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Title:
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A Proton NMR Approach to the Culture and Extracellular Metabolomics of Trypanosoma congolense

2018

The Other Trypanosome: A Proton NMR Approach to the Culture and Extracellular Metabolomics of *Trypanosoma congolense*

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A DISSERTATION SUBMITTED TO THE UNIVERSITY OF BRISTOL IN ACCORDANCE WITH THE REQUIREMENTS FOR AWARD OF THE DEGREE OF MASTERS BY RESEARCH INTHE FACULTY OF SCIENCE

Abstract

Trypanosoma congolense has fallen by the wayside as a parasite of interest and yet remains a critical threat to animals and economic stability across the African continent. The present study aimed to refocus research on this clinically relevant species and establish improved culture methodology based upon a quantitative proton Nuclear Magnetic Resonance (NMR) approach for each life stage of *T. congolense*. In doing so the metabolism of *T. congolense* was re-examined after decades of neglect and utilising modern techniques minimal media was obtained for procyclic culture. This led onto metabolic investigations of epimastigote and bloodstream form *T. congolense* and further media revisions were recommended for these lifecycle stages. In each instance significant differences were observed in the substrate preferences of *T. congolense* and the metabolic end products generated when compared with historical observations, as well as contemporary research into the closely related *Trypanosoma brucei* and other trypanosomatids. The simple and relatively inexpensive process of ^{1}H NMR has proven an invaluable tool to achieve these results and could be applied in the culture and investigation of other clinically relevant species such as *Trypanosoma vivax* and *Trypanosoma evansi*.

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I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE: .....

Acknowledgements

Firstly, I would like to thank my supervisor Professor Wendy Gibson for the encouragement and guidance you provided throughout my project, as well as the kindness and consideration you gave me at a time when I needed it most. Dr Tim Woodward I also thank for being a generally wonderful person who spent so much time answering my questions and helping me understand the intricacies of NMR and the importance of never doing something on a Friday afternoon. Thanks also go to the Wickstead Laboratory for the gracious treatment I received when I visited your facilities, and especially for the IL3000 bloodstream form you provided for my project. This not only helped me enormously, but has also opened an entirely novel line of enquiry for the Gibson group. I hope the cooperation that exchange began continues long into the future and is fruitful for all involved.

Special thanks and appreciation goes to Dr Chistopher Kay for the generosity he displayed time and again, not only with the sharing of his broad expertise, but also the honest and enthusiastic exchange of ideas that made every day a pleasure to come in and work. Further thanks go to Rachel Hutchinson for her support and providing an empathetic ear on the days when nothing worked the it was meant to.

Finally, I give my heartfelt thanks to my family and to those closest to me who have provided such unerring support and kindness, it is through your combined efforts my sanity has remained intact.

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I -
I took the one less traveled by,
And that has made all the difference.

- Robert Frost

Introduction

Animal African Trypanosomiasis

Figure 1. Distribution Map of Animal African Trypanosomiasis (AAT)

Animal African trypanosomiasis (AAT) is a wasting disease caused by extracellular flagellate *Trypanosoma* parasites and significantly affects livestock in 37 countries of sub-Saharan Africa (Fig.1) (Auty *et al.*, 2015; Giordani *et al.*, 2016; Morrison *et al.*, 2016; Yaro *et al.*, 2016). The host range of *T. congolense* (savannah subtype) is wide ranging and includes a variety of mammals including domestic livestock species such as cattle and goats vital to daily life in sub-Saharan Africa, as well as significant reservoir of other wild mammals (Katunguka-Rwakishaya *et al.*, 1992; Biryomumaisho *et al.*, 2003; Auty *et al.*, 2015; Yaro *et al.*, 2016). Historically *Trypanosoma congolense* was regarded as the primary cause of AAT in domestic animals in Eastern Africa, as well as a significant cause in Western Africa in combination with *T. vivax* (Losos and Ikede, 1972). The most recent literature indicates that while *T. congolense* remains the primary cause of AAT in sub-Saharan Africa (Auty *et al.*, 2015), the geographical boundaries are less pronounced and mixed infections of *T.* congolense, *T. vivax,* and to a lesser degree *T. brucei* sensu lato are commonplace (Morrison *et al.*, 2016). Notably while *T. brucei* s. l. is commonly detected in cattle and other livestock it is not regarded as being a significant contributor to AAT, with the notable exception of *T. evansi* as a well-recognised pathogen of cattle, water buffalo, horses and camels (Auty *et al.*, 2015; Morrison *et al.*, 2016).

