into acetyl-CoA and acetate. Taken together, these data suggest that
343 BSF *T. congolense* both from *in vitro* cultures and *in vivo* infection metabolically resemble an
344 intermediate between BSF and PCF *T. brucei*, with moderate glycolytic capacity and
345 significant levels of succinate shunt activity (glycosomal, rather than mitochondrial; S1
346 Table) as well as a highly active mitochondrial acetate generating pathway.

347 Previous work has shown that reduction of glucose concentrations in BSF *T. brucei* culture
348 from 10 mM to 5 mM leads to decreased cellular motility, reduction in growth and cell body
349 rounding morphology within 8 hours [67]. Given that glucose was not substantially depleted
350 in *T. congolense* cultures after 56 h, we tested the effect of reduced glucose concentrations
351 on *T. congolense* viability. Unlike *T. brucei*, *T. congolense* was able to maintain a growth
352 rate equal to controls at concentrations as low as 2 mM (Fig 4A) when continuously
353 passaged with no observable change in morphology or motility. To test whether glucose
354 uptake was essential in *T. congolense*, cells were incubated with D-glucose in addition to
355 varying concentrations of 2-deoxy-D-glucose (2DG), which can be internalised, but not
356 metabolised further than 2-deoxy-D-glucose 6-phosphate, thereby inhibiting glycolysis (Fig
357 4B). Incubation of *T. congolense* in medium supplemented with 2DG (in addition to 10 mM
358 glucose) led to growth defects in a dose dependent manner, likely due to 2DG being
359 outcompeted by glucose at lower concentrations (Fig 4B). Although the growth defect was
360 minor in the presence of 1 mM 2DG, there was a more pronounced reduction with 5 mM
361 2DG. When equimolar concentrations of glucose and 2DG were used, growth was repressed
362 and cell death occurred within 48 hours (Fig 4B). *T. congolense* viability was also tested in
363 SCM-6 in the presence of N-acetyl-D-glucosamine (GlcNAc), a sugar that inhibits glucose
364 uptake [68] (S2 Fig). In the presence of 60 mM GlcNAc with 10 mM glucose, there was a

365 moderate, yet significant ($P < 0.0001$ at 96 h, t -test of cell densities) growth defect in *T.*
366 *congolense* (S2 Fig). Viability was further reduced when the same concentration GlcNAc
367 was used alongside 2 mM glucose ($P < 0.0001$ at 96 h, t -test of cell densities), the lowest
368 concentration *T. congolense* could tolerate (S2 Fig). The rate of glucose consumption was
369 measured by assaying glucose concentrations in cell culture supplemented with 4 mM
370 glucose, and shown to be $47.17 \pm 27.91 \text{ nmol}^{-1} \text{ min}^{-1} 10^8 \text{ cells}$ in *T. congolense*, significantly
371 lower than the rate ($132.18 \pm 16.31 \text{ nmol}^{-1} \text{ min}^{-1} 10^8 \text{ cells}$) in *T. brucei* ($n = 4$, $P = 0.0039$; t -
372 test).

373 To further probe glycolytic metabolism in BSF *T. congolense*, several targets were selected
374 for RNAi-mediated knock-down, using a tetracycline-inducible *T. congolense* line expressing
375 T7 polymerase and Tet repressor under puromycin selection (TcoSM [69]). Given that the
376 majority of both malate and succinate appear to originate from glucose catabolism, the effect
377 of reducing expression levels of two proteins involved in the succinate shunt, PEPCK and
378 PPDK (TcIL3000.A.H_000922100 – both expressed at high levels in *T. congolense*; Fig 3I),
379 was tested in separate experiments (Fig 4C, D). RNAi was induced by addition of 1 $\mu\text{g}/\text{mL}$
380 tetracycline, and cell growth in culture and transcript abundance measured by qPCR were
381 monitored every 24 hours (Fig 4C, D). Creek et al showed that PEPCK is essential in BSF *T.*
382 *brucei*, even though the levels of succinate generated through this pathway are negligible
383 [42]. In BSF *T. congolense*, RNAi targeting the five copies of PEPCK reduced overall
384 *PEPCK* transcript abundance by approximately 50% (mean transcript levels of 60%, 46%
385 and 63% compared to uninduced controls at 24, 48 and 72 h post-induction, respectively;
386 Fig 4E), leading to a small but non-significant reduction in growth rate (Fig 4C ($P=0.0689$, t -
387 test at the 96 h time-point)). PPDK expression is not detected in BSF *T. brucei* but is
388 expressed in the PCF stage [44]. Knock-down of PPDK in *T. congolense* did not affect
389 parasite viability or growth rate (Fig 4D), although similar levels of transcript knockdown
390 were observed (mean transcript levels of 67%, 64% and 50% compared to uninduced
391 controls at 24, 48 and 72 h post-induction, respectively; Fig 4F).

392 RNAi was also used to knock down expression of the hexose transporter (HT) array,
393 specifically those matching the THT1 and THT2 array in *T. brucei*
394 (TcIL3000.A.H_000260500, TcIL3000.A.H_000260600, TcIL3000.A.H_000794500,
395 TcIL3000.A.H_000794600, TcIL3000.A.H_000794700.1), which has been shown to
396 significantly restrict growth of BSF *T. brucei* [70]. Whilst growth rate was unaffected in BSF
397 *T. congolense* (Fig 4G), induction of HT RNAi led to a reduction in transcript abundance at
398 all time points (mean transcript levels of 83%, 75%, 68% and 65% compared to uninduced
399 controls at 24, 48, 72 and 96 h post-induction, respectively; Fig 4H). Glucose uptake was
400 decreased (mean reduction of 37% in uptake compared to uninduced controls after 72 h; Fig

401 4l), suggesting that either lower levels of glucose are sufficient for energy generation in *T.*
402 *congolense*, or the parasite can utilize other carbon sources for ATP production. These
403 alternatives sources could include serum components such as fatty acids or amino acids,
404 both of which trypanosomatids have been reported to utilise [71, 72].

405 PCF *T. brucei* express most components of the electron transport chain (ETC) to generate
406 ATP through oxidative phosphorylation, in contrast to BSF *T. brucei*, which do not detectably
407 express any ETC components with the exception of the reversed ATPase and alternative
408 oxidase [73]. As mentioned previously, BSF *T. congolense* is thought to express a complex I
409 NADH dehydrogenase, but it is not known whether BSF *T. congolense* has capacity for
410 oxidative phosphorylation. Transcriptomics analysis of the ETC was attempted, using a gene
411 list generated by Zikova and colleagues [73], but no significant patterns could be discerned
412 (S1 Table, S3 Fig).

413 **Nucleotide metabolism**

414 Metabolomic analysis of BSF *T. congolense* culture supernatants indicated a significant
415 uptake of exogenous ribose, a contributor to nucleotide metabolism via uptake, or via the
416 pentose phosphate pathway (PPP; Fig 5A and Fig 2A). Whilst guanosine was not detected
417 in the supernatant, significant accumulation of guanine (Fig 5B) was observed, suggesting
418 either excretion of this metabolite, or, hydrolysis of guanosine through parasite-secreted
419 hydrolases/nucleosidases (previously identified in BSF *T. brucei* secretomes [74, 75]). This
420 mechanism would enable uptake of guanine and other nucleobases through nucleobase
421 transporters, for which multiple orthologues have been identified in the *T. congolense*
422 genome [18] through homology with known *T. brucei* nucleobase transporters TbNT8.1 and
423 TbNBT1 [76, 77]. In addition, there was an accumulation of xanthine, a product of
424 xanthosine hydrolysis, and depletion of inosine, an important nucleoside composed of
425 hypoxanthine and ribose (Fig 5C and 5D). The nucleoside cytidine and the nucleobase
426 hypoxanthine were also detected, but appeared to remain unchanged during the time
427 course, although the latter was a medium supplement potentially added in excess (S4
428 Table). It is noteworthy that only a single nucleoside transporter gene (TbTc_1072; *T.*
429 *congolense* gene IDs: TcIL3000.A.H_000665800 and the pseudogene
430 TcIL3000.A.H_000679300; S2 Table) can be identified in *T. congolense*, a syntenic
431 homologue of TbNT10 [18], functionally characterized as a P1-type purine nucleoside
432 transporter [78], and is thus unlikely to transport cytidine [79].

433 Purine salvage is an essential process in trypanosomatids, as they lack the *de novo*
434 synthesis pathway for the purine ring [80], and previous analysis of cell pellets to investigate
435 intracellular nucleotide metabolism utilizing ¹³C-U-D-glucose in BSF *T. brucei* showed purine

436 salvage pathways incorporating 5-carbon labelled ribose derived from glucose [42] (Fig. 5F).
437 Whilst the ribose incorporated into these nucleosides originates almost exclusively from
438 glucose in *T. brucei* (Fig 5F), *T. congolense* appears to use far less glucose-derived ribose
439 to make purine nucleosides such as adenosine, guanosine and inosine (Fig 5F).

440 Transcriptomics analyses indicated upregulation of genes associated with generation of
441 adenosine nucleotides (Fig 5G; red vertical bar), especially in *ex vivo* *T. congolense*, as well
442 as hypoxanthine-guanine phosphoribosyltransferase and uracil phosphoribosyltransferase.
443 Upregulation of nucleoside hydrolases and phosphoribosyltransferases supports previous
444 theories based upon genome content that *T. congolense* has a capacity for nucleobase
445 uptake [18].

446 The purines guanosine and inosine, which incorporate glucose-derived ribose in *T. brucei*,
447 were almost entirely unlabelled in *T. congolense* (Fig 5F). However, the phosphorylated
448 nucleosides GMP, GDP and GTP all incorporate glucose-derived carbon atoms, presumably
449 through ribose. Given the labelling patterns seen in adenosine, one possible explanation
450 could be conversion of AMP to inosine monophosphate (adenosine monophosphate
451 deaminase; TbTc_0145), IMP to xanthosine monophosphate (IMP dehydrogenase;
452 TbTc_1648) and XMP to GMP (GMP synthase; TbTc_1452). However, only one of these
453 enzymes, GMP synthase, was expressed at higher abundance in *T. congolense* (Log₂ fold
454 change: 1.56 and 2.02 for *ex vivo* and *in vitro*, respectively). Overall, incorporation of
455 glucose-derived carbons into purine nucleosides is reduced in *T. congolense* compared to *T.*
456 *brucei*. It should be noted that in both experiments, there was no ribose supplementation in
457 the media

458 Of the pyrimidines, uracil and its derivatives were detected during the glucose labelling
459 experiment (S4 Fig). Uracil is known to be the main pyrimidine salvaged by other
460 kinetoplastids including *T. brucei* [81-83]. Whilst the majority of the uridine, UMP, UDP and
461 UTP pools incorporate glucose-derived ribose (five ¹³C labels), 5-carbon isotopologues of
462 these pyrimidines were reduced in abundance in *T. congolense* compared to *T. brucei*.
463 Instead, 2-carbon labelled isotopologues appeared to comprise the majority of uridine, uracil
464 and their nucleotides (S4 Fig).

465 Whilst uracil biosynthesis is not essential in *T. brucei* [84], the uracil pool in *T. congolense*
466 appears to derive almost entirely from glucose, when corrected for 50% glucose labelling
467 (76% in *T. congolense* vs 44% in *T. brucei* [42]; S4 Fig), suggesting that this species
468 predominantly synthesizes uracil from orotate to UMP (orotate
469 phosphoribosyltransferase/orotidine 5-phosphate decarboxylase; TbTc_0735) and from
470 UMP to uracil (uracil phosphoribosyltransferase; TbTc_4220), as can occur in *T. brucei* [42].

471 Both these genes are expressed at higher abundance in *T. congolense*, both *in vitro* and *ex*
472 *vivo*, compared to *T. brucei* (Fig 5G, S1 Table), which could explain the increased
473 isotopologue labelling. Uridine nucleosides (UMP, UDP, UTP) all show a similar pattern, with
474 significant 2-carbon labelling, as well as moderate levels of 5-carbon labelling from
475 incorporation of glucose-derived ribose (S4 Fig).

476 These data indicate that, at least under the growth conditions used here, BSF *T. congolense*
477 favours purine nucleoside salvage in lieu of biosynthesis, in addition to *de novo* synthesis of
478 orotate, uracil and uridine nucleosides. However, the difference in serum requirements for
479 the two organisms is a confounding factor to the interpretation of this difference.

480 **Amino acid metabolism**

481 It is well established that trypanosomatid parasites scavenge amino acids, key nutrients for
482 survival, from their hosts [85, 86]. Therefore, comparative analyses of *T. congolense* and *T.*
483 *brucei* amino acid metabolism were undertaken. Whilst the majority of amino acids were
484 detected during the supernatant time course, relative abundances in the medium did not
485 vary greatly after 56 hours of *in vitro* culture (Fig 6A–C, S4 Table). The greatest reductions
486 were observed in threonine (Log₂ FC after 56 hours: -0.89; Fig 6A), tryptophan (Log₂ FC: -
487 0.74; Fig 6B), glutamine (Log₂ FC: -0.39), asparagine (Log₂ FC: -0.35) and phenylalanine
488 (Log₂ FC: -0.35). Interestingly, cysteine, an essential factor for the *in vitro* culture of *T.*
489 *brucei*, was not significantly consumed by 56 hours (Log₂ FC: -0.07; Fig 6C). However, at
490 least low-level exogenous cysteine is still required to sustain parasite growth *in vitro*, as
491 viability was significantly affected in the absence of cysteine (for both 1.5 mM and 1 mM vs 0
492 mM cysteine, $P < 0.0001$, *t*-test of cells densities at 96 h; S5 Fig). Experiments were carried
493 out to test the essentiality of all other individual amino acids (with the exception of glutamine,
494 known to be an important amino donor in trypanosomatid metabolism). Using the minimal
495 medium SCM-6, cell viability was monitored for 72 hours in the absence of specific amino
496 acids. Removal of the following amino acids from culture medium led to defects in growth
497 over 72 hours: asparagine, histidine, isoleucine, leucine, methionine, proline, serine, tyrosine
498 and valine (Fig 6D–G). Whilst aspartate appeared to be depleted in spent culture
499 supernatants (S4 Table), this also occurred in the medium only control. Furthermore,
500 removal of aspartate did not lead to reduced cell viability or growth rate in culture (Fig 6F).
501 Long term culture was impossible without the addition of phenylalanine and threonine,
502 leading to a final culture formulation, SCM-7 (S6 Table) containing a total of 14 amino acids.
503 Therefore, BSF *T. congolense* appears to require a higher number of amino acids than BSF
504 *T. brucei*, at least *in vitro*, with CMM containing only 8 amino acids in total, including cysteine
505 and glutamine [56]. To further probe amino acid metabolism, pathway analysis was carried
506 out on the transcriptome (S6 Fig) and metabolome (Fig 6; S6, S7 Fig).

507 BSF *T. brucei* utilizes exogenous L-glutamine as the primary source of intracellular
508 glutamate and 2-oxoglutarate and produce significant levels of glutamine-derived succinate
509 [42, 85] (Fig 6I). Given the high levels of succinate excreted by *T. congolense*, stable isotope
510 labelling was used to determine the contribution of L-glutamine to this pool. *T. congolense*
511 was incubated for 48 hours with 1 mM ¹³C-U-L-glutamine and cell pellets analysed by LC-
512 MS. Results indicated the presence of biochemical activities consistent with those observed
513 in *T. brucei*. Significant glutamine-derived carbon labelling was detected after 48 h
514 incubation for succinate (41.3%, 48.5% corrected), glutamate (76.1%, 89.2% corrected), 2-
515 oxoglutarate (80.5%, 94.3% corrected) and succinate semialdehyde (94.7% corrected; Fig
516 6I). As would be anticipated, labelling of glutathione (86.1%) and trypanothione (98.4%) from
517 glutamine through glutamate were also observed (S7 Fig). No labelling of malate or
518 aspartate was seen in this study, despite the use of high concentrations of ¹³C-U-L-
519 glutamine compared to the equivalent study performed in *T. brucei* with a 50:50 ratio of ¹³C-
520 U-L-glutamine [85].

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

532 As in *T. brucei*, glucose-derived carbon usage was detected in several amino acids in *T.*
533 *congolense* (S6A Fig). Aspartate (a precursor for pyrimidine nucleotide biosynthesis) and
534 alanine (a by-product of a pyruvate-utilising aminotransferase reaction) (S6A Fig) exhibited
535 3-carbon isotopologues derived from ¹³C-U-D-glucose. However, in *T. brucei*, a small
536 proportion of L-asparagine labelling was observed (1.2% 3-carbon labelling) [42], whilst none
537 was observed in *T. congolense* (S6A Fig). The metabolism of asparagine has not been
538 studied in African trypanosomes; given the reduction of cell growth in the absence of this
539 amino acid (Fig 6F), labelling with ¹³C-U-L-asparagine was performed, but no other labelled
540 metabolites were detected (S8 Fig). This indicates that, as with proline, protein synthesis is
541 the sole role of asparagine in *T. congolense*. The reduced expression of asparagine
542 synthetase (TbTc_4894; TcIL3000.A.H_000497800), which converts aspartate to asparagine

543 (S6 Fig), suggests that BSF *T. congolense* may rely upon scavenging of exogenous
544 asparagine.

545 Serine was also shown to be essential to *T. congolense* (Fig 6F), in contrast to minimal
546 culturing requirements for *T. brucei* [56]. ¹³C-U-L-serine labelling indicated that *T.*
547 *congolense* L-serine metabolism mirrors that of *T. brucei* in several aspects, such as *de*
548 *novo* sphingolipid biosynthesis, with 70.0% 2-carbon labelling of sphinganine and
549 downstream labelling of ceramide and sphingomyelin species (S8 Fig). Similarly,
550 phosphatidylserine decarboxylase activity was evidenced at both transcript and metabolite
551 levels, with 40.1% 2-carbon labelling of glycerol-phospho-ethanolamine (S1 Table; S8 Fig).
552 However, L-serine also has a minor role in S-adenosyl-L-homocysteine detoxification, where
553 serine-derived carbon ultimately contributes to cysteine biosynthesis. In *T. congolense*,
554 serine-derived carbon labelling can be detected in cystathionine (18.1%) and cysteine
555 (16.7%), through to glutathione (4.1%) and trypanothione disulfide (3-carbon labelled, 6.8%;
556 6-carbon labelled, 0.02%; S7 Fig). Therefore, the inability to exclude L-serine from *T.*
557 *congolense in vitro* culture media may primarily be attributable to lipid metabolism and an
558 increased demand for serine-derived cysteine, potentially over exogenously obtained
559 cysteine, depending on bioavailability. Indeed, metabolomics analysis of culture medium
560 indicates that the ability of *T. congolense* to take up cysteine from its environment may be
561 lower than in *T. brucei* (Fig 6C).