Despite this *Trypanosoma brucei* has been the primary focus of research for the past several decades. *T. b. brucei* as a non-infective strain can be used as an effective and safe proxy organism for

the potentially more hazardous research of human infective *T. b. gambiense* and *T. b. rhodesiense* as model organisms to research Human African Trypanosomiasis (HAT). Considering the close phylogenetic relationship of *T. b. brucei* and *T. congolense* it is perhaps an understandable assumption that the results of this research could also be simply generalised and therefore benefit AAT control efforts as well. Whether this assumption is correct however has not been comprehensively challenged but is likely overly optimistic. The early investigations of *T. congolense* metabolic pathways highlighted clear differences in the compounds produced *in vitro* (Agosin and Von Brand, 1954; Ryley, 1956; von Brand and Tobie, 1959; Lumsden and Evans, 1976; Mazet *et al.*, 2013; Bringaud *et al.*, 2015). The differences in life stage differentiation to diverse compartments of the mammalian host and tsetse host with the concomitant changes in environmental cues are well established (Sharma *et al.*, 2009; Peacock *et al.*, 2012). As are the differences in antigenic variation (Cross, 1996; Jackson *et al.*, 2012), drug uptake and resistance mechanisms (Delespaux *et al.*, 2008; Munday *et al.*, 2013). It is curious then that the research into *T. congolense* metabolism and research generally has been so limited since the turn of the millennium. As a modern researcher looking back across the literature, it appears that both species were given equal attention for much of the last century, but since the year 2000 *T. brucei* has been taken forward, and *T. congolense* and *T. vivax* largely set aside.

The Economic Impacts of AAT

In recent years the argument has been put forward that the impacts of trypanosomiasis on animals may have far more significant and extensive effects than had been previously thought (Auty *et al.*, 2015; Giordani *et al.*, 2016; Morrison *et al.*, 2016). Like many neglected tropical diseases AAT disproportionately affects poorer countries, with 26 of the affected 37 countries having been identified by the International Development Association (IDA) and International Monetary Fund (IMF) (2016) as Heavily Indebted Poor Countries. The Food and Agriculture Organization of the

United Nations (2016) classifies 28 as Low Income Food-Deficit Countries. Economic estimates of the impacts of AAT indicate that 4.75 Billion USD in economic losses per annum occur within sub-Saharan Africa (Otte *et al.*, 2004), with as much as 2.8 Billion USD loss estimated in Eastern Africa alone (Shaw *et al.*, 2014). These losses represent an extraordinary economic constraint for the countries directly affected and negatively impact the entire continent. The nature of this economic cost can be the outright loss of 3 million cattle per annum, or indirectly due to the cost of trypanosomiasis prevention such as the 35 million doses of preventative chemotherapy and treatment administered (Mattioli *et al.*, 2004). AAT also impacts at the micro level as the loss of animals as efficient sources of traction and fertiliser indirectly lowers crop yield and cultivation area (Jahnke, 1983; Swallow, 1999). When considering the more direct benefits of milk and meat production, there is a clear cost attributable to animal trypanosomiasis. Cattle raised expressly for the production of milk suffer substantially reduced milk offtake from infected individuals and increased mortality within the herd (Jahnke, 1983; Swallow, 1999). Cattle raised for the purpose of meat production exhibit lower weight of individuals and increased calf mortality in infected individuals thereby significantly lowering the value for each individual and decreasing the overall theoretical output by limiting the total number of offspring per generation (Jahnke, 1983; Swallow, 1999). The wider ranging impacts of AAT are more insidious and difficult to model but the landscape of the trypanosome affected areas must be considered. The majority of the affected fertile arable land is underutilised due to the reasons above (Jahnke, 1983; Otte *et al.*, 2004) and in mountainous, hilly, arid or otherwise non-viable regions that are not suitable for arable farming, the grazing of animals as a source of revenue or subsistence in these areas is therefore severely hindered (Jahnke, 1983; Jahnke *et al.*, 1988).