562 Although L-cysteine is primarily a source of sulphur for trypanosomatids, we also
563 investigated the carbon contribution of this amino acid in *T. congolense*, and in particular,
564 whether L-cysteine-derived carbon atoms contribute to the biosynthesis of glutathione and
565 trypanothione. ¹³C-U-L-cysteine stable isotope labelling experiments were performed (S7
566 and S8 Fig). Direct replacement of the 1.5 mM L-cysteine present in SCM with ¹³C-U-L-
567 cysteine led to high levels of labelling in glutathione and trypanothione disulfide (S7B Fig).
568 This indicates that *T. congolense* can readily take up and metabolize exogenous cysteine,
569 even though abundance of the amino acid is not reduced significantly over 56 hours of
570 parasite *in vitro* culture. Although no clear pattern could be observed in transcriptomic
571 analysis of the trypanothione biosynthesis pathway, both trypanothione synthase (TRYS;
572 TbTc_1359) and trypanothione reductase (TRYR; TbTc_4239) were expressed at high
573 levels in *in vitro T. congolense* cells relative to *ex vivo* cells, indicating that under *in vitro*
574 conditions, cells may be subjected to higher levels of oxidative stress (S7C Fig).

575 **Fatty acid metabolism in *T. congolense***

576 Lipids have a variety of crucial roles in trypanosomes, as a major constituent of membranes
577 and under certain conditions, for energy [72]. BSF *T. brucei* require large quantities of

578 myristic acid in particular, for the synthesis of glycosylphosphatidylinositol (GPI) that anchors
579 the parasite's major surface glycoprotein antigens [88]. To do this, BSF *T. brucei* both
580 synthesises and scavenges myristic acid. Glucose labelling experiments in *T. brucei* have
581 shown that myristic acid is partially synthesized from glucose-derived carbon through acetyl-
582 CoA, using a system of fatty acid elongases [89] (Fig 7A). However, no fatty acid carbon
583 labelling was detected after incubation of *T. congolense* with ^{13}C -U-D-glucose (Fig 7A).
584 Carbon dissemination was also investigated from threonine, which is used as a source of
585 acetate, and thus, lipids [90] (Fig 7B). Similarly, no saturated lipid carbon labelling was
586 observed, suggesting that *T. congolense* either uses alternative sources of carbon for lipid
587 biosynthesis, or does not rely on acetate as a source of lipids in the same way as *T. brucei*
588 [30].

589 While acetate/acetyl-CoA metabolism is highly active at the level of gene expression in *T.*
590 *congolense* compared to *T. brucei* (Fig 7C), consistent with metabolic data, expression of
591 acetyl-CoA synthetase (TbTc_0318), a key enzyme in lipid biosynthesis from acetate, is
592 reduced in both *ex vivo* and *in vitro* *T. congolense* (Fig 7C). Furthermore, an acetyl-CoA
593 thioesterase (TbTc_5515) that is involved in ATP synthesis-uncoupled acetate production in
594 PCF *T. brucei* [91] is also expressed at lower levels in *T. congolense* (Fig 7B). Other
595 enzymes involved in fatty acid biosynthesis, namely acetyl-CoA carboxylase (TbTc_0754),
596 β -ketoacyl-CoA synthase (TbTc_3372) and β -ketoacyl-CoA reductase (TbTc_1241), were all
597 expressed at lower abundance in *T. congolense* than *T. brucei*, in particular in *ex vivo* cells
598 (Fig 7C). Of the four elongases, ELO1 (TbTc_0159) and ELO2 (TbTc_1882) were expressed
599 at equal levels in BSF *T. congolense*, compared to BSF *T. brucei* (S1 Table). Whilst
600 expression of ELO3 (TbTc_0235) appeared to be reduced in *T. congolense* (Log₂ fold
601 change of -1.98 and -1.62 compared to *T. brucei* for *in vitro* and *ex vivo*, respectively; S1
602 Table), *T. congolense* cells expressed higher levels of ELO4 (TbTc_0737) in both *in vitro*
603 and *ex vivo* conditions, compared to *T. brucei* (Log₂ fold change: 1.39 and 1.38 for *in vitro*
604 and *ex vivo* comparisons, respectively)

605 The variation in observed gene expression associated with the sterol pathway appeared to
606 correlate with sample condition rather than species (Fig 7C). However, *T. congolense*
607 transcripts for genes involved in lanosterol synthesis were reduced, especially under *in vitro*
608 conditions (squalene synthase, SQase, TbTc_2577; squalene monooxygenase, SM,
609 TbTc_3357; lanosterol synthase, LSS, TbTc_4540; Fig 7C).

610 Fatty acid oxidation was recently confirmed to be an energy source for *T. brucei* residing in
611 adipose tissue [72]. Transcripts associated with this pathway were less abundant in *T.*
612 *congolense* compared to *T. brucei* under both conditions (Fig 7C), suggesting this may not

613 be an energy-generating pathway in glucose-rich culture medium, or under the *in vivo*
614 conditions from which they were sampled. However, capacity for ATP generation from fatty
615 acid oxidation should not be ruled out.

616 **Exploiting differences in metabolism for pharmacological intervention**

617 Differences in metabolism between *T. congolense* and *T. brucei* have implications for
618 differential drug efficacy between the two species. To validate our findings in key areas of
619 metabolism, pharmacological inhibition was attempted for specific targets in trypanosome
620 metabolism, in order to compare inhibitory concentrations (EC_{50}).

621 To assess whether areas of mitochondrial metabolism were more essential in BSF *T.*
622 *congolense* than in BSF *T. brucei*, both species were treated with FCCP, an uncoupling
623 agent that depolarises the mitochondrial membrane. However, there was no difference in
624 sensitivity between the species (EC_{50} : $13.0 \pm 5.0 \mu\text{M}$ and $12.6 \pm 5.3 \mu\text{M}$ for *T. brucei* and *T.*
625 *congolense*, respectively; Table 1). Given both metabolic and transcriptomic data indicated
626 no increased electron transport chain activity, we also treated with the complex III inhibitor
627 antimycin A, with again no significant differences seen between the species (Table 1). In
628 addition, there was no change in sensitivity to azide, an inhibitor of ATP hydrolysis by the F_1 -
629 ATPase (Table 1). However, *T. congolense* appeared to be more resistant to rotenone, a
630 complex I NADH dehydrogenase inhibitor (Table 1). Previous data inferred complex I activity
631 in BSF *T. congolense* based on nitroblue tetrazolium staining [48]. Rotenone resistance
632 could indicate NADH dehydrogenase activity of a rotenone-insensitive NADH
633 dehydrogenase, such as the inner membrane space-facing NDH2 [92]. *T. congolense* also
634 showed enhanced sensitivity to salicylhydroxamic acid (SHAM), an inhibitor of the
635 trypanosome alternative oxidase (TAO; Table 1). Taken together, these data indicate that,
636 like *T. brucei*, *T. congolense* does not rely on oxidative phosphorylation for ATP production,
637 as indicated by transcriptomics analysis, and that, as previously reported, TAO is the
638 terminal oxidase [48, 51].

639 Metabolomics and transcriptomics data indicated that *T. congolense* direct pyruvate towards
640 mitochondrial metabolism, with high transcript levels in PDH and enzymes involved in
641 acetate generation, compared to *T. brucei* (Fig 3 and 7). We therefore hypothesised *T.*
642 *congolense* to be more sensitive to inhibition of mitochondrial pyruvate uptake and to
643 investigate this further, we tested drug sensitivities for UK5099, an inhibitor of mitochondrial
644 pyruvate transport [93]. As expected, *T. congolense* (EC_{50} : $82.1 \mu\text{M}$) was significantly more
645 sensitive ($P = 0.0091$, unpaired *t*-test) to UK5099 compared to *T. brucei* ($130.0 \mu\text{M}$; Table 1).

646 Whilst acetate generation appears to be important in *T. congolense*, our data suggest that
647 the acetate does not appear to be utilised for the biosynthesis of fatty acids, in contrast to

648 what has been shown for *T. brucei*. To probe this further, we compared drug sensitivity of
649 the two species with compounds targeting fatty acid synthesis (Fig 8). Indeed, *T. congolense*
650 was significantly more resistant than *T. brucei* to an acetyl-CoA synthetase inhibitor (ACS
651 inhibitor; 1-(2,3-di(thiophen-2-yl)quinoxalin-6-yl)-3-(2-methoxyethyl)urea, [94]; Fig 8A; Table
652 1), indicating that acetyl-CoA synthetase is far less essential to this species. ACS is
653 essential to both BSF and PCF *T. brucei* [30, 95], thus indicating a key metabolic difference
654 between the species.

655 We next compared drug sensitivity to Orlistat, an inhibitor of fatty acid synthase and
656 phospholipase [32]. Here, a striking difference was found, with *T. congolense* exhibiting
657 significant resistance (780-fold increase in EC₅₀) to the compound compared to *T. brucei*
658 (Fig 8B; Table 1), providing further evidence that *T. congolense* primarily relies on fatty acid
659 scavenging, instead of synthesis, as predicted by the combination of metabolomics and
660 transcriptomics.

661 **Discussion**

662 The protozoan parasite *T. congolense* is a principal cause of AAT, but crucially, *T. brucei*
663 remains the dominant model for laboratory-led studies of African trypanosomes, even in the
664 face of mounting evidence that *T. brucei* and *T. congolense* differ profoundly in many facets
665 of their biology. In order to facilitate the identification and development of potential drug
666 targets for *T. congolense*, a detailed understanding of the fundamental cellular metabolism,
667 leading to an understanding of both the differences and commonalities between *T.*
668 *congolense* and *T. brucei*, would be a significant step forward.

669 Thus, this study aimed to generate a detailed comparison of metabolism in *T. congolense*
670 and *T. brucei*, through a combination of metabolomics, transcriptomics and gene knockdown
671 approaches. Transcriptomic data was generated from *T. congolense* and *T. brucei* with
672 parasite samples isolated from both *in vitro* culture and purified from *in vivo* murine infections
673 (*ex vivo*). Crucially, there were high levels of correlation between *ex vivo* and *in vitro* *T.*
674 *congolense* samples, indicating that the cultured form of the parasite closely resembles the
675 *in vivo* situation, at a transcriptomic level. In contrast, there was lower inter-species
676 correlation between *T. brucei* and *T. congolense*.

677 Our data demonstrate that BSF *T. congolense*, while possessing some metabolic similarities
678 with BSF *T. brucei* (as expected), differs substantially in several core components, including
679 in having a reduced reliance on glucose, excretion of distinct glycolytic end products
680 (acetate, malate and succinate in *T. congolense* compared to pyruvate in *T. brucei*), and
681 increased gene expression and metabolic signatures of specific mitochondrial pathways, in

682 particular pyruvate to acetate conversion. Additionally, we show increased reliance on
683 exogenous substrates such as ribose for nucleotide synthesis as demonstrated by reduced
684 glucose-derived carbon labelling in nucleoside species in addition to upregulation of
685 hydrolases and phosphoribosyltransferases. Furthermore, while there is overlap in amino
686 acid utilisation (e.g. glutamine), *T. congolense* relies on more exogenous amino acids than
687 *T. brucei*. Surprisingly, this included serine which, in the case of *T. congolense*, appears to
688 be important in the transsulfuration pathway that is geared towards trypanothione
689 biosynthesis. This may also explain the observed decreased reliance on exogenous L-
690 cysteine. Unlike *T. brucei*, *T. congolense* also requires asparagine and proline for viable *in*
691 *vitro* culture, although carbon usage from these amino acids is minimal. Finally, *T.*
692 *congolense* exhibits increased acetate/acetyl-CoA metabolism compared to *T. brucei*,
693 despite a reduction in fatty acid biosynthesis through the classical trypanosomatid pathways
694 involving acetyl-CoA synthase, acetyl-CoA carboxylase, β -ketoacyl-CoA synthase and β -
695 ketoacyl-CoA reductase, the expression of which are reduced in *T. congolense* (both in *ex*
696 *vivo* and *in vitro* conditions). This is further underlined by lack of glucose-derived 2-carbon
697 labelling of fatty acids, most notably myristic acid, a key GPI anchor component of variant
698 surface glycoproteins of *T. brucei*. However, fatty acid elongase 4, previously shown to
699 extend exogenously scavenged arachidonic acid (C22:4) to docosatetraenoic acid (C22:4)
700 [96], is upregulated under *in vitro* conditions, compared to *T. brucei*, which may indicate a
701 reliance on long-chain polyunsaturated fatty acids. These findings are shown in a summary
702 figure of *in vitro* transcriptomics data (Fig 9)

703 Analyses of culture supernatants showed that 10 mM glucose was not substantially depleted
704 after *T. congolense* cultures reached high cell density, as would be expected from an
705 equivalently dense *T. brucei* culture [56]. *T. brucei* requires at least 5 mM glucose in culture
706 [67], whereas BSF *T. congolense* were viable and maintained doubling times in levels as low
707 as 2 mM. Furthermore, confirming conclusions from one previous study on BSF *T.*
708 *congolense* [43], the primary metabolic outputs *in vitro* were (S)-malate, succinate and
709 acetate, in contrast to *T. brucei*, in which the main output is pyruvate, which is excreted in
710 large amounts [42, 56]. Interestingly, we observed a reproducible reduction in pyruvate
711 levels in *T. congolense* supernatants over time, before abundance of this metabolite
712 returned to levels similar to those observed in negative controls. A recent study in PCF *T.*
713 *brucei* demonstrated that these parasites can re-metabolize glycolytic end products such as
714 pyruvate and succinate [37]. Stable isotope labelling patterns in catabolic products derived
715 from glucose do not support cyclical TCA activity, nor re-uptake of excreted metabolites in
716 BSF *T. congolense*. However, it would be of interest to determine whether this species can
717 recycle the aforementioned metabolites.

718 *T. congolense* exhibits high levels of expression in genes involved in the glycosomal
719 succinate shunt (PEPCK, glycosomal malate dehydrogenase and fumarate hydratase; Fig
720 9). In *T. brucei* these phenotypes are associated with PCF rather than BSF; thus, to further
721 dissect glycolytic metabolism, RNAi was employed to investigate the essentiality of PPK
722 and PEPCK in *T. congolense*. In *T. brucei*, PPK is only expressed in the PCF stage, and is
723 absent in the BSF stage. In contrast, BSF *T. congolense* expresses PPK at both transcript
724 (Fig 3) and protein [44] levels, although our initial analyses suggest that the protein is not
725 essential for growth *in vitro*. PEPCK was previously found to be essential in BSF *T. brucei*
726 [42]; though in BSF *T. congolense*, PEPCK knock-down only led to a mild reduction in
727 growth rate. Previous studies in PCF *T. brucei* demonstrated that individual null mutants of
728 PEPCK and PPK showed no change in growth rate, with moderate reductions in glycolytic
729 flux [40]. However, a PEPCK/PPK null mutant did exhibit reduced growth rates, with further
730 data showing that PPK functions in a glycolytic direction and contributes to glycosomal
731 ATP/ADP balance [40]. Further work is required to establish the roles of PEPCK and PPK
732 in BSF *T. congolense*. Gene knock-out has not been previously attempted for *T. congolense*,
733 and consistent with other studies RNAi penetrance does not appear as efficient as in *T.*
734 *brucei* [69]. Techniques such as CRISPR/Cas9 and conditional knock-out would greatly
735 enhance our capabilities to study this parasite.

736 Whilst the major PGK isoform in BSF *T. brucei* is expressed in the glycosome, a previous
737 study suggested that the major isoform of phosphoglycerate kinase in BSF *T. congolense*
738 lacks the glycosomal targeting signal present in *T. brucei*, and is thus expressed in the
739 cytosol, akin to PCF *T. brucei* [46]. This has significant implications for glycosomal ADP/ATP
740 balance, as the expression of cytosolic PGK in BSF *T. brucei* is lethal [39]. Taken together,
741 these data suggest that *T. congolense* appears to carry out glycolytic metabolism in the
742 same fashion as PCF, not BSF *T. brucei*, including in *ex vivo* cells.

743 Whilst 2-deoxy-D-glucose does cause *T. congolense* death *in vitro* and supplementation of
744 cultures with GlcNAc also has a detrimental impact on viability, knock-down of the glucose
745 transporter array did not affect growth, even though glucose uptake appeared to be reduced
746 by 37% subsequent to 72 h of RNAi induction. These experiments highlight a crucial
747 difference between BSF *T. congolense* and *T. brucei* in a pathway that has become a
748 metabolic paradigm in the latter species. Whilst *T. brucei* requires high levels of glucose to
749 sustain a significant glycolytic flux, *T. congolense* remains viable in significantly lower
750 glucose concentrations, with a reduced flux, more similar to PCF *T. brucei*. However,
751 glucose remains an essential carbon source in this species, as growth is abolished in the
752 absence of glucose. Of particular interest is whether the parasite generates the majority of
753 ATP from this reduced glucose intake, or if it can thrive on other carbon sources such as

754 amino acids or even fatty acids. If the latter, this adaptation could be due to the reduced
755 bioavailability of glucose in the ruminant host bloodstream. Blood concentrations of glucose
756 in humans are approximately 5.5 mM [97]. Glucose concentrations in ruminants are typically
757 lower (2–4 mM [98-100]), and primary sources of energy are typically volatile fatty acids in
758 the form of acetic, propionic and butyric acid [101, 102]. To date, products of volatile fatty
759 acid metabolism, such as 2-methylcitrate and 2-methyl-cis-acnitase, have not been
760 reported in *T. congolense*. As such, the ability of *T. congolense* to utilise other products only
761 available in adult ruminant blood merits further investigation.

762 RNAseq analyses of *T. congolense* indicate high levels of expression of mitochondrial
763 pathways associated with glucose catabolism, specifically acetate and acetyl-CoA
764 metabolism involving pyruvate dehydrogenase, acetate:succinate CoA transferase and
765 succinyl-CoA synthetase (Fig 9). Given that the large amounts of acetate generated by the
766 parasite appear not to be required for fatty acid synthesis, these findings could suggest
767 significant reliance on mitochondrial substrate level phosphorylation for growth, similar to
768 PCF *T. brucei* cultured in glucose-rich medium [103, 104]. Interestingly, *T. congolense* does
769 not appear to encode a homologue of MPC1, and therefore likely relies on MPC2 for
770 pyruvate transport into the mitochondrion. The lack of multiple pyruvate transporters
771 combined with the importance of mitochondrial pyruvate catabolism likely explains the
772 increased sensitivity of *T. congolense* to UK5099, a mitochondrial pyruvate transport
773 inhibitor, compared to *T. brucei*.