There is also the concern that tsetse distribution and the effects described previously will only worsen in the future due to the effects of climate change, with trypanosomiasis highlighted as one of the 'Deadly Dozen' by a Wildlife Conservation Society (2008) report. Rogers and Randolph (1993) suggested that tsetse distribution ranges may expand due to temperature increases in some regions,

but that vegetation cover is a significant factor and the distribution data for *G. pallidipes* was likely incomplete due to poor sampling methodology.

By comparison the more recent model by Moore *et al.* (2012) indicates that the total area infested by *G. morsitans* and *G. pallidipes* will only increase modestly by 2055 and there is unlikely to be a substantial increase even up to 2090. It is likely that large areas of current infestation will become non-viable for tsetse and new areas of Southern and Eastern Africa will be infested. These areas will previously have been unaffected by trypanosomiasis and therefore will have no or limited infrastructure in place to control the spread of disease. Uninfested areas such as the densely populated highland areas contain the largest concentration of susceptible livestock (Jahnke, 1983; Jahnke *et al.*, 1988). A more short term report produced by Thornton *et al.* (2006) considers the impacts up to 2030 and indicated that climate change would only have a minor impact but did raise the issue of increased population that would likely decrease tsetse distribution due to change in land use and deforestation. This is not a clear-cut situation, however, as deforested areas can simply be replaced by palm crops or other crops that would provide shaded areas ideal for tsetse habitation (Jahnke, 1983; Jahnke *et al.*, 1988).

The majority of climate change papers have focused primarily on the effect on Human African trypanosomiasis; however, as the tsetse distributions outlined above also carry *T. congolense* and *T. vivax,* climate change would have serious implications on the control of AAT and the burgeoning African economies at a time where populations are predicted to be at their peak. Trypanosomiasis and other neglected diseases primarily affect the poorer countries of sub-Saharan Africa. Consequently, the development of more effective trypanocidal drugs or vaccines against AAT has been limited, and is largely the result of philanthropic funding. At present only two drugs are available: diminazene aceturate and isometamidium chloride. Yet these drugs were developed decades ago and while reports of resistance are not widespread it is profoundly short-sighted to have control and treatment efforts dependent on only these compounds (Delespaux *et al.*, 2008).

Trypanosoma congolense Biology

T. congolense (Savannah) is the most pathogenic and widespread of the *T. congolense* sub-types and as such is the focus of this investigation. In common with all salivarian trypanosomes *T. congolense* has a complex lifecycle involving a mammalian host in which trypanosomiasis occurs, and a largely asymptomatic infection of the tsetse insect vector *Glossina* (Figure 2). The bloodstream form of *T. congolense* are primarily observed in the capillaries and venules of the skeletal muscles, the myocardium and particularly in the brain, and can be regarded as a solely plasma trypanosome with no invasion of the host tissues (Losos and Ikede, 1972; Losos *et al.*, 1973). This is in stark contrast to *T. brucei* bloodstream form whereby there is extensive extravascular tissue invasion, accompanied by substantial inflammation and lesion formation (Losos, 1970). *T. congolense* variant surface glycoproteins (VSG) (Ross *et al.*, 1987; Strickler *et al.*, 1987; Jackson *et al.*, 2012) fulfil the same role of immune evasion as those found in *T. brucei* (Cross, 1996; Horn, 2014) with VSG densely covering the entirety of the parasite, and as demonstrated for *T. brucei* the VSG coat allows nutrients access while still maintaining an effective barrier to immune effectors (Mehlert *et al.*, 2012).

While the procyclic forms *of T. congolense* establish and proliferate within the tsetse posterior midgut and ectoperitrophic space, the differentiation to procyclic is substantially different with *T. brucei* utilising a non-proliferative short stumpy bloodstream form (Sharma *et al.*, 2009), while there is no evidence for such an intermediate form in *T. congolense*. The long-slender *T. congolense* bloodstream form is either already competent for the insect host environment, or capable of readily switching upon entering the host (Silvester *et al.*, 2017). *T. congolense* and *T. brucei* later differ in the areas of epimastigote attachment and proliferation, as well as the accompanying metacyclogenesis that occur in the proboscis and salivary gland respectively (Sharma *et al.*, 2009; Peacock *et al.*, 2012).

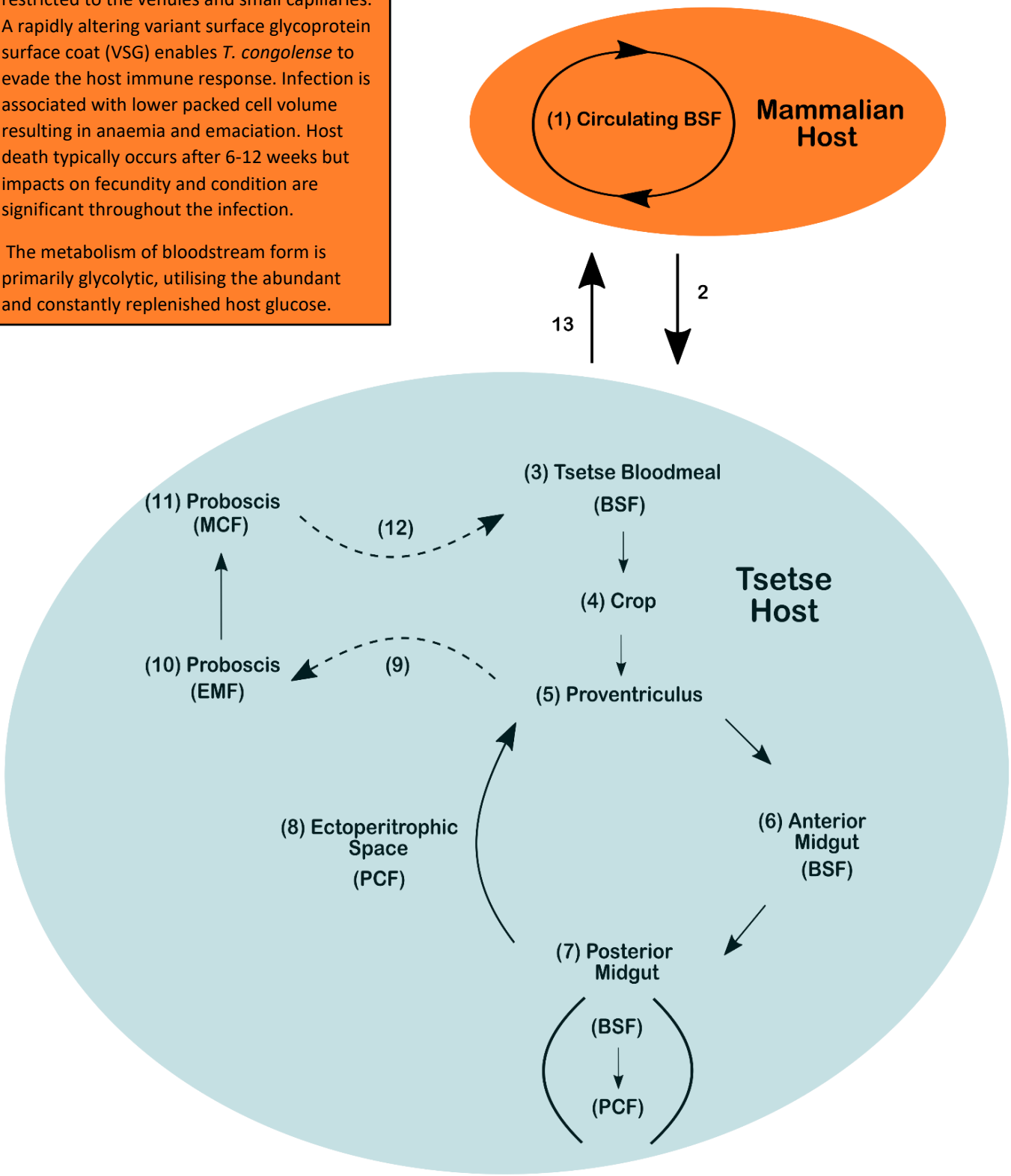
T. congolense Lifecycle

Figure 2 – Data obtained from (Peacock *et al.*, 2012)

Mammalian Lifestage

As a strictly plasma parasite *T. congolense* is restricted to the venules and small capillaries. A rapidly altering variant surface glycoprotein surface coat (VSG) enables *T. congolense* to evade the host immune response. Infection is associated with lower packed cell volume resulting in anaemia and emaciation. Host death typically occurs after 6-12 weeks but impacts on fecundity and condition are significant throughout the infection.