774 Our data are consistent with the absence of oxidative phosphorylation, based on
775 transcriptomics and lack of sensitivity to chemical inhibition, compared to *T. brucei*.
776 Interestingly, a previous study reported NADH dehydrogenase (complex I) activity in *T.*
777 *congolense*, offering the possibility of ATP generation via complex V [48]. However, there
778 was no change in rotenone sensitivity in *T. congolense*, suggesting that the NADH
779 dehydrogenase activity may originate from a rotenone-insensitive NADH dehydrogenase
780 such as NDH2, known to be important for acetate production in BSF *T. brucei* [92, 105, 106].

781 Rather than oxidative phosphorylation, we propose it is likelier that considerable ATP
782 production occurs in the acetate:succinate CoA transferase – succinyl-CoA synthetase
783 (SCS) cycle, which would explain the high levels of acetate generated by *T. congolense*, in
784 addition to increased sensitivity to inhibition of mitochondrial uptake of pyruvate, the key
785 metabolic precursor. Given that 2-oxoglutarate dehydrogenase complex expression appears
786 to be less than, or equal to, that in *T. brucei* (under *in vitro* culturing conditions, Fig 9), it is
787 likely that SCS activity occurs in the acetate-generating pathway rather than in the TCA
788 cycle, which is not thought to be fully functional in BSF African trypanosomes [35], although

789 recent data have challenged this paradigm in PCF *T. brucei* [37]. The mechanisms proposed
790 here bear some similarities to the scheme proposed by Dewar and colleagues for stumpy-
791 form *T. brucei* metabolism, which also exhibit increased mitochondrial metabolism compared
792 to BSF *T. brucei* [107].

793 In *T. brucei*, carbon atoms from glucose disseminate through multiple pathways in the cell
794 [42] and, using stable isotope-labelled glucose, our data demonstrate that this pattern is also
795 seen in *T. congolense*, in particular through the glycolytic pathway, suggesting some of the
796 key metabolic differences observed are quantitative, rather than qualitative. However, there
797 were key differences in glucose-derived carbon usage. In particular, a reduction in labelling
798 was observed in purine nucleotides in *T. congolense*. In both species, carbon labelling is
799 likely due to generation of ribose phosphate sugars via the PPP and these data suggest that
800 *T. congolense* does not obtain its ribose through the pentose phosphate pathway (from
801 glucose), to the same extent that *T. brucei* does. Interestingly, *T. congolense* appears to
802 express higher levels of APRT1 (cytosolic) compared to APRT2 (glycosomal) to synthesise
803 adenosine (Fig 5). This discrepancy could underpin the reduced fraction of glucose-derived
804 purine labelling, with a reliance on ribose from alternative sources (for example,
805 exogenously).

806 Whilst the majority of pyrimidine labelling is 5-carbons in *T. brucei*, indicating labelled ribose,
807 there is decreased 5-carbon labelling and higher abundance of 2-carbon labelling in *T.*
808 *congolense*, likely through uridine generated from aspartate through orotate, again
809 highlighting a reduction in glucose-derived ribose, but conversely, an increase in glucose-
810 derived UMP and its derivatives.

811 There was also a reduced abundance of glucose-derived fatty acid labelling in *T. congolense*
812 relative to *T. brucei*. Coupled with a decreased abundance of acetyl-CoA synthetase mRNA,
813 these results suggest that *T. congolense* may scavenge exogenous lipids in favour of
814 carrying out fatty acid biosynthesis, for which it must break down extracellular lipids to their
815 constituent parts. Indeed, supernatant metabolomics showed accumulation of both choline
816 and choline phosphate, with a corresponding decrease in the LysoPC lipids, which appears
817 to indicate activity of the phospholipases which *T. congolense* is known to secrete [58, 108].
818 It is unknown whether *T. congolense* is able to generate cytosolic acetyl-CoA for fatty acid
819 biosynthesis through the action of citrate lyase, although transcript abundance of this gene
820 was reduced compared to *T. brucei*. Analysis of drug sensitivity supports these conclusions,
821 as *T. congolense* is significantly less sensitive to acetyl-CoA synthetase inhibition, as well as
822 Orlistat, and inhibitor of fatty acid synthase, suggesting that fatty acid scavenging (e.g. lipid
823 or fatty acid transporters) could be a viable therapeutic target for this species.

824 BSF *T. brucei* growth in CMM required only cysteine and glutamine when supplemented with
825 FBS gold, although a further 6 amino acids (Tyr, Phe, Trp, Leu, Met and Arg) were required
826 when supplemented with standard FBS [56]. As part of this study, 14 amino acids essential
827 for *T. congolense* growth were identified. Tryptophan and arginine, essential to *T. brucei*,
828 were not required to sustain *T. congolense* growth in 10% goat serum. Conversely, several
829 amino acids considered not essential to *T. brucei* were crucial for *T. congolense* growth *in*
830 *vitro* (Asp, His, Ile, Pro, Ser and Val). Proline is a well-established carbon source for PCF *T.*
831 *brucei* [87]. However, based on stable isotope labelling experiments, this amino acid is solely
832 used for protein synthesis in BSF *T. congolense*, as there was no evidence of carbon
833 dissemination from proline into the metabolome (likewise for asparagine). Unlike BSF *T.*
834 *congolense*, BSF *T. brucei* must be able to synthesise sufficient amounts of these amino
835 acids from alternative sources, or obtain them from the serum supplement.

836 One metabolic area of interest in trypanosomatids is trypanothione biosynthesis, a crucial
837 pathway for parasite response to oxidative stress. Indeed, trypanothione synthase, as well
838 as proteins involved in the trypanothione biosynthesis pathway, such as ornithine
839 decarboxylase (targeted by Eflornithine), have long been considered prime
840 chemotherapeutic targets due to their absence from other organisms [109]. Whilst cysteine
841 was previously known to be a main carbon contributor to trypanothione synthesis in *T. brucei*
842 along with glutamine and methionine [85], we show here that L-serine, an amino acid
843 essential to *T. congolense*, also contributes to the generation of this metabolite. Interestingly,
844 L-cysteine was not significantly depleted from *T. congolense* culture supernatants and future
845 work should ascertain whether the presence of L-serine in medium can compensate for
846 reduced L-cysteine levels in *T. congolense* culture.

847 The data presented here have led to the generation of a novel semi-defined medium for
848 culturing the strain IL3000, which must be further optimized for the culture of multiple strains
849 of *T. congolense*. Of interest is the peculiar requirement of adult bovine or goat serum for *in*
850 *vitro* culture of *T. congolense*, rather than foetal bovine serum (FBS) which is typically used
851 to culture *T. brucei* [15, 59]. Whilst this study made no attempts to adapt *T. congolense* to
852 FBS-supplemented medium (indeed, even in SCM-7, growth rate is drastically reduced in
853 the presence of FBS after 2-3 passages), this is of crucial importance, as it would allow the
854 study of multiple species of African trypanosome under the same *in vitro* conditions. Analysis
855 of metabolism presented here indicates that this phenomenon is likely to centre on the lipid
856 requirements of *T. congolense*, although it remains to be seen if this requirement is for
857 energy generation or synthesis of lipids in general. Furthermore, adult ruminant serum
858 composition drastically differs from that of non-ruminants and of foetal ruminants [101, 102],

859 and this likely has significant implications on the extracellular environment faced by livestock
860 trypanosomes.

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

867 **Materials and Methods**

868 **Compounds and reagents**

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

872 **Cell lines and *in vitro* culture**

873 In all cases, *T. congolense* strain IL3000 [48] was used (originally received from Theo Baltz,
874 University of Bordeaux). For RNAi experiments a *T. congolense* IL3000 single marker line,
875 TcoSM, was used [69]. For *in vitro* experiments, cells were grown at 34°C, 5% CO₂ and
876 routinely cultured in either TcBSF3 [14] or HMI-93 [15], in both cases without a serum plus
877 supplement, with 20% goat serum (Gibco). For global metabolite analysis of culture
878 supernatant, an experimental medium (SCM-3) was used with the following components: 77
879 mM NaCl, 1.5 mM CaCl₂, 4.5 mM KCl, 0.8 mM MgSO₄, 36 mM NaHCO₃, 25 mM HEPES,
880 0.05 mM bathocuproinedisulfonic acid, 0.22 mM 2-mercaptoethanol, 50 U/mL
881 penicillin/streptomycin, 2.5 mM glucose, 1 mM pyruvate, 10 % goat serum, 10% TcBSF3
882 [14], 1 mM each of L-cysteine and L-glutamine, and 100 µM L-tyrosine, L-phenylalanine, L-
883 tryptophan, L-leucine, L-methionine and L-arginine. BSF *T. congolense* in exponential
884 growth phase were centrifuged at 1,500 × *g* for 10 minutes, washed with PBS and inoculated
885 into this medium (0 h time point).

886 For stable isotope labelling experiments, as well as experiments involving the removal or
887 addition of specific medium components, a custom medium, Steketee's Congolense
888 Medium-6 (SCM-6) was used (S6 Table). The final medium formulation based on this study's
889 findings, SCM-7, is provided in S6 Table. This medium is essentially HMI-93, although i)
890 vitamins (with the exception of folate) were removed, ii) D-glucose concentrations were
891 modified depending on experimental procedure, but was routinely kept at 10 mM, iii) goat
892 serum levels were reduced to 10% and, iv) of the 20 amino acids, 14 were added. Increasing

893 the temperature to 37°C led to a detrimental effect on cell viability after several passages, as
894 previously reported [15].

895 For experiments involving *T. brucei*, either the monomorphic Lister 427 (*in vitro* experiments
896 and growth curves) or pleomorphic STIB 247 (RNAseq experiments, both *in vitro* and *ex vivo*
897 sample groups) strains were used. Lister 427 cells were grown in HMI-11 [110], whilst STIB
898 247 were grown in modified HMI-9 containing 1.1% methylcellulose and 20% serum plus
899 (Sigma) [111, 112]. In both cases, cells were incubated at 37°C, 5% CO₂.

900 For both species, cell counts were carried out using a haemocytometer, and in the case of *T.*
901 *congolense*, cells were mechanically detached from the culturing plasticware by pipetting
902 prior to counting. Growth curves were routinely carried out in 2 mL samples incubated in 24-
903 well plates, resuspended using a P1000. For detachment of cells from flasks, 10 mL plastic
904 pipettes were used. In cases where cells were harvested for experiments other than those
905 involving metabolomics, cells could also be detached by replacing the medium with PBS for
906 incubating at room temperature for several minutes, prior to vigorously tapping the flask to
907 detach parasites.

908 RNAi experiments using TcoSM were carried out in HMI-93 in 20 mL cultures. Cells were
909 seeded at 7×10^5 cells/mL and RNAi induction was initiated with the addition of 1 µg/mL
910 tetracycline (Sigma) and 1×10^7 cells were isolated every 24 hours for RNA analysis
911 (outlined below) before cells were passaged

912 **Ethics statement**

913 All animal experiments were performed in accordance with the Animals (Scientific
914 Procedures) Act 1986 and the University of Glasgow care and maintenance guidelines. All
915 animal protocols and procedures were approved by The Home Office of the UK government
916 and the University of Glasgow Ethics Committee.

917 **Animal experiments**

918 Adult female CD-1 mice (20–30 g body weight; Charles River Laboratories) were infected
919 with 5×10^4 wild-type *T. brucei* STIB 247 or 1×10^5 wild-type *T. congolense* IL3000 by
920 intraperitoneal injection. Parasitaemia was monitored daily by venesection of the lateral tail
921 vein [113]. At first peak of parasitaemia ($>10^7$ cells/mL) mice were euthanised and blood
922 isolated. Parasites of both species were purified from blood by anion exchange using DEAE
923 cellulose [114]. Purified cells were counted, and a total of 1×10^8 cells were centrifuged for
924 10 minutes at $1,500 \times g$ prior to RNA extraction.

925 **RNA extraction**

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

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

941 **Metabolomics sample preparation**

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

951 For analysis of intracellular metabolites, cells were grown to a final density of 2×10^6
952 cells/mL and a total of 10^8 cells isolated. Cells were quenched in 50 mL falcon tubes to 4°C
953 using a dry ice/ethanol bath (stirred and measured by thermometer) and all subsequent
954 steps were carried out at 4°C . Cells were centrifuged at $1,500 \times g$ for 10 minutes and if
955 supernatant samples were required in addition to cell pellets, 5 μ L was transferred to an
956 eppendorf containing 200 μ L extraction solvent. Cells were resuspended in residual medium
957 before transfer to eppendorf tubes. Cells were then centrifuged ($1,500 \times g$, 5 minutes) and
958 washed twice with ice-cold phosphate buffered saline (PBS) before resuspension in 200 μ L
959 extraction solvent (chloroform:methanol:water in a 1:3:1 ratio). Samples were vortexed at

960 4°C for 1 hour, and then centrifuged for 5 minutes at 13,000 × *g*. Supernatants were
961 transferred to clean eppendorf tubes. For all experiments, a quality control sample was
962 generated by pooling 10 µL from each sample and samples were stored under argon gas at
963 -80°C.

964 **Primers and plasmids**

965 RNAi experiments were carried out using a *T. congolense* single marker line, TcoSM [69]
966 that expresses Tet repressor and T7 polymerase, maintained in 0.5 µg/mL puromycin, and
967 gene specific RNAi constructs were introduced with a *T. congolense* specific plasmid, p3T7-
968 TcoV [69]. Primers carrying a HindIII (5'-AAGCTT-forward) or an FseI (5'-GGCCGGCC-
969 reverse) restriction site were used to amplify *TcoPEPCK*, *TcoPPDK* and *TcoHT* (S7 Table).
970 Gene fragments were amplified using a HiFi polymerase master mix (NEB) and cloned into
971 pGEM-T easy (Promega) and sequenced to confirm correct sequence identity of each
972 fragment. The constructs were then digested with HindIII and FseI and ligated into the p3T7-
973 TcoV vector using T4 DNA ligase (Promega). The final plasmid was linearised with NotI
974 before purification by ethanol precipitation prior to electroporation into TcoSM cells.

975 **Transfections/electroporations**

976 *T. congolense* IL3000 electroporation experiments and selection experiments were
977 performed as developed by [69]. A total of 4×10^7 cells were used per transfection, including
978 a negative (buffer only) control. A transfection buffer previously published for use with *T.*
979 *brucei* was used for *T. congolense* transfections [115]. Cells were centrifuged at 1,500 × *g*
980 for 10 minutes, pellets resuspended in residual medium and transferred to eppendorf tubes
981 for a further centrifugation step. Cells were subsequently washed in transfection buffer prior
982 to final resuspension in 100 µL buffer per transfection. Up to 12 µg linearised plasmid DNA
983 was added to an electroporation cuvette (Sigma), and 100 µL cells were subsequently
984 added. Electroporation was carried out using a Nucleofector II (Lonza) programme Z-001.
985 Transfected cells were then incubated overnight in 25 mL warm medium in the absence of
986 selective antibiotics, prior to their addition and plating out at dilutions of 1:50, 1:100 and
987 1:200 in 96-well plates. Antibiotics were added at the following concentrations: Puromycin:
988 0.5 µg/mL; Neomycin (G418): 0.4 µg/mL. Clones were retrieved after 7-10 days, and these
989 were maintained in 0.25 µg/mL puromycin and 0.2 µg/mL G418.

990 **Drug sensitivity assays**

991 Drug sensitivity assays were carried out using the alamar blue method developed by Raz
992 and colleagues [116]. Briefly, Compounds were diluted to 2x starting concentration in SCM-6
993 (with 10% goat serum for *T. congolense* IL3000 or 10% FBS for *T. brucei* Lister 427) and
994 200 µL was transferred to the first well of a solid white flat-bottomed 96-well plate. 100 µL

995 medium was then added to 23 further wells and compounds were diluted 1:2 over this series
996 of wells, with the exception of the last well, for a negative control. Subsequently, 100 μ L cells
997 were added at 2 \times starting density (4×10^4 cells/mL for *T. brucei* and 5×10^5 cells/mL for *T.*
998 *congolense*). Plates were incubated for 48 hours (37°C or 34°C for *T. brucei* and *T.*
999 *congolense*, respectively, 5% CO₂ in both cases), prior to addition of 20 μ L resazurin sodium
1000 salt (0.49 mM in 1 \times PBS, pH 7.4) to each well. Plates were then incubated for a further 24
1001 hours before measurements of cell viability.

1002 Reduction of the resazurin salt was measured as a function of cell viability. Fluorescence of
1003 each plate was read using a Cytation 5 imaging reader (BioTek) and GEN5 software.
1004 Parameters were as follows: $\lambda_{\text{excitation}} = 540$ nm and $\lambda_{\text{emission}} = 590$ nm. Raw values were
1005 plotted against concentrations (converted to Log₁₀ values) and normalised (0% defined as
1006 smallest mean in the dataset; 100% defined as largest mean in the dataset) using Graphpad
1007 Prism version 8.4.0. EC₅₀ values for each compound were calculated using a non-linear
1008 sigmoidal dose-response curve. Each assay was performed in duplicate and each EC₅₀
1009 value represents a mean of three independent experiments.

1010 **Real-time quantitative PCR (RT-qPCR)**

1011 RNA was extracted as described above, and reverse transcription was carried out in 20 μ L
1012 using 1 μ g RNA, using a high capacity cDNA kit (Applied Biosystems). Primers for RT-qPCR
1013 analysis were designed using Primer 3 [117], and primer efficiency was tested using serial
1014 dilutions of *T. congolense* IL3000 genomic DNA by plotting Ct value against Log₁₀(DNA
1015 concentration). Real-time PCR was carried out using the SensiFAST SYBR Hi-ROX kit
1016 (Bioline, BIO92005). Briefly, a 20 μ L reaction was set up using 10 μ L SYBR mix, RT
1017 template and 400 nM of each primer. Cycling conditions were: 96°C, 120 seconds, followed
1018 by 40 cycles of 95°C for 5 seconds, 62°C for 10 seconds and 72°C for 20 seconds.
1019 Previously published endogenous control primers for TcoTERT were used for within sample
1020 normalisation [118], and normalised transcript level was calculated using the delta delta Ct
1021 method [119].