The metabolism of bloodstream form is primarily glycolytic, utilising the abundant and constantly replenished host glucose.



Tsetse Lifestages

Bloodstream form (BSF) persist in the crop and anterior midgut compartments of the tsetse and rapidly differentiate to procyclic form in the posterior midgut whereby VSG is shed in favour of a cascade of surface proteins (7). Procyclics (PCF) establish and proliferate in the posterior midgut for approximately 2 weeks, solely reliant upon amino acid metabolism. After 6 days non-dividing long-slender procyclics are observed in the ectoperitrophic space having crossed the peritrophic membrane (8). forms travel anteriorly, cross the PM once again to invade the proventriculus (9) with and differentiate to adherent epimastigote forms (EMF) (10). These colonies go on to shed motile metacyclic trypanosomes expressing VSG proteins (11) which are finally delivered alongside the anti-coagulant saliva (12) into the mammalian host during tsetse feeding to complete the lifecycle (13).

Pathology in the Mammalian Host

Animal trypanosomiasis due to *T. congolense* in cattle has been an area of extensive research in the past and has seen a revival in recent years (Auty *et al.*, 2015; Giordani *et al.*, 2016; Morrison *et al.*, 2016). The clinical presentation of the disease is characterised by an acute phase of fever with the destruction of red blood cells correlating to higher parasitaemia and rapid decrease in packed cell volume (PCV), as well as decreased levels of cholesterol, total plasma protein, serum albumin and Hb; mortality at this stage has been observed but is uncommon, whereas lowered reproductive output is pronounced (Fiennes *et al.*, 1946; Losos and Ikede, 1972; Losos *et al.*, 1973; Wellde *et al.*, 1974; Valli *et al.*, 1978; Auty *et al.*, 2015). A chronic phase follows whereby the fever is intermittent, but the anaemia is maintained despite lower parasitaemia, resulting in: emaciation, cachexia, polioencephalomalacia, and ultimately leading to host death after 6 – 12 weeks (Fiennes *et al.*, 1946; Losos and Ikede, 1972; Losos *et al.*, 1973; Wellde *et al.*, 1974; Valli *et al.*, 1978; Kristjanson *et al.*, 1999; Auty *et al.*, 2015). Clinical post mortems of infected cattle indicate trypanosomes are concentrated in the capillaries and venules of the brain and heart, and are attached anteriorly to the walls of these small vessels, only entering the peripheral circulation periodically (Losos and Ikede, 1972; Losos *et al.*, 1973; Banks, 1978). The clinical presentation in other important livestock species such as goats, sheep and pigs has received less attention than bovine trypanosomiasis, however the gross clinical presentation is similar in these host species (Losos and Ikede, 1972; Katunguka-Rwakishaya *et al.*, 1992; Biryomumaisho *et al.*, 2003; Faye *et al.*, 2005; Omeje and Anene, 2012). Of note however is the hypophospholipidaemia observed by Katunguka-Rwakishaya *et al.* (1992) in sheep, and mirrored in goats (Biryomumaisho *et al.*, 2003).

Considering the significant impacts of *T. congolense* as outlined above, the successful reproduction of the lifecycle of *T. congolense in vitro* is of the utmost importance. A better understanding of the biochemical and immunological mechanisms of trypanosomiasis during all life stages of the parasite would be beneficial.

Trypanosome Metabolism and Biochemistry

Bloodstream Form

The bloodstream form of *T. congolense* are primarily observed in the capillaries and venules of the skeletal muscles, the myocardium and particularly in the brain, and can be regarded as a solely plasma trypanosome with no invasion of the host tissues (Losos and Ikede, 1972; Losos *et al.*, 1973). This is in stark contrast to *T. brucei* bloodstream form whereby there is extensive extravascular tissue invasion, accompanied by substantial inflammation and lesion formation (Losos, 1970). *T. congolense* variant surface glycoproteins (VSG) (Ross *et al.*, 1987; Strickler *et al.*, 1987; Jackson *et al.*, 2012) fulfil the same role of immune evasion as those found in *T. brucei* (Cross, 1996; Horn, 2014). The VSG densely covers the entirety of the parasite, thereby disguising the parasite and the surface proteins required for metabolism and uptake of key resources. By necessity these receptors cannot vary considerably, but as demonstrated for *T. brucei* the VSG coat allows nutrient access while still maintaining an effective barrier to immune effectors (Mehlert *et al.*, 2012).

The level of free amino acids in bovine plasma has been well characterised and is summarised in Table 1 (Shimbayashi *et al.*, 1967). The parasite benefits from a plentiful supply of glucose (Fiennes *et al.*, 1946) and glucose is not limited by chronic infection as the host will utilise glycogen stores via glycogenolysis evident by the absence or inconsistent reporting of hypoglycaemia (Fiennes *et al.*, 1946; Faye *et al.*, 2005). Amino acids are freely available (Table. 1), as well as cholesterol and other lipids. The bloodstream form of *T. congolense* therefore enjoys a complete repertoire of nutrient sources. The metabolism of bloodstream forms *T. brucei* has been the area of extensive and recent research

Table. 1 Bovine Plasma Amino Acids (Shimbayashi *et al.*, 1967)

Amino Acid	Concentration (uM)
Threonine	136.00
Valine	227.91
Methionine	26.81
Isoleucine	151.12
Leucine	118.93
Phenylalanine	72.64
Lysine and Ornithine	247.62
Histidine	101.19
Arginine	146.38
Serine	119.90
Aspartate	49.58
Glutamate	97.87
Citrulline	115.87
Proline	74.70
Glycine	416.96
Alanine	298.57
Tyrosine	63.46
Taurine	63.92

(Flynn and Bowman, 1973; Michels *et al.*, 2006; Mazet *et al.*, 2013; Bringaud *et al.*, 2015), while the metabolism of *T. congolense* bloodstream form has received some attention in the past (Agosin and von Brand, 1954; Ryley, 1956; Flynn and Bowman, 1973) but little recent interest.

The long-slender bloodstream forms of *T. brucei* solely utilises the readily available and rapidly replenished glucose available within the mammalian host, generating ATP via the glycolytic pathway. The glycosome is a modified peroxisome which contains the first seven enzymes of the glycolytic pathway to convert glucose to 3-phosphoglycerate; the remaining glycolytic enzymes are cytosolic (Opperdoes and Borst, 1977). The mitochondrion is largely inactive in the bloodstream form with the TCA cycle non-functioning; the oxidation of glucose is therefore incomplete, and pyruvate produced as a major metabolic end-product along with small amounts of glycerol, acetate, and succinate. The mitochondrion is still utilised at this stage to maintain the redox balance of the glycosome. Glycerol-3-phosphate is exchanged for dihydroxyacetone phosphate via a FAD-linked glycerol-3-phosphate dehydrogenase, ubiquinone and the trypanosome terminal oxidase, (Michels *et al.*, 2006). Experiments conducted *in vitro* demonstrate *T. brucei* long-slender bloodstream form loses motility and ceases respiration if extracellular glucose is unavailable (Ryley, 1956).

In contrast the scant knowledge of *T. congolense* bloodstream form indicates a mitochondrion with tubular cristae and greater activity than in *T. brucei,* as observed by Vickerman (1965 and 1969) and later confirmed by Bienen *et al.* (1991). Curiously despite this higher level of mitochondrial activity presence of cytochromes was negative in *T. congolense* bloodstream forms in the investigations carried out by Bienen *et al.* (1991). Other differences observed in the metabolism of these two species is in the acquisition of essential haem during the bloodstream form life stage. The well characterised haptoglobin-haemoglobin receptor in *T. brucei* is absent in *T. congolense* bloodstream form and is in fact only expressed in the later epimastigote (Yamasaki *et al.*, 2016). Whether this is achieved by another receptor is unknown, and regrettably beyond the scope of this investigation, but represents a fundamental difference in the metabolism of these species.