1022 **Glucose uptake assays**

1023 For analysis of wild-type *T. congolense* and *T. brucei* glucose uptake, cells were seeded in
1024 10 mL cultures of SCM-6 at an initial density of 2×10^5 cells/mL (four cultures per species),
1025 with 10 mM glucose added separately at the start of the experiment. Upon the addition of
1026 glucose, 1 mL supernatant was immediately centrifuged (1,500 \times g, 10 minutes) and
1027 supernatant stored at -80°C. This process was repeated at 12, 15, 18, 21 and 24 h, and cell
1028 density measured by haemocytometer. A medium-only control (4 replicates) was also
1029 incubated alongside *in vitro* cultures. Glucose concentration of each supernatant sample

1030 was analysed using the Glucose (GO) assay kit (GAGO-20; Sigma) in a 96-well format.
1031 Briefly, 40 μ L supernatant sample (diluted if necessary) was incubated with 80 μ L assay
1032 reagent for 30 minutes at 37°C, after which 80 μ L 12 N sulphuric acid was added and
1033 absorbance measured at 540 nm using a spectrophotometer. A standard curve was also run
1034 to calculate glucose concentration. Rate of glucose consumption was calculated using a
1035 custom script [57].

1036 For glucose consumption of the TcoHT RNAi line, the Glucose Uptake-Glo kit (Promega)
1037 was used. RNAi was induced for 72 hours prior to carrying out the assay. Cells were
1038 centrifuged, washed in PBS and resuspended in assay buffer (77 mM NaCl, 1.5 mM CaCl₂-
1039 2H₂O, 4.5 mM KCl, 0.8 mM MgSO₄-7H₂O, 36 mM NaHCO₃, 25 mM HEPES and 0.02 mM
1040 bathocuproinedisulfonic acid), as it was determined *T. congolense* viability is reduced in PBS
1041 alone. Density was adjusted to 10⁸ cells/mL, and three 100 μ L replicates of each sample
1042 were added to wells of a black flat-bottomed 96-well plate. The uptake reaction was started
1043 by the addition of 50 μ L 1 mM 2-deoxy-D-glucose. Plate was shaken for 15 minutes at 34°C
1044 prior to addition of 25 μ L stop buffer, 25 μ L neutralisation buffer and 100 μ L pre-prepared
1045 2DG6P detection reagent. Plates were shaken in between addition of the buffers. Finally, the
1046 plate was read with 0.3–1 second integration on a luminometer (Cytation 5 Imaging reader,
1047 BioTek). Wild-type *T. congolense*, and *T. congolense* supplemented with glucose were used
1048 as controls, in addition to cells without 2-deoxy-D-glucose and assays in the absence of
1049 cells.

1050 **Metabolomics – Liquid chromatography mass spectrometry**

1051 Hydrophilic interaction liquid chromatography (HILIC) was carried out by Glasgow Polyomics
1052 (Glasgow, UK), using a Dionex UltiMate 3000 RSLC system (Thermo Fischer Scientific)
1053 coupled to a ZIC-pHILIC column (150 mm \times 4.6 mm, 5 μ m column, Merck Sequant). The
1054 column was maintained at 30°C and samples were eluted with a linear gradient (20 mM
1055 ammonium carbonate in water and acetonitrile) over 26 minutes with a flow rate of 0.3
1056 mL/minute.

1057 Sample injection volume was 10 μ L and samples were maintained at 4°C before injection. A
1058 Thermo Orbitrap Exactive (Thermo Fischer Scientific) was used to generate mass spectra,
1059 and was operated in polarity switching mode with the following settings: Resolution: 50,000;
1060 AGC: 106; m/z range: 70–1,400; sheath gas: 40; auxiliary gas: 5; sweep gas: 1; probe
1061 temperature: 150°C; capillary temperature: 275°C. Samples were run in both positive and
1062 negative polarity with the following ionisation: source voltage +4.5 kV, capillary voltage +50
1063 V, tube voltage +70 kV and skimmer voltage +20 V for positive mode; source voltage -3.5
1064 kV, capillary voltage -50 V, tube voltage -70 V and skimmer voltage -20 V for negative mode.

1065 Mass calibration was performed for each polarity immediately prior to each analysis batch.
1066 The calibration mass range was extended to cover small metabolites by inclusion of low-
1067 mass contaminants with the standard Thermo calmix masses (below m/z 1400), $C_2H_6NO_2$ for
1068 positive ion electrospray ionisation (PIESI) mode (m/z 76.0393) and $C_3H_5O_3$ for negative ion
1069 electrospray ionisation (NIESI) mode (m/z 89.0244). To enhance calibration stability, lock-
1070 mass correction was also applied to each analytical run using these ubiquitous low-mass
1071 contaminants. A set of authentic standards was run prior to the sample set for each
1072 experiment.

1073 **Metabolomics data analysis**

1074 RAW spectra were converted to mzXML files (mzML files for fragmentation data) using
1075 XCMS for untargeted peak detection [120]. The resultant files were further processed using
1076 mzMatch [121] for peak matching and annotation, resulting in a tabular output that was
1077 analysed using IDEOM with default settings [122]. For stable-isotope assisted metabolomics
1078 experiments, mzMatch output (in .peakml format) was analysed using mzMatch-ISO to
1079 extract all carbon isotopologue abundances from putative metabolites [123]. Data analysis of
1080 stable isotope-labelled metabolomics was based on a 48 hour time-point in all experiments.
1081 Data was further analysed using Microsoft Excel or Metaboanalyst v4.0 [124]. The mzXML
1082 files from all metabolomics analyses are available in Metabolights.

1083 **RNA sequencing and data processing**

1084 RNA sequencing was carried out by Edinburgh Genomics (Edinburgh, UK). Libraries were
1085 prepared from 8 samples (4x *T. brucei*, 4x *T. congolense*) using the TruSeq Stranded
1086 mRNA kit (Illumina) and 2 × 75 bp paired-end sequencing was carried out using a HiSeq
1087 4000 system (Illumina). Sequencing reads were aligned to the corresponding genome
1088 sequence using HiSat2 (--no-spliced-alignment) [125]. For *T. brucei*, the TREU 927
1089 reference genome sequence was used (v34.0 from TriTrypDB [126]), whilst a PacBio
1090 assembly of *T. congolense* IL3000 was used for *T. congolense* [65]. The resulting SAM files
1091 were converted to BAM files using samtools [127], and subsequently filtered for quality and
1092 primary alignment (-q 1 -F 0x100), the latter to reduce the effects of multimapping. Read
1093 counts were extracted from the filtered BAM files using HTSeq-count (-s reverse -f bam -t
1094 CDS -i ID -m union -a 0 --nonunique all).

1095 For all samples, transcripts per million (TPM) values for each gene were calculated manually
1096 using Microsoft Excel as follows: 1) Reads per kilobase (RPK) were calculated by dividing
1097 the read counts by the length of gene in kilobases; 2) All RPK values in a sample were
1098 summed and divided by 1 million as a scaling factor; 3) Each RPK value was divided by the
1099 scaling factor to yield TPM values [53]. To compare transcript abundances between the two

1100 species, Orthofinder [54] was used to infer orthologue genes or gene groups. Default
1101 parameters were used to compare the TriTrypDB v34.0 TREU 927 annotated proteins and
1102 the PacBio *T. congolense* IL3000 annotated proteins (S2 Table). A custom MATLAB
1103 (version R2019a) was used to combine the Orthofinder dataset and the TPM values for 1-to-
1104 1 orthologues, as well as “sum of TPM” values for groups containing multiple genes, where
1105 TPM value for each gene was summed, resulting in a final dataset (S1 Table). Raw RNA-
1106 seq data is deposited at GEO. Transcriptomics data were cross-referenced with the
1107 TrypanoCyc database (vm-trypanocyc.toulouse.inra.fr/; [60]) to enable pathway analysis of
1108 the data.

1109 **Computation**

1110 Figures were generated using Graphpad Prism version 8.4.0 (www.graphpad.com) with the
1111 exception of scatter plots and heatmaps, which were generated using R [128]. Heatmaps
1112 were generated using the R packages pheatmap and ComplexHeatmap [129]; scatter plots
1113 were generated using GGplot2 and GGally; and pathway maps were generated with
1114 Inkscape v1.0.

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1129 **Figure legends**

1130 **Figure 1: Overview of comparative transcriptomics analysis of *T. brucei* and *T.***
1131 ***congolense*, isolated from *ex vivo* and *in vitro* conditions.** RNAseq data from *T.*
1132 *congolense* (IL3000) and *T. brucei* (STIB247) in both *in vitro* and *ex vivo* (from mouse

1133 infections) conditions was aligned to the species' respective genome sequence and read
1134 counts were normalised by the transcripts per million (TPM) method. To directly compare the
1135 species, a pseudogenome was generated using the Orthofinder tool [54]. TPM values from
1136 the 4 sample groups were plotted against each other to analyse correlation between
1137 conditions (A and B) and between species in the same conditions (C and D). Correlation was
1138 assessed using both Spearman's rank correlation (ρ) and Pearson correlation (ρ ; Pearson's
1139 r) coefficients.

1140 **Figure 2: Analysis of supernatant metabolites after *T. congolense* culture.** A heatmap
1141 covering the 80 putative medium components judged to be significantly altered after 56
1142 hours of *in vitro* cell culture containing *T. congolense* strain IL3000, as calculated by a one-
1143 way repeated measures ANOVA ($P < 0.05$). Peak abundances were log transformed and
1144 mean centred and metabolites were clustered based on Pearson correlation. Two clusters of
1145 interest were identified, which are shown in a larger format on the right. Metabolites in the
1146 top cluster were observed to increase significantly over time, whilst those in the bottom
1147 cluster decreased. Metabolite names follow by [*] were matched to an authentic standard. B)
1148 Comparison of metabolite changes in medium supernatants after 56 hours between *T.*
1149 *brucei* [56] and *T. congolense* (S4 Table). Relative changes in metabolite abundance were
1150 calculated as Log_2 fold change of 56 h vs 0 h. Key differences are highlighted numerically: 1,
1151 guanine; 2, N6-acetyl-L-lysine; 3, succinate; 4, 4-hydroxy-4-methylglutamate; 5, N6,N6,N6-
1152 trimethyl-L-lysine; 6, choline; 7, 2-oxoglutarate; 8, L-1-pyrroline-3-hydroxy-5-carboxylate; 9,
1153 D-glycerate; 10, pyruvate; 11, 12-hydroxydodecanoic acid; 12, L-cystine; 13, diacetyl; 14,
1154 [PC (18:0)] 1-octadecanoyl-sn-glycero-3-phosphocholine; 15, LysoPC(17:0); 16, [PC (16:0)]
1155 1-hexadecanoyl-sn-glycero-3-phosphocholine; 17, inosine; 18, [PC (16:1)] 1-(9Z-
1156 hexadecenoyl)-sn-glycero-3-phosphocholine; 19, [FA trihydroxy(18:1)] 9S,12S,13S-
1157 trihydroxy-10E-octadecenoic acid; 20, inosine.

1158 **Figure 3: Energy metabolism in *T. congolense*.** A-E) Supernatant metabolomics analysis
1159 of metabolites involved in glycolytic metabolism in *T. congolense*. Grey bars indicate a
1160 negative medium control incubated for 56 hours. F) A commercial kit was used to measure
1161 acetate concentration during *T. congolense* culture, with supernatant samples analysed at
1162 the same time points as the supernatant metabolomics experiment. G) A simplified overview
1163 of the glycolytic pathway. Typically, the succinate shunt is only active in PCF *T. brucei*, with
1164 low levels of activity in BSF *T. brucei*. Numbers refer to the following proteins: 1, glucose
1165 transporters; 2, hexokinase; 3, glucose 6-phosphate isomerase; 4, phosphofructokinase; 5,
1166 aldolase; 6, triosephosphate isomerase; 7, glycerol 3-phosphate dehydrogenase; 8, glycerol
1167 kinase; 9, glyceraldehyde 3-phosphate dehydrogenase; 10, phosphoglycerate kinase; 11,
1168 phosphoglycerate mutase and enolase; 12, phosphoenolpyruvate carboxykinase; 13, malate

1169 dehydrogenase; 14, fumarate hydratase; 15, NADH-dependent fumarate reductase; 16,
1170 pyruvate kinase; 17, alanine aminotransferase; 18, pyruvate dehydrogenase complex; 19,
1171 acetate:succinate CoA-transferase and acetyl-CoA thioesterase. H) Tracing glucose derived
1172 carbon usage through glycolytic metabolism. *T. congolense* were incubated with a 50:50 mix
1173 of ¹²C-D-glucose:¹³C-U-D-glucose before cell pellets were isolated for metabolomics
1174 analysis. Results were compared to those generated in *T. brucei* by Creek and colleagues
1175 [42]. Colours indicate the number of ¹³C atoms in each metabolite. I) Comparative analysis
1176 of transcript level activity of glycolysis in *T. brucei* and *T. congolense* from both *in vitro* and
1177 *ex vivo* conditions. Gene IDs: HK1 & 2, hexokinase, TbTc_0341; GPI, glucose 6-phosphate
1178 isomerase, TbTc_1840; PFK, phosphofructokinase, TbTc_1399; ALDA, aldolase,
1179 TbTc_0358; TPI, Triosephosphate isomerase, TbTc_1075; GPDH, glycerol 3-phosphate
1180 dehydrogenase, TbTc_2722; GK, glycerol kinase, TbTc_0392; GAPDH, glyceraldehyde 3-
1181 phosphate dehydrogenase, TbTc_0377; PGK, phosphoglycerate kinase, TbTc_6030; PGKA,
1182 phosphoglycerate kinase A, TbTc_0241; PGKB/C, phosphoglycerate kinase B & C,
1183 TbTc_0240, ENO1, enolase, TbTc_0465; ENO2, enolase, putative, TbTc_3614, PK1,
1184 pyruvate kinase 1, TbTc_0372; FBPase, fructose-1,6-bisphosphatase, TbTc_1967; PEPCK,
1185 phosphoenolpyruvate carboxykinase, TbTc_0348; gMDH, glycosomal malate
1186 dehydrogenase, TbTc_0642, FH, fumarate hydratase, TbTc_0242; Frd, NADH-dependent
1187 fumarate reductase, TbTc_0141; PPDK, pyruvate phosphate dikinase, TbTc_1304; AAT,
1188 alanine aminotransferase, TbTc_0675; PDH E1 α , pyruvate dehydrogenase E1 alpha
1189 subunit, TbTc_4169; PDH E1 β , pyruvate dehydrogenase E1 beta subunit, TbTc_5437.

1190 **Figure 4: *In vitro* analysis of glycolytic metabolism.** To further probe glycolytic
1191 metabolism in *T. congolense*, novel RNAi technology was employed to knock-down key
1192 glycolytic and gluconeogenic steps. A) *T. congolense* remains viable in reduced glucose
1193 concentrations. A growth defect was only observed when glucose concentrations were
1194 reduced to <2 mM. B) Supplementation with increased concentrations of 2-deoxy-D-glucose
1195 leads to *T. congolense* cell death (red dotted line indicates detection limit by
1196 haemocytometer). C) Growth analysis of RNAi-mediated knock-down of PEPCK in *T.*
1197 *congolense* IL3000 single marker induced with 1 μ g/mL tetracycline. D) Growth analysis of
1198 RNAi-mediated knock-down of PPDK in *T. congolense* IL3000 single marker induced with 1
1199 μ g/mL tetracycline. E-F) Transcript abundance over time, following tetracycline-mediated
1200 RNAi induction of PEPCK and PPDK. G) Knock-down of the entire glucose transporter (HT)
1201 array does not affect *in vitro* cell viability. H) Normalised HT mRNA abundance over time
1202 after RNAi induction. I) Changes in glucose uptake in RNAi-induced cells were detected via
1203 an enzyme-linked luminescence assay coupled to 2-deoxy-D-glucose uptake over a period

1204 of 30 minutes. The assay was carried out 72-hours post-induction. Of the three RNAi lines, 2
1205 showed a significant reduction in glucose uptake capability ($*P < 0.05$; $***P < 0.001$)

1206 **Figure 5: Nucleotide metabolism in *T. congolense*.** Supernatant analysis of *T.*
1207 *congolense* *in vitro* cultures showing changes in abundance of D-ribose (A), guanine (B),
1208 xanthine (C) and inosine (D) over 56 hours. Grey bar indicates a negative medium control
1209 group E) Simplified overview of purine salvage and synthesis in trypanosomatids adapted
1210 from [130]. Numbers indicate the following enzymes: 1, APRT; 2, AD; 3, HGPRT; 4, IMPD;
1211 5, HGXPRT; 6, GMPR; 7, GMPS; 8, HGPRT. Red cross indicates guanine deaminase,
1212 which is not encoded/annotated in the *T. congolense* genome. F) Comparison of glucose-
1213 derived purine carbon labelling in *T. congolense* and *T. brucei* [42]. Colours indicate the
1214 number of ^{13}C atoms in each metabolite. D) Comparative RNAseq analysis of *T. congolense*
1215 and *T. brucei* under both *in vitro* and *ex vivo* conditions. Gene IDs from top to bottom: P121-
1216 PWY (adenine/adenosine salvage): IMPDH1, inosine-5'-monophosphate dehydrogenase,
1217 TbTc_1648; ADSS, adenylosuccinate synthetase, TbTc_1142; APRT-1, cytosolic adenine
1218 phosphoribosyltransferase, TbTc_3522; HGPRT, hypoxanthine-guanine
1219 phosphoribosyltransferase, TbTc_0726; GMPR, GMP reductase, TbTc_4627; HGXPRT,
1220 hypoxanthine-guanine-xanthine phosphoribosyltransferase, TbTc_3696; APRT-2,
1221 glycosomal adenine phosphoribosyltransferase, TbTc_5918; ADSL, adenylosuccinate lyase,
1222 TbTc_1986. PWY0-162 (pyrimidine biosynthesis): DHODH, dihydroorotate dehydrogenase
1223 (fumarate), TbTc_0620; PYR1A-B, glutamine hydrolysing carbomoyl phosphate synthase,
1224 TbTc_1631; PYR2, aspartate carbamoyltransferase, TbTc_1630; PYR3, dihydroorotase,
1225 TbTc_3801; CTPS, cytidine triphosphate synthase, TbTc_0920; OMPDC/OPRT, orotidine-5-
1226 monophosphate decarboxylase/orotate phosphoribosyltransferase, TbTc_0735; CMF40a,
1227 nucleoside diphosphate kinase, TbTc_5784. PWY0-163 (pyrimidine salvage): UP, uridine
1228 phosphorylase, TbTc_5794; CDA, cytidine deaminase, TbTc_3318; UPRT, uracil
1229 phosphoribosyltransferase, TbTc_4220; NDPK, nucleoside diphosphate kinase, TbTc_0593;
1230 CMF40a, nucleoside diphosphate kinase, TbTc_5784; NDPK3, nucleoside diphosphate
1231 kinase 3, TbTc_2560.

1232 **Figure 6: Amino acid metabolism in *T. congolense* IL3000.** A-C) Analysis of indicated
1233 amino acids in *T. congolense* IL3000 culture supernatants over a 56 h time course. Grey
1234 bars indicate a negative medium control group. D-F) Growth curves in SCM-6 excluding one
1235 amino acid at a time, to determine those essential to *T. congolense* viability. In each
1236 experiment, full SCM-6 was used as a positive control. Legends indicate which amino acid
1237 was removed in each experiment. G) Growth analysis of SCM-6 and SCM-7, the latter
1238 containing only amino acids deemed essential, compared to HMI-93 [110]. H) Simplified map
1239 of intracellular glutamine metabolism. Numbers refer to the following enzymes: 1,

1240 glutaminase; 2, glutamate decarboxylase; 3, 4-aminobutyrate aminotransferase; 4, succinate
1241 semialdehyde dehydrogenase; 5, glutamate dehydrogenase; 6, 2-oxoglutarate
1242 dehydrogenase; 7, Succinyl-CoA synthetase; 8, isocitrate dehydrogenase; 9 & 10,
1243 aconitase. I) Carbon utilisation from L-glutamine was analysed in *T. congolense* (100% ¹³C-
1244 U-L-glutamine) and compared to that in *T. brucei* (50:50 ratio of L-glutamine and ¹³C-U-L-
1245 glutamine) [85].

1246 **Figure 7: Fatty acid metabolism in *T. congolense*.** A) Glucose-derived ¹³C carbon
1247 labelling of saturated fatty acids in *T. congolense* and *T. brucei* [42]. Colours correspond to
1248 the number of ¹³C labels detected in each metabolite. B) L-threonine-derived saturated fatty
1249 acid ¹³C labelling in *T. congolense*. Fatty acid systematic names and numbers: lauric acid:
1250 dodecanoic acid, C12:0; myristic acid: tetradecanoic acid, C14:0; palmitic acid:
1251 hexadecanoic acid, C16:0; nonadecyclic acid: nonadecanoic acid, C19:0. C) Transcriptomics
1252 analysis of acetate and lipid metabolism. Gene names and IDs: ACH, acetyl-CoA hydrolase,
1253 TbTc_5515; ACS, acetyl-CoA synthetase, TbTc_0318; AKCT, 2-amino-3-ketobutyrate-CoA
1254 ligase, TbTc_6236; TDH, L-threonine 3-dehydrogenase, TbTc_5991; PDHe1 α , pyruvate
1255 dehydrogenase E1 α subunit, TbTc_4169; PDHe1 β , pyruvate dehydrogenase E1 β subunit;
1256 SCS α , succinyl-CoA synthetase α subunit, TbTc_0813; PPK, pyruvate phosphate dikinase,
1257 TbTc_1304; PDHe2, dihydrolipoamide acetyltransferase, TbTc_1015; PDHe3, pyruvate
1258 dehydrogenase E3, TbTc_4765; PYK1, pyruvate kinase, TbTc_0372; BKR, β -ketoacyl-ACP
1259 reductase, TbTc_1241; BKS, β -ketoacyl synthase, TbTc_3372; ACC, acetyl-CoA
1260 carboxylase, TbTc_0754; HMGCL, hydroxymethylglutaryl-CoA lyase, TbTc_6160; FPPS,
1261 farnesyl pyrophosphate synthase, TbTc_5375; LSS, lanosterol synthase, TbTc_4540; MVK,
1262 mevalonate kinase, TbTc_3761; SM, squalene monooxygenase, TbTc_3357; MDD,
1263 mevalonate diphosphate decarboxylase, TbTc_0546; SMT, sterol 24-c methyltransferase,
1264 TbTc_0387; CYP51A1, lanosterol 14 α demethylase, TbTc_4837; SQase, squalene
1265 synthase, TbTc_2577; SPPS, solanesyl-diphosphate synthase, TbTc_3025; IDI, isopentenyl-
1266 diphosphate delta-isomerase, TbTc_1099; PTase, prenyltransferase, TbTc_1352; GGTase-
1267 II β , geranylgeranyl transferase type II β subunit, TbTc_0680; SCP2, 3-ketoacyl-CoA
1268 thiolase, TbTc_4024; PMVK, phosphomevalonate kinase, TbTc_3039; HMGR, 3-hydroxy-3-
1269 methylglutaryl-CoA reductase, TbTc_3189; LACS5, fatty acyl-CoA synthetase, TbTc_0099;
1270 ACSL_0688, long-chain-fatty-acid-CoA ligase, TbTc_0688; ECHD, enoyl-CoA hydratase,
1271 TbTc_3283; ACS3/ACS4, fatty acyl-CoA synthetase 3 & 4, TbTc_0101; ACS1, fatty acyl-
1272 CoA synthetase 1, TbTc_0100; ACS2, fatty acyl-CoA synthetase 2, TbTc_0102; ECI_4184,
1273 3,2-trans-enoyl-CoA isomerase, TbTc_4184; ACSL_2381, long-chain-fatty-acid-CoA ligase,
1274 TbTc_2381; TFE α 1, enoyl-CoA hydratase/enoyl-CoA isomerase, TbTc_3362; SCP2, 3-

1275 ketoacyl-CoA thiolase, TbTc_4024; ECI_0360, 3,2-trans-enoyl-CoA isomerase, TbTc_0360;
1276 ACAD, acyl-CoA dehydrogenase, TbTc_4954.

1277 **Figure 8: Pharmacological inhibition of fatty acid synthesis in *T. brucei* and *T.***
1278 ***congolense*.** Dose-response curves to determine differential sensitivity of the two species of
1279 parasite to inhibition of an ACS inhibitor (panel A) and Orlistat (B).

1280 **Figure 9: Summary of *T. congolense* and *T. brucei* in vitro transcriptome.** Log₂ fold
1281 change (*T. congolense*/*T. brucei*) was calculated for each gene (for ratio changes, see the key
1282 on the bottom-left). Dashed lines represent transport processes. Genes: 1, hexose
1283 transporters, TbTc_0095; 2, hexokinase, TbTc_0341; 3, glucose-6-phosphate isomerase,
1284 TbTc_1840; 4, phosphofructokinase, TbTc_1399; 5, fructose-1,6-bisphosphatase,
1285 TbTc_1967; 6, aldolase, TbTc_0358; 7, triosephosphate isomerase, TbTc_1075; 8, glycerol-
1286 3-phosphate dehydrogenase, TbTc_2722; 9, glycerol kinase, TbTc_0392; 10,
1287 glyceraldehyde 3-phosphate dehydrogenase, TbTc_0377; 11, phosphoglycerate kinase,
1288 TbTc_0240; 12, phosphoglycerate mutase, TbTc_5039; 13, enolase, TbTc_0465; 14,
1289 pyruvate kinase 1, TbTc_0372; 15, alanine aminotransferase, TbTc_0675; 16, pyruvate
1290 phosphate dikinase, TbTc_1304; 17, Phosphoenolpyruvate carboxykinase, TbTc_0348; 18,
1291 glycosomal malate dehydrogenase, TbTc_0642; 19, glycosomal fumarate hydratase,
1292 TbTc_0242; 20, glycosomal NADH-dependent fumarate reductase, TbTc_0140; 21, glucose-
1293 6-phosphate dehydrogenase, TbTc_0931; 22, 6-phosphogluconolactonase, TbTc_4165; 23,
1294 6-phosphogluconate dehydrogenase, TbTc_2025; 24, ribulose-5-phosphate epimerase,
1295 TbTc_4356; 25, ribose 5-phosphate isomerase, TbTc_3090; 26, transketolase, TbTc_1701;
1296 27, transaldolase, TbTc_1823; 28, ribokinase, TbTc_5212; 29, malic enzyme, TbTc_0296;
1297 30, Mitochondrial pyruvate carrier 2, TbTc_2668; 31, FAD-dependent glycerol-3-phosphate
1298 dehydrogenase, TbTc_2282; 32, NADH dehydrogenase (NDH2), TbTc_5033; 33, Alternative
1299 oxidase, TbTc_6589; 34, mitochondrial fumarate hydratase, TbTc_0243; 35, mitochondrial
1300 NADH-dependent fumarate reductase, TbTc_0141; 36, mitochondrial malate
1301 dehydrogenase, TbTc_0256; 37, citrate synthase, TbTc_0486; 38, aconitase, TbTc_5765;
1302 39, isocitrate dehydrogenase, TbTc_0510; 40, 2-oxoglutarate dehydrogenase E1
1303 component, TbTc_2864; 41, 2-oxoglutarate dehydrogenase E1 component, TbTc_3111; 42,
1304 2-oxoglutarate dehydrogenase E2 component, TbTc_3057; 43, succinyl-CoA synthetase α ,
1305 TbTc_0813; 44, succinyl-CoA ligase β , TbTc_3392; 45, glutamine synthetase, TbTc_2226;
1306 46, glutamate dehydrogenase, TbTc_0872; 47, pyruvate dehydrogenase E1 α subunit,
1307 TbTc_4169; 48, pyruvate dehydrogenase E1 β subunit, TbTc_5437; 49, dihydrolipoamide
1308 acetyltransferase, TbTc_1015; 50, pyruvate dehydrogenase complex E3, TbTc_4765; 51, L-
1309 threonine 3-dehydrogenase, TbTc_5991; 52, 2-amino-3-ketobutyrate coenzyme A ligase,
1310 TbTc_6236; 53, Acetyl-CoA hydrolase (ACH), TbTc_5515; 54, Succinyl-CoA:3-ketoacid

1311 coenzyme A transferase (ASCT), TbTc_0236; 55, Acyl carrier protein, TbTc_5262; 56, beta-
1312 ketoacyl-ACP synthase, TbTc_3372; 57, beta-ketoacyl-ACP reductase, TbTc_1241; 58,
1313 Trans-2-enoyl-ACP reductase 1, TbTc_5269; 59, acetyl-CoA synthetase, TbTc_0318; 60,
1314 acetyl-CoA carboxylase, TbTc_0754; 61, Fatty acid elongase (ELO1), TbTc_0159; 62, Fatty
1315 acid elongase (ELO2), TbTc_1882; 63, Fatty acid elongase (ELO3), TbTc_0235; 64,
1316 elongation of very long chain fatty acids protein (ELO4), TbTc_0737; 65, aspartate
1317 aminotransferase, TbTc_0799; 66, aspartate carbamoyltransferase, TbTc_1630; 67,
1318 dihydroorotase, TbTc_3801; 68, dihydroorotate dehydrogenase, TbTc_0620; 69, orotidine-5-
1319 phosphate decarboxylase/orotate phosphoribosyltransferase, TbTc_0735; 70, uracil
1320 phosphoribosyltransferase, TbTc_4220; 71, Adenine phosphoribosyltransferase (APRT-2),
1321 TbTc_3522; 72, inosine-adenosine-guanosine-nucleoside hydrolase, TbTc_4998; 73,
1322 adenosine kinase, TbTc_1024; 74, AMP deaminase, TbTc_5808; 75, hypoxanthine-guanine
1323 phosphoribosyltransferase (HGPRT), TbTc_0726; 76, inosine-guanine nucleoside hydrolase,
1324 TbTc_0808; 77, inosine-5'-monophosphate dehydrogenase, TbTc_1648; 78, Hypoxanthine-
1325 guanine-xanthine phosphoribosyltransferase (HGXPRT), TbTc_3696; 79, GMP reductase,
1326 TbTc_4627; 80, GMP synthase, TbTc_1452. Abbreviations: PUFA, polyunsaturated fatty
1327 acid.

1328 **S1 figure: comparative analysis of published *T. congolense* RNAseq data and data**
1329 **generated in this study.** Scatter matrix of *T. congolense* datasets from this study compared
1330 to ascending and peak parasitaemia *in vivo* transcriptomics data generated by Silvester and
1331 colleagues [55]. TPM values were calculated for each gene in the *T. congolense* genome
1332 and Log₂ TPM was plotted. Lower panels: Scatter plots of individual comparisons of the 4
1333 datasets. Red dots correspond to genes associated with glycolysis; Diagonal panels: sample
1334 names; Upper panels: Pearson correlation coefficients for comparisons of entire datasets
1335 (black), glycolytic pathway (“Glyc”, green) and proteins with predicted transmembrane
1336 domains (“Trans”, red).

1337 **S2 figure: Growth of *T. congolense* IL3000 in absence or presence of N-acetyl-D-**
1338 **glucosamine.** Parasites were cultured in SCM-6 supplemented with 10 mM or 2 mM
1339 glucose in the presence or absence of 60 mM GlcNAc and density monitored by
1340 haemocytometer every 24 hours.

1341 **S3 figure: Comparative transcriptomics analysis of the electron transport chain in *T.***
1342 ***congolense* and *T. brucei*.** A heatmap of all ETC complexes based on a table generated by
1343 Zikova and colleagues [73]. Heatmaps are divided into the alternative oxidases (AOX),
1344 NADH dehydrogenase 2 (NDH2), complex I, II, III, IV and ATPase (complex V).

1345 **S4 figure: Stable isotope labelled (¹³C)-glucose derived pyrimidine labelling.**

1346 Comparative analysis of glucose-derived pyrimidine labelling in *T. congolense* and *T. brucei*
1347 (taken from [42]).

1348 **S5 figure: Effect of cysteine exclusion on *T. congolense* growth.** Parasites were grown

1349 in SCM-6 supplemented with 1.5 mM, 1.0 mM or absence of L-cysteine. Cell density was
1350 monitored every 24 hours.

1351 **S6 figure: Comparison of amino acid metabolism in *T. congolense* and *T. brucei*.** A)

1352 glucose-derived carbon labelling of amino acids B) Transcriptomics pathway analysis. Gene
1353 IDs: A) ARG+POLYAMINE-SYN: AdoMetDC_3193, AdoMet decarboxylase, TbTc_3193;
1354 ODC, ornithine decarboxylase, TbTc_5903; AdoMetDC_0696, AdoMet decarboxylase,
1355 TbTc_0696; SpSyn, spermidine synthase, TbTc_1034. B) ASPASN-PWY: cASAT, cytosolic
1356 aspartate aminotransferase, TbTc_0799; ASNS, asparagine synthetase, TbTc_4894;
1357 mASAT, mitochondrial aspartate aminotransferase, TbTc_5877. C) GLUCAT-PWY: OGDH-
1358 E1, 2-oxoglutarate dehydrogenase E1, TbTc_2864; GDH, glutamate dehydrogenase,
1359 TbTc_0872; SCS α , succinyl-CoA synthetase, TbTc_0813; SUCLG2, succinyl-CoA ligase,
1360 TbTc_3392; OGDH-E2, 2-oxoglutarate dehydrogenase E2, TbTc_3057. D) ILEUDEG-PWY:
1361 ECH, enoyl-CoA hydratase, TbTc_3283; BCAAT, branched-chain amino acid
1362 aminotransferase, TbTc_0559; SCP2, 3-ketoacyl-CoA thiolase, TbTc_4024. E) LEUDEG-
1363 PWY: ECH, enoyl-CoA hydratase, TbTc_3283; BCKDH α , 2-oxoisovalerate dehydrogenase
1364 α , TbTc_1182; BCKDH β , 2-oxoisovalerate dehydrogenase β , TbTc_0682; AUH,
1365 methylglutaconyl-CoA hydratase, TbTc_5348; HMGCL, hydroxymethylglutaryl-CoA lyase,
1366 TbTc_6160; BCAAT, branched-chain amino acid aminotransferase, TbTc_0559; MCC β , 3-
1367 methylcrotonyl-CoA carboxylase β , TbTc_5385; MCC α , 3-methylcrotonyl-CoA carboxylase
1368 α , TbTc_1670; SCP2, 3-ketoacyl-CoA thiolase, TbTc_4024; IVDH, isovaleryl-CoA
1369 dehydrogenase, TbTc_3112. F) PWY0-781: cASAT, cytosolic aspartate aminotransferase,
1370 TbTc_0799; MTR - 5-methyltetrahydropteroyltriglutamate-homocysteine S-
1371 methyltransferase, TbTc_5805; NMNAT, nicotinamide/nicotinic acid mononucleotide
1372 adenyltransferase, TbTc_4133; NADSYN, NAD⁺ synthase, TbTc_2404; mASAT,
1373 mitochondrial aspartate aminotransferase, TbTc_5877; METK1, AdoMet synthase,
1374 TbTc_0178. G) PWY1V8-11: AKCT, 2-amino-3-ketobutyrate-CoA ligase, TbTc_6236; TDH,
1375 L-threonine dehydrogenase, TbTc_5991. H) VALDEG-PWY: ECH, enoyl-CoA hydratase,
1376 TbTc_3283; HOPR, 2-hydroxy-3-oxopropionate reductase, TbTc_2903; BCAAT, branched-
1377 chain amino acid aminotransferase, TbTc_0559. I) PROLINE-DEG2-PWY: P5CDH, delta-1-
1378 pyrroline-5-carboxylate dehydrogenase, TbTc_1695; GDH, glutamate dehydrogenase,
1379 TbTc_0872; ProDH, proline dehydrogenase, TbTc_1591.