The partially oxidised end products of *T. congolense* bloodstream form indicate that metabolic pathways extend beyond the typical end product of the carbohydrate metabolism of long-slender *T. brucei* in which 85.1% is excreted pyruvate (Mazet *et al.*, 2013). Instead previous investigations have indicated the major *T. congolense* end products are acetate, succinate, glycerol and CO_2 (Agosin and Von Brand, 1954; Ryley, 1956; Bowman and Flynn, 1976). This may be indicative of a metabolism closer to procyclic *T. brucei* and utilising a glycosomal fumarate reductase, or perhaps more similar *to* bloodstream form *T. lewisi* whereby pyruvate is degraded in the mitochondrion (Reid, 1950; Lumsden and Evans, 1976; Tielens and Van Hellemond, 1998), but due to the enigmatic mitochondrial activity of *T. congolense* (Vickerman, 1965, 1969) this has not yet been established. The *de novo* biosynthesis of sterols is not entirely absent in *T. brucei* bloodstream form (Nes *et al.*, 2012), but it contributes only a minor amount; instead a mechanism of receptor-mediated endocytosis of low-density lipoproteins carrying esterified cholesterol and phospholipids fulfils the parasites requirements (Coppens and Courtoy, 2000). It is unknown whether *T. congolense* functions in the same manner, however this process can be inferred by the substantial decrease of cholesterol and phospholipids observed in livestock (Fiennes *et al.*, 1946; Dargie *et al.*, 1979; Katunguka-Rwakishaya *et al.*, 1992; Biryomumaisho *et al.*, 2003). Regrettably further research into *T. congolense* bloodstream form metabolism has not been undertaken and remains an important avenue of investigation.

Procyclic Form

Trypanosomes are detected only periodically in the peripheral bloodstream but when this coincides with tsetse feeding patterns, they are readily taken up with the bloodmeal (Figure 2), moving first to the anterior midgut, via the proventriculus, and with any excess blood temporarily stored in the crop (Leak, 1998). The blood ingested by the tsetse is comparatively heavy relative to the fly and therefore to counteract this, rapid diuresis occurs within the anterior midgut; some water is retained for hydration and the rest excreted (Bursell, 1960; Lumsden *et al.*, 1978; Brown, 1980; Leak, 1998;

Aksoy *et al.*, 2003). At this stage the bloodstream form trypanosomes will have experienced a prolonged temperature drop from the relatively stable 36-37^{O}C of the mammalian host to approximately 24^{o}C in the insect, as well as a pronounced increase in pH from the stable 7.4 pH of the mammalian bloodstream to the variable and highly alkali conditions of the insect host (Liniger *et al.*, 2003). Simultaneously the *T. congolense* VSG, which are essential for survival within the mammalian host (Strickler *et al.*, 1987; Cross, 1996; Horn, 2014), are shed via an endogenous proteinase (Grandgenett *et al.*, 2007).

T. congolense utilises a distinct species-specific repertoire of surface coat proteins. Initially VSG is replaced by protease-resistant surface molecule (PRS) (Bütikofer *et al.*, 2002) offering protection from the hostile, proteolytic tsetse midgut environment. PRS expression then falls and subsequent expression of a glutamic-acid and alanine-rich protein (GARP) is detected in established early procyclic populations (Bayne *et al.*, 1993; Beecroft *et al.*, 1993; Loveless *et al.*, 2011), observable as a dense, protective layer (Loveless *et al.*, 2011). Both PRS and GARP differ substantially from the EP or GPEET pentapeptide repeat procyclins identified in *T. brucei* (Roditi *et al.*, 1989). In fact the expression of PRS and GARP is absent in later stage midgut infections (Bütikofer *et al.*, 2002). This mystery was resolved by Utz *et al.* (2006) who identified the *T. congolense* heptapeptide repeat proteins, *T. congolense* procyclins, which are expressed throughout the midgut and more closely resemble the *T. brucei* EP procyclin in sequence and structure.