1380 **S7 figure: Carbon utilisation for trypanothione biosynthesis in *T. congolense*.**

1381 Metabolomics and transcriptomics analyses were carried out to analyse trypanothione
1382 biosynthesis. A) A simplified map of trypanothione biosynthesis as known in *T. brucei*.
1383 Numbers refer to the following enzymes: 1, S-adenosyl-L-methionine synthase, METK1; 2,
1384 S-adenosyl-L-methionine decarboxylase, AdoMetDC; 3, spermidine synthase, SpSyn; 4,
1385 methyltransferase reaction, MTase; 5, S-adenosyl-L-homocysteine dehydrolase,
1386 AdoHycase; 6, cystathionine beta synthase, CBS; 7, cystathione gamma lyase, CTH; 8,
1387 glutaminase/amidase, AM; 9, gamma-glutamylcysteine synthetase, GCS; 10, glutathione
1388 synthetase, GSS; 11, ornithine decarboxylase, ODC; 12, spermidine synthase, SpSyn; 13,
1389 glutathionylspermidine synthase, GSP; 14, trypanothione synthetase, TRYS; 15,
1390 tryparedoxin peroxidase, TXN1b; 16, trypanothione reductase, TRYR. B) Isotopologue
1391 labelling experiments using 100% ¹³C-L-serine, ¹³C-L-glutamine, ¹³C-L-methionine or ¹³C-L-
1392 cysteine, showing the abundance of carbon labelling derived from these amino acids in
1393 components of the trypanothione biosynthesis pathway. C) Transcriptomics analysis using
1394 the following TrypanoCyc pathways: PWY1V8-6 (trypanothione biosynthesis),
1395 HOMOCYSDESGR-PWY1 (homocysteine degradation/cysteine biosynthesis) &
1396 METHIONINE-DEG1-PWY (methionine degradation I). GeneIDs: TNX1b, tryparedoxin 1b,
1397 TbTc_0324; TRYS, trypanothione synthetase, TbTc_1359; SpSyn, Spermidine synthase,
1398 TbTc_1034; TRYR, trypanothione reductase, TbTc_4239; AdoMetDC_0696, S-
1399 adenosylmethionine decarboxylase, TbTc0696; GCS, gamma-glutamylcysteine synthetase,
1400 TbTc_3424; METK1, S-adenosylmethionine synthetase, TbTc_0178; GSS, glutathione
1401 synthetase, TbTc_3678; AdoMetDC_3193, S-adenosylmethionine decarboxylase,
1402 TbTc_3193; AM, amidase, TbTc_5549; ODC, ornithine decarboxylase, TbTc_5903; CTH,
1403 cystathione gamma lyase, TbTc_1051; CBS, cystathionine beta synthase, TbTc_0413;
1404 AdoHycase, S-adenosylhomocysteine hydrolase, TbTc_0685; METK1, S-
1405 adenosylmethionine synthase, TbTc_0178.

1406 **S8 Figure: Analysis of LC-MS utilising stable isotope labelled amino acids.** Percentage
1407 total labelling of metabolites identified in data from 6 stable isotope labelling experiments
1408 using ¹³C-L-asparagine, ¹³C-L-cysteine, ¹³C-L-glutamine, ¹³C-L-methionine, ¹³C-L-proline and
1409 ¹³C-L-serine. Colour intensity correlates to the total fraction of the metabolite that was ¹³C-
1410 labeled.

1411 **Supplementary Data**

1412 **S1 Table:** RNAseq dataset – *T. congolense ex vivo*, *T. congolense in vitro*, *T. brucei ex vivo*,
1413 *T. brucei in vitro*, Silvester *et al* dataset

1414 **S2 Table:** Orthofinder output comparing *T. congolense* TriTrypDB (v34.0), *T. congolense*
1415 Liverpool pacbio, *T. brucei* TriTrypDB (v34.0) and other trypanosomatids

1416 **S3 Table:** RNAseq dataset – *T. congolense* only, Pacbio assembly, single genes.

1417 **S4 Table:** Supernatant metabolomics dataset for *in vitro* cultured *T. congolense* over a
1418 period of 56 hours. Metabolites highlighted in yellow were confidently predicted using a set
1419 of metabolite standards run alongside the experimental samples. Results of statistical
1420 analysis by means of a one-way repeated measures ANOVA (false discovery rate-adjusted
1421 P value, FDR) is also shown for metabolites that were taken forward for downstream
1422 analysis

1423 **S5 Table:** TrypanoCyc pathways and linked Orthogroup gene IDs

1424 **S6 Table:** Formulation of Steketee's congolense medium (SCM)-6 & -7

1425 **S7 table:** List of primers used in this study

1426

Compound	Target	<i>T. congolense</i> EC ₅₀ Mean ± SEM	<i>T. brucei</i> 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

1427 **Table 1: Comparative analysis of sensitivity to metabolic inhibitors in *T. congolense***
 1428 **and *T. brucei*.** Abbreviations: SHAM, salicylhydroxamic acid; LCFA, long-chain fatty acid

1429

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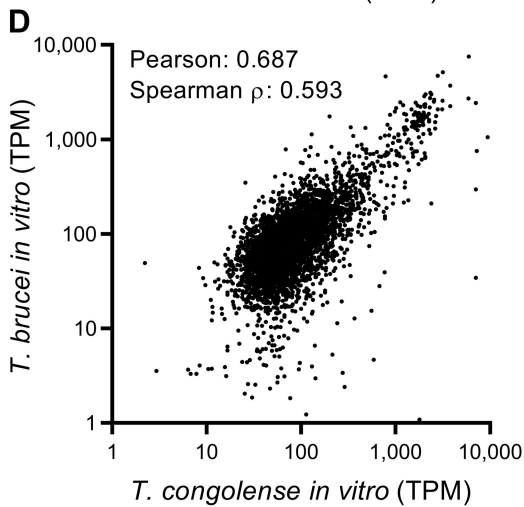
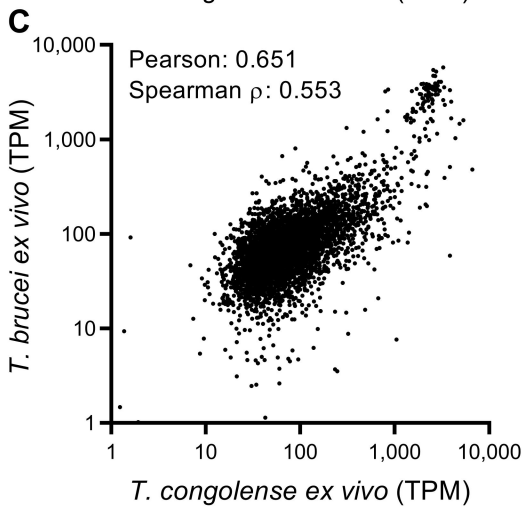
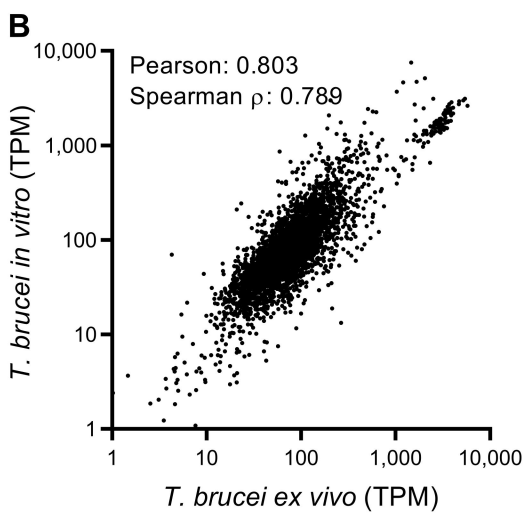
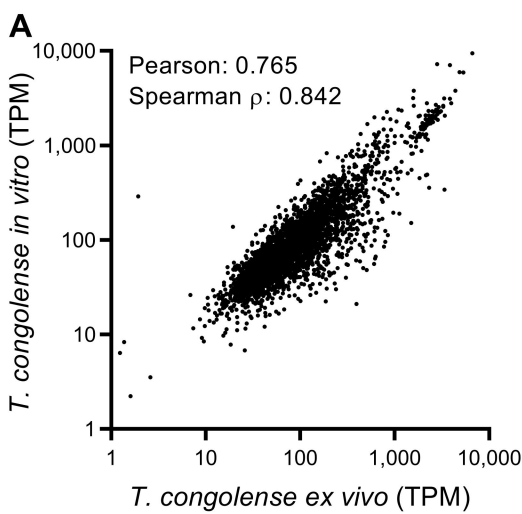
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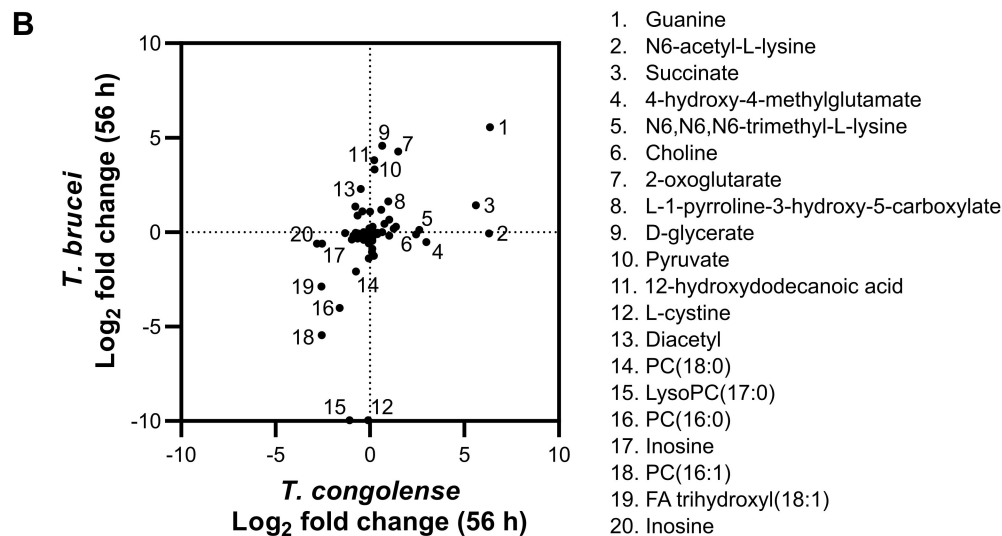
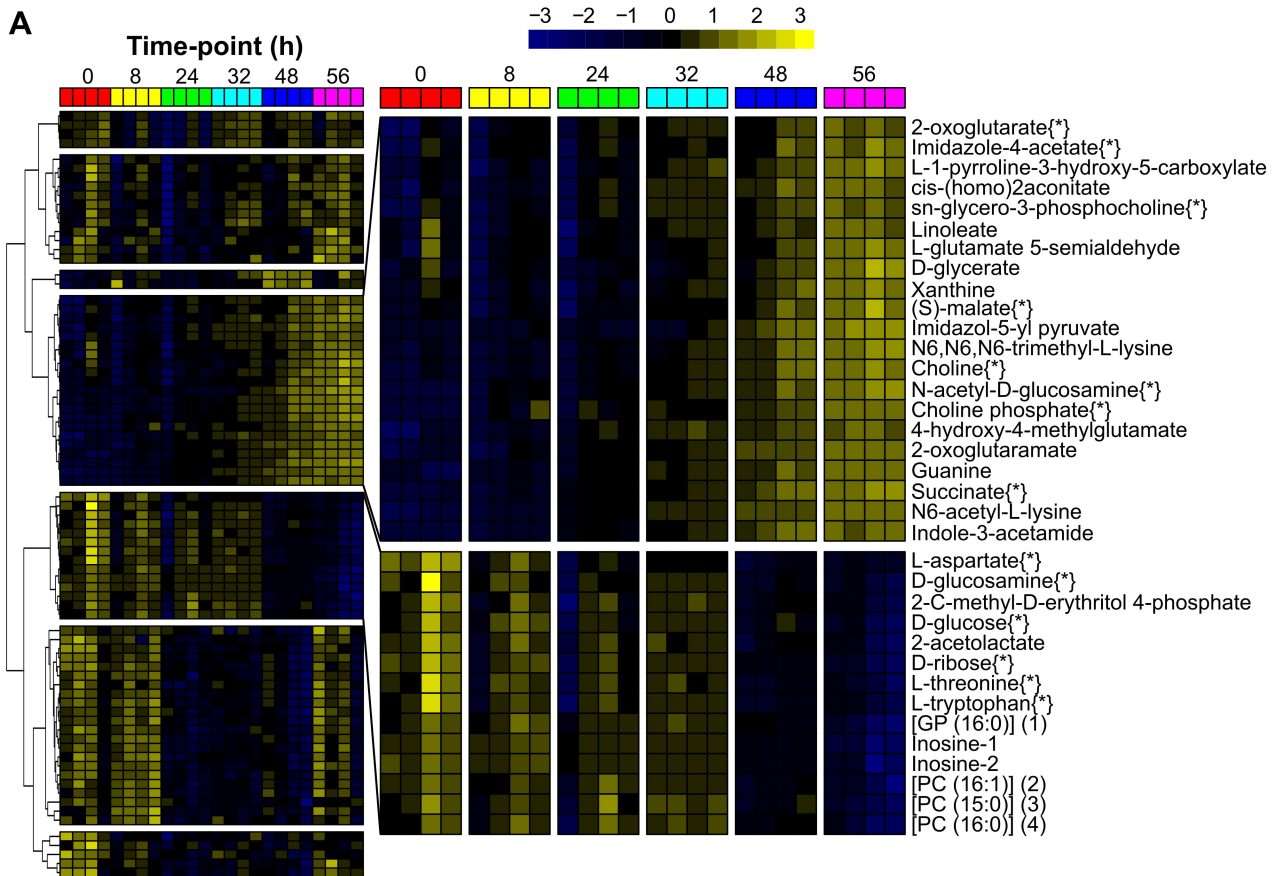
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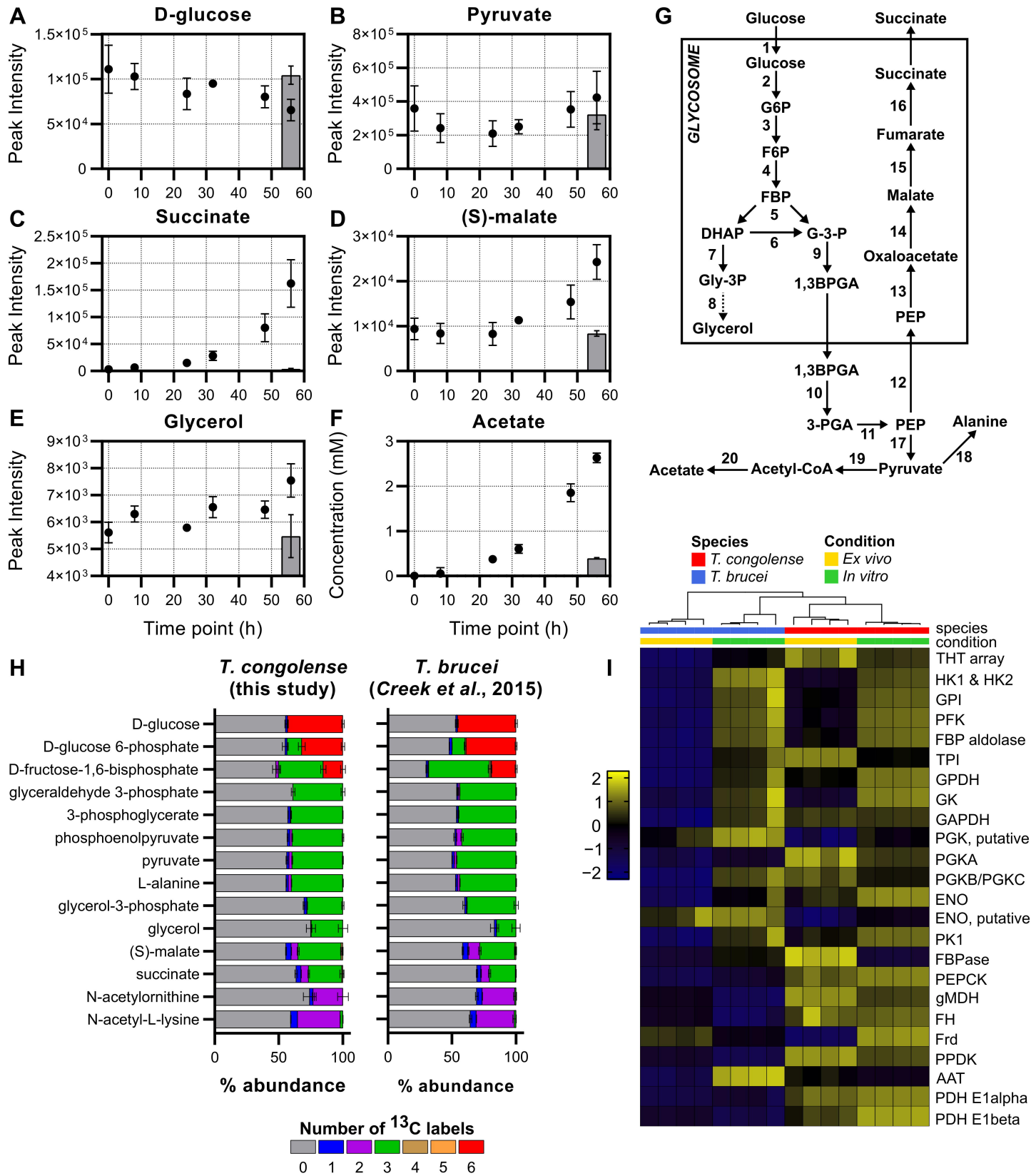
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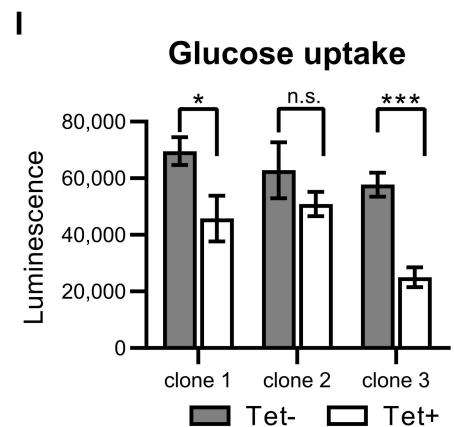
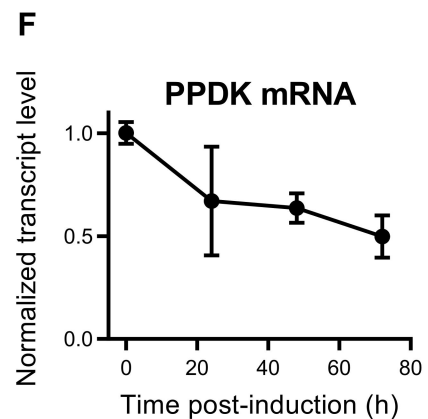
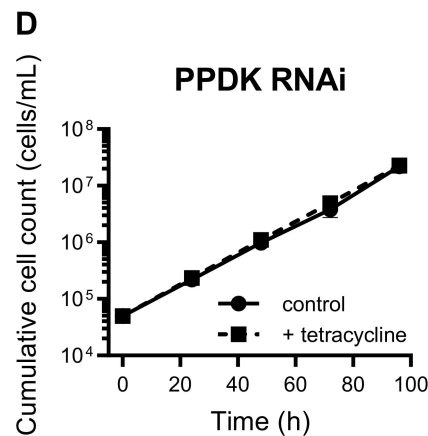
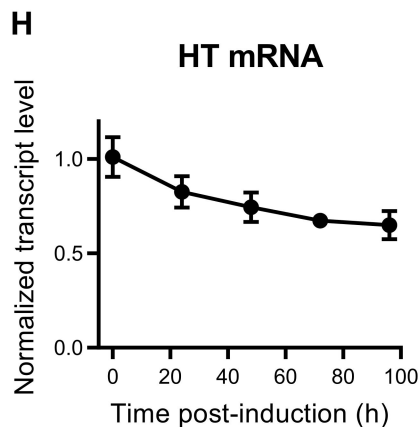
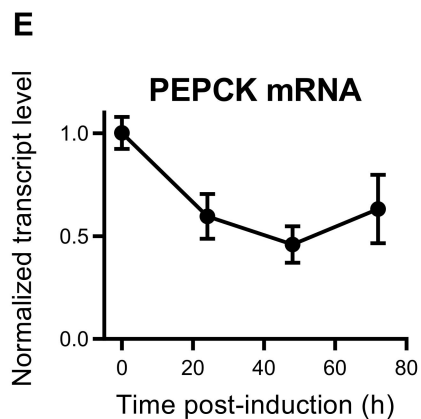
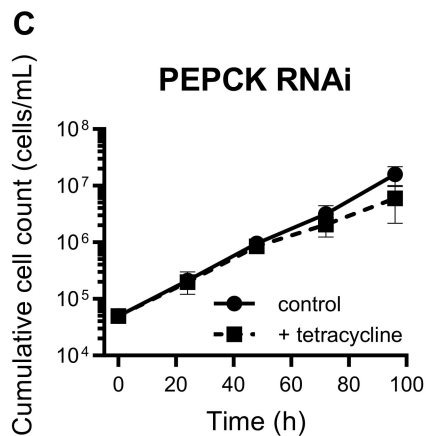
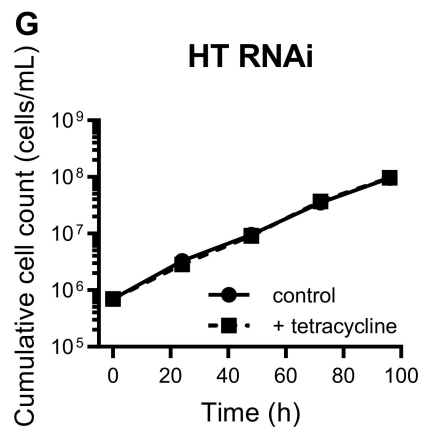
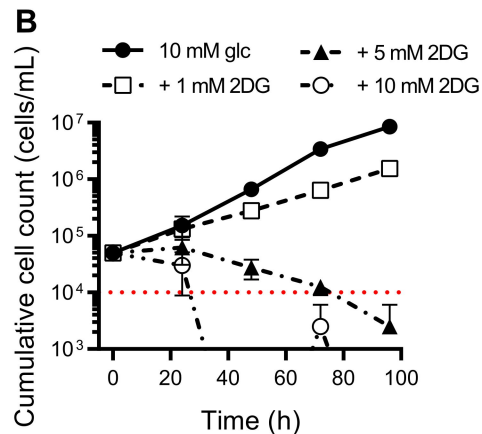
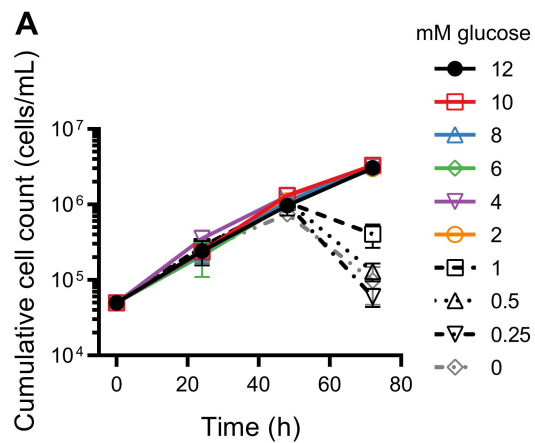
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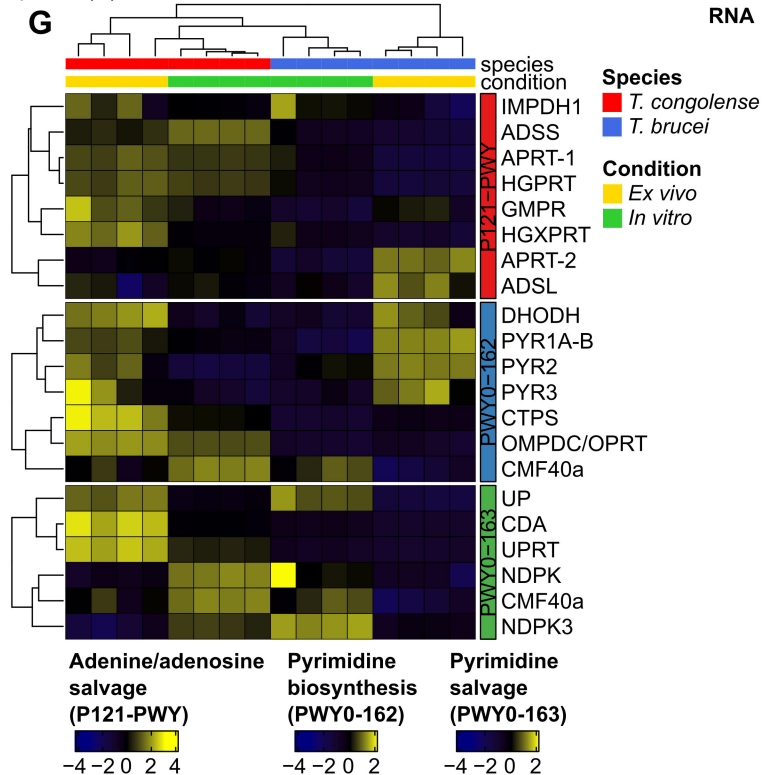
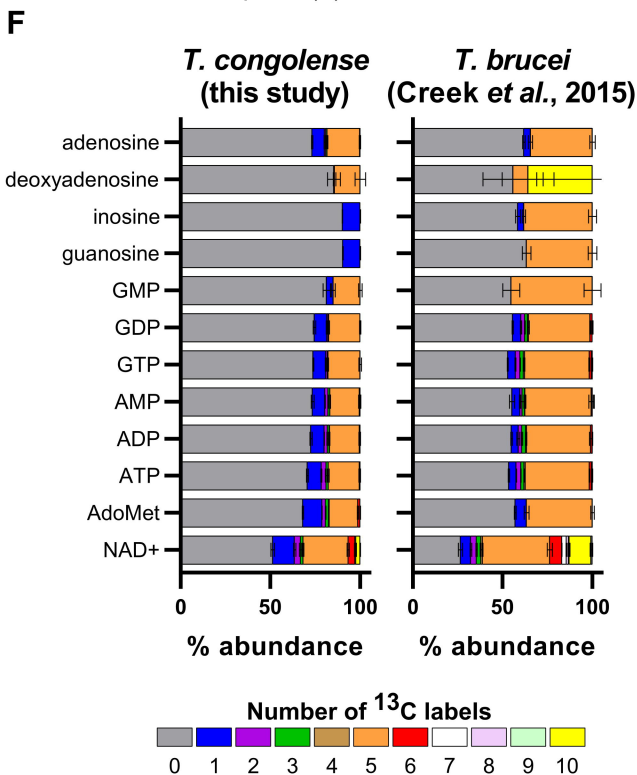
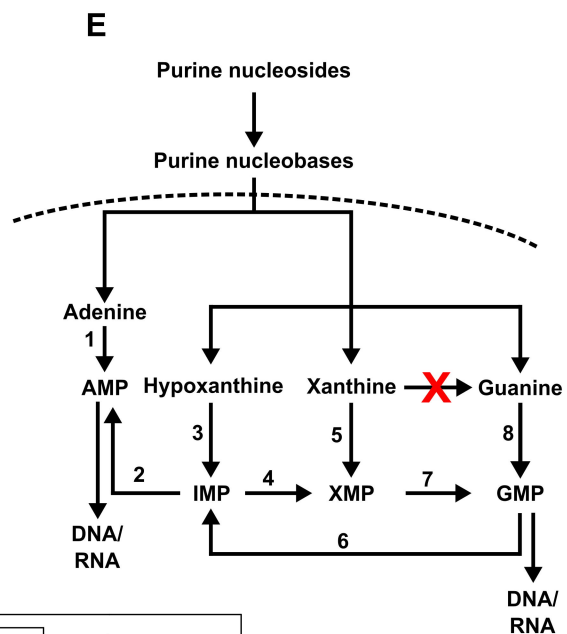
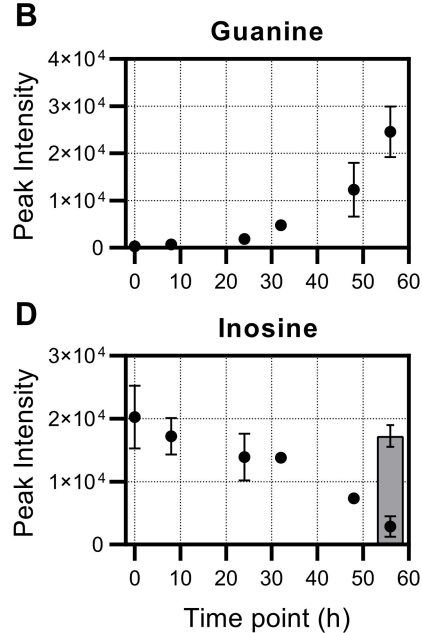
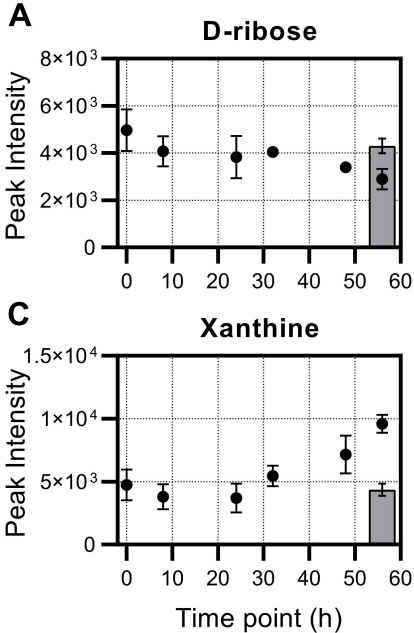
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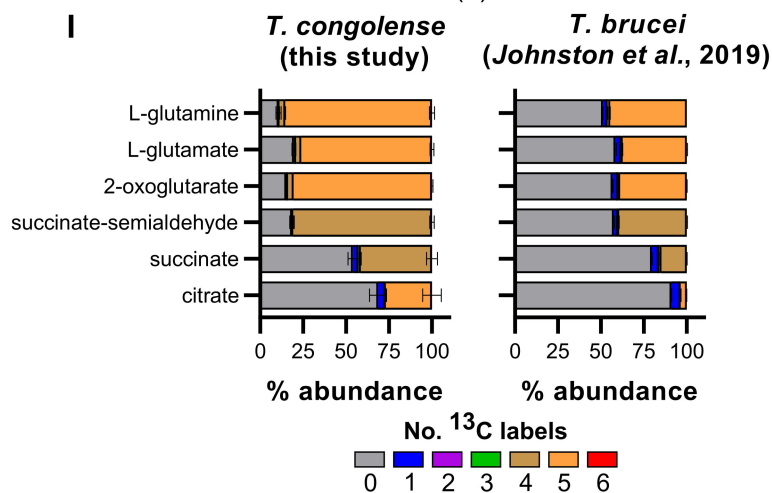
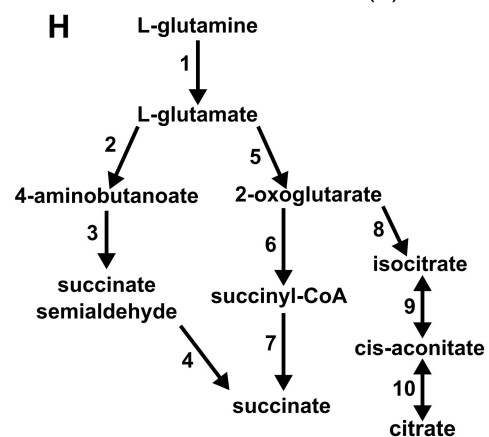
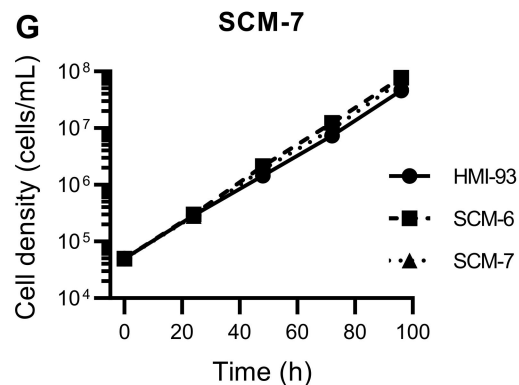
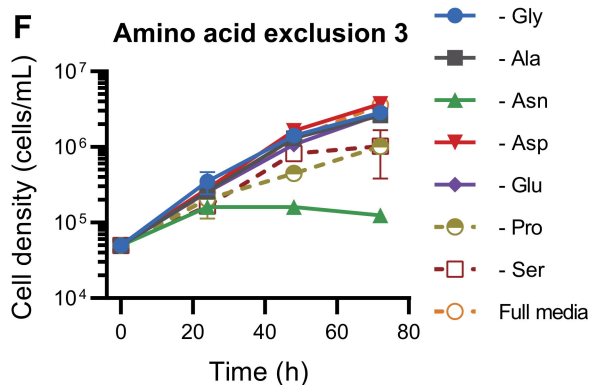
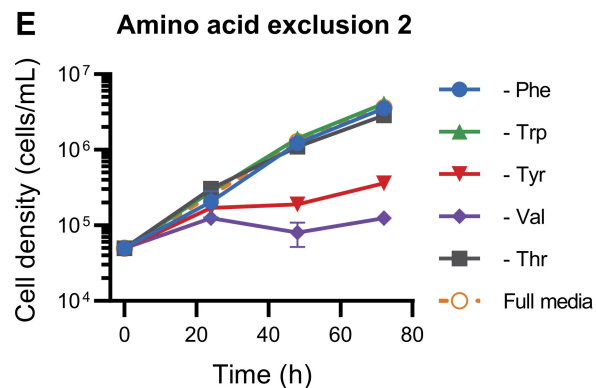
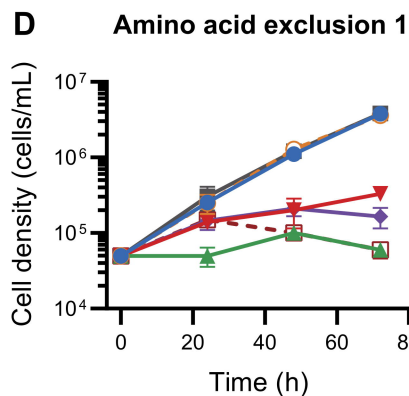
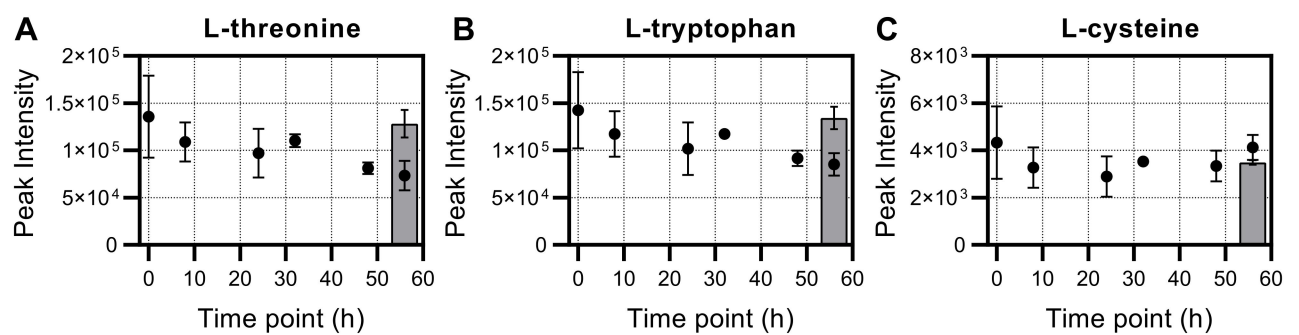