Based upon *in vitro* experiments with *T. brucei,* the temperature drop alone can lead to the cessation of VSG transcription and significant upregulation of procyclin surface proteins (Matthews and Gull, 1994). While many other triggers of differentiation have been identified (Sbicego *et al.*, 1999), it seems likely the temperature drop most closely represents the *in vivo* condition. In *T. brucei* a differentiation step to the short stumpy bloodstream form within the mammalian host is required for the transformation to procyclic form. This is achieved via a pre-adapted shift away from glycolysis towards an amino-acid based metabolism and is consistent with the decreased availability

of glucose within the insect midgut. *In vitro* this mechanism requires the presence of cis-aconitate and citrate in order for the differentiation step to occur, although there is no evidence this reflects the *in vivo* condition (Overath *et al.*, 1986; Matthews and Gull, 1997). In *T. congolense* however there is no evidence of polymorphism within the mammalian host and indeed the mitochondrion remains active through this lifecycle stage, therefore no additional lifecycle stage shift is necessary (Reid, 1950; Ryley, 1956; Vickerman, 1965).

The bloodmeal taken in by tsetse flies is enclosed by a type-II peritrophic matrix (PM) that is produced continuously in the proventriculus. The PM encloses the incoming bloodmeal and in mature flies runs the full length of the alimentary tract from the proventriculus to the hindgut, terminating as a ragged aperture (Fig.3). The PM carries out the dual function of protecting the midgut epithelial cells from undigested food and representing a barrier to pathogen infection (Richards and Richards, 1977). Pores within the membrane allow digestive enzymes and free amino acids to be transported across the PM surface to the gut epithelium whereby they are absorbed and subsequently transported into the tsetse circulating haemolymph (Richards and Richards, 1977; Simpson and Casas, 2009).

In the anterior portion of the midgut the bloodmeal would largely consist of whole erythrocytes and undigested protein, with low concentrations of free amino acids or glucose available (Balogun, 1974; Aksoy *et al.*, 2003; Hegedus *et al.*, 2009). In the posterior midgut that tsetse haemolysin lyses the erythrocytes and it is within this compartment that trypsin, carboxypeptidases A and B, proteinases VI and VII, and aminopeptidase begin to break down macromolecules such as lipids, proteins and carbohydrates to free amino acids and fatty acids (Leak, 1998; Aksoy *et al.*, 2003; Hegedus *et al.*, 2009). It is therefore in this comparatively nutrient-rich region of the fly that *T. congolense* infection is first established (Peacoc*k et al*, 2012).

To complete their lifecycle *T. congolense* procyclics must cross or circumnavigate the peritrophic matrix to enter the ectoperitrophic space; although the mechanism of invasion remains unknown. *T.*

brucei trypanosomes have been observed to associate with the PM within the midgut (Gibson and Bailey, 2003) and direct invasion seems likely. An elegant model has been proposed by Aksoy *et al.* (2016) whereby the shed VSG is taken up by the PM-producing tsetse cardia and inhibits the miRNA mir-275, thereby disrupting signalling and transcription factors such that the PM synthesis is compromised. Proliferating forms are observed within the ectoperitrophic region as well as within the endoperitrophic space, although eventually procyclics travel anteriorly towards the proventriculus requiring a second crossing of the PM in order to invade and establish as epimastigotes within the cibarium and proboscis (Peacock *et al.*, 2012).

Procyclic forms of *T. congolense* are capable of degrading glucose via aerobic fermentation, utilising cytochrome oxidase and trypanosome alternate oxidase via similar processes to *T. brucei* within the glycosome (von Brand and Tobie, 1959; Obungu *et al.*, 1999a). Curiously the three genes within the PGK complex of *T. congolense* exhibit high cross-species similarity at the amino acid and nucleotide level with those of *T. brucei*, but only a single phosphoglycerate kinase isoform (56PGK) is responsible for PGK glycosomal activity in *T. congolense*. 56PGK is constitutively expressed during all lifecycle stages, while the two downstream genes are likely the result of a gene conversion event and have lost any glycosomal targeting and remain cytoplasmic (Parker *et al.*, 1995). A metabolic pathway was outlined by Obungu *et al.* (1999) (Figure 3). The partially oxidised end products succinate and acetate are therefore generated within the mitochondrion via fumarate dehydrogenase and pyruvate dehydrogenase respectively, although an additional source of succinate may occur via a fumarate reductase in the glycosome as outlined in a later study by Besteiro *et al.* (2002). An additional step occurs whereby