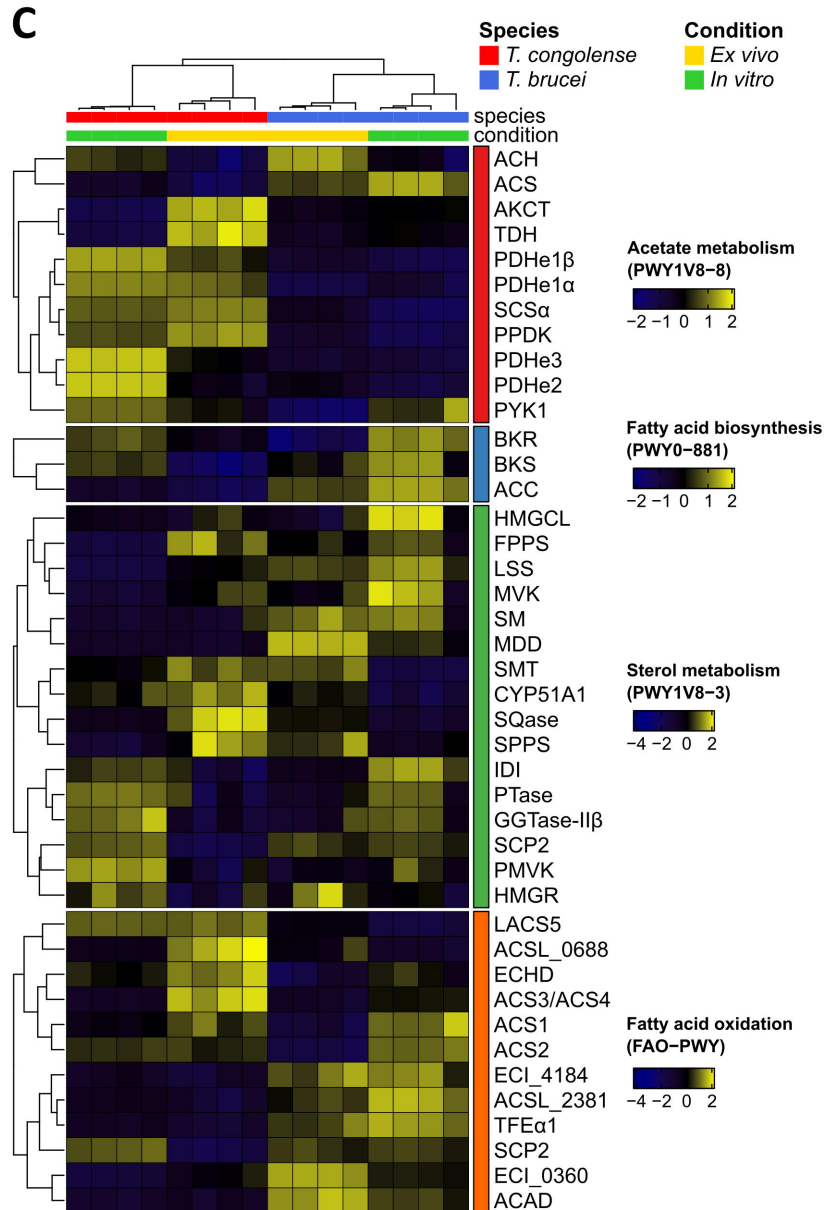
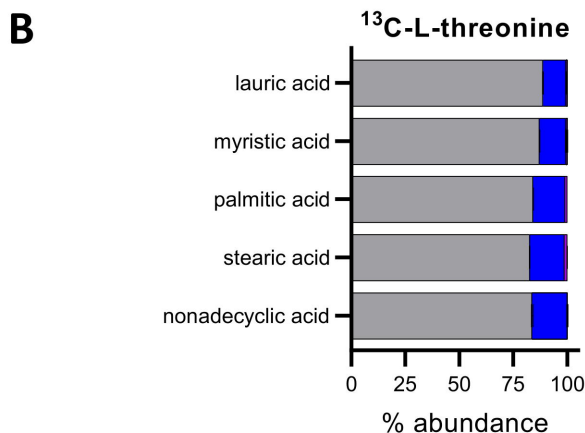
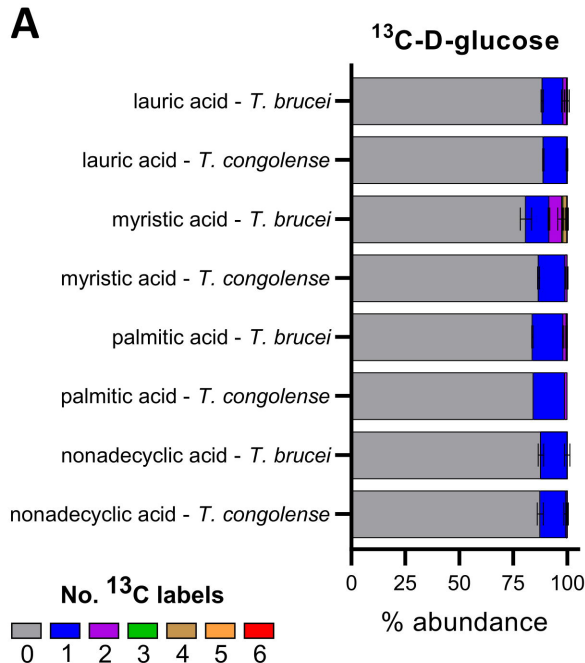


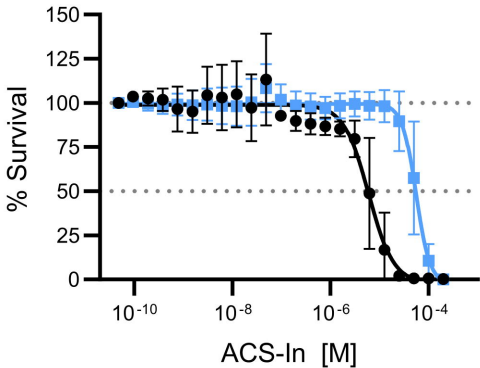
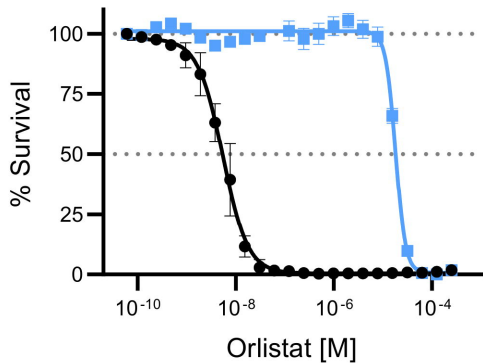


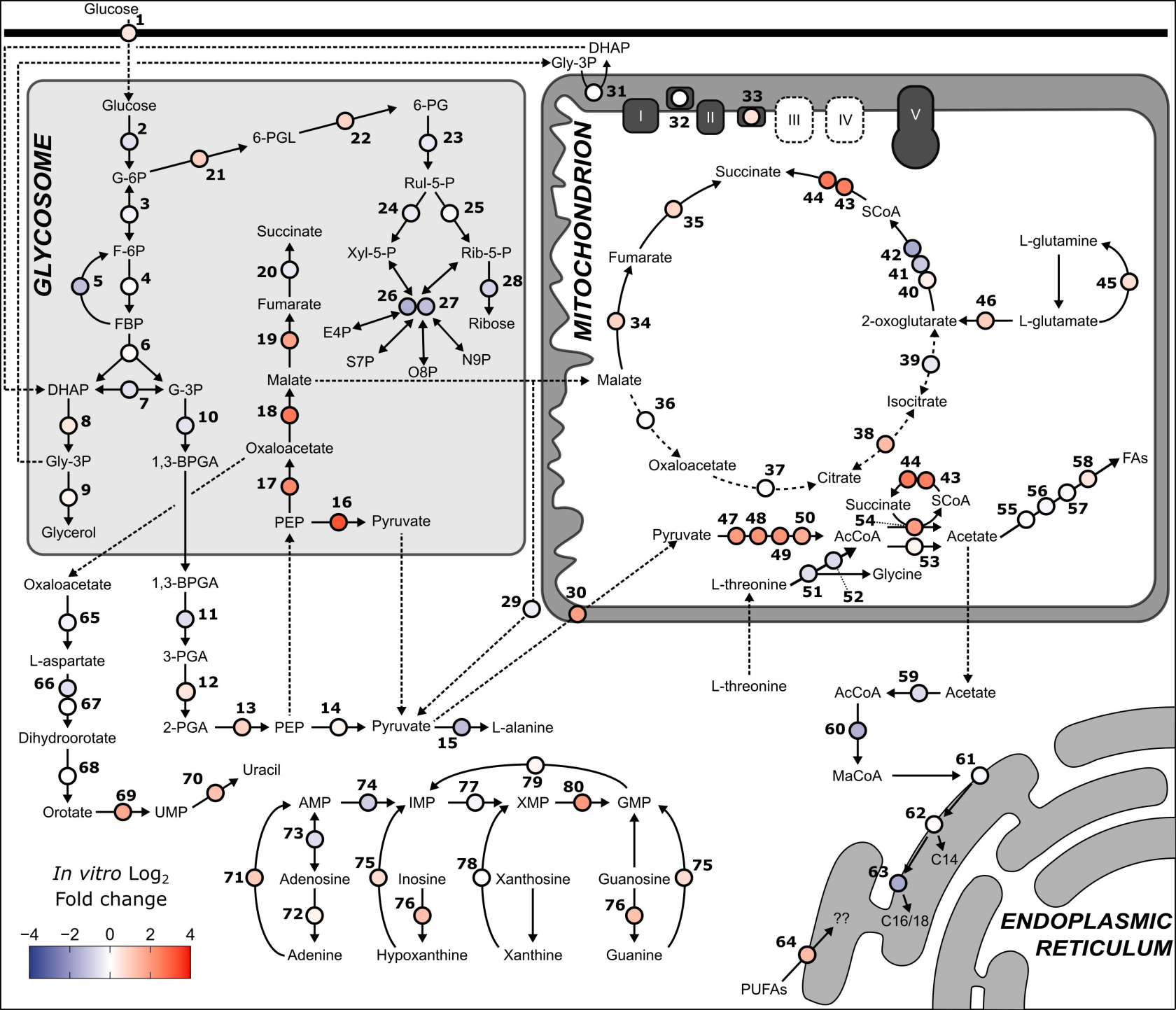


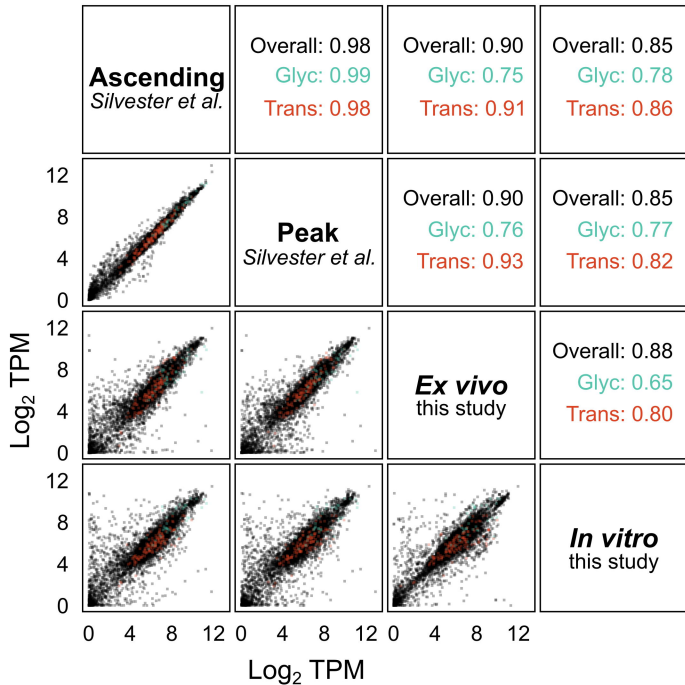


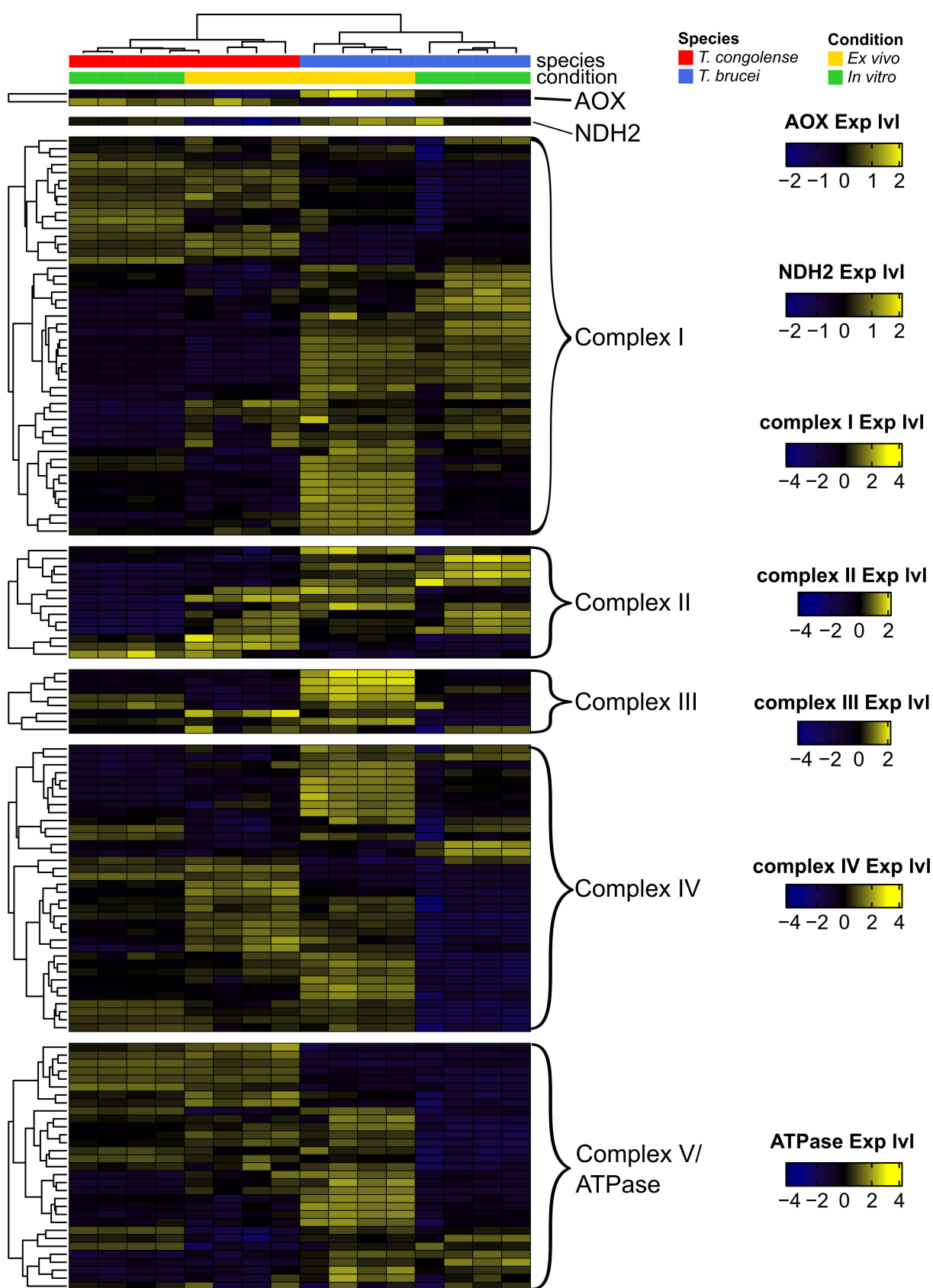




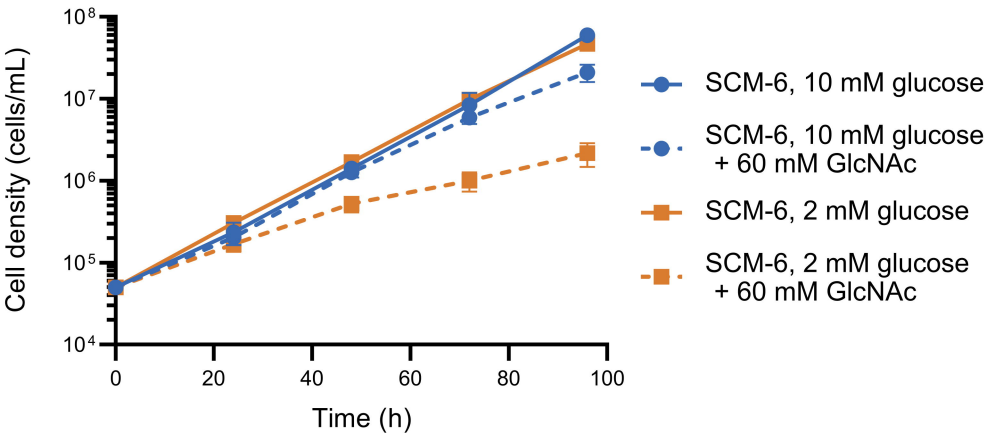
A**ACS inhibitor**● *T. brucei*■ *T. congolense***B****Orlistat**





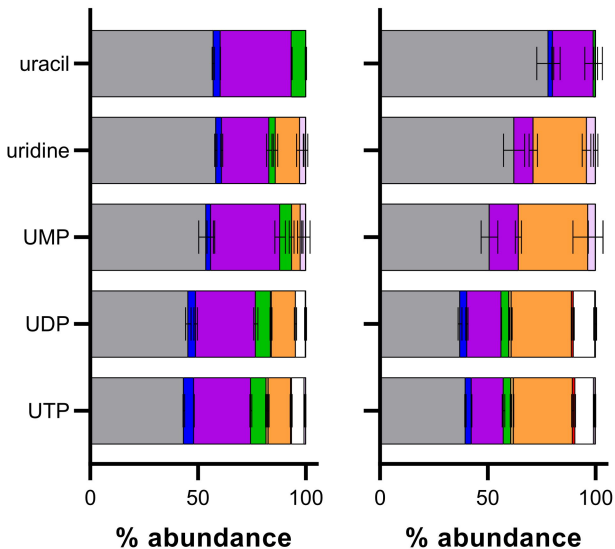


T. congolense growth ± N-acetyl-D-glucosamine



T. congolense
(this study)

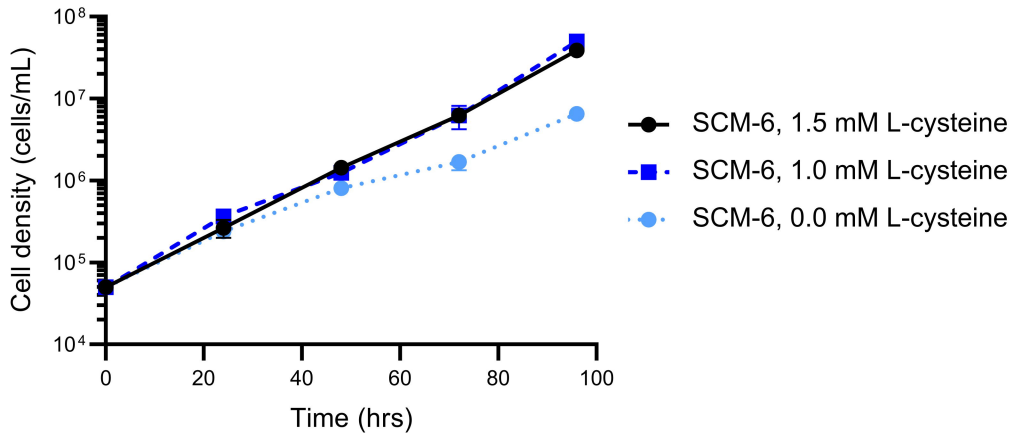
T. brucei
(Creek et al., 2015)

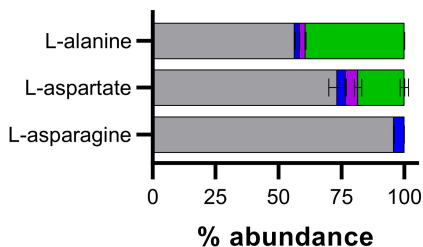
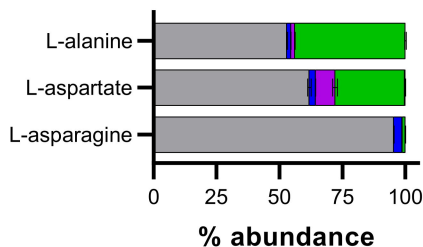


No. ¹³C labels



T. congolense growth ± L-cysteine



A***T. congolense***
(this study)***T. brucei***
(Creek et al., 2015)**Number of ^{13}C labels****B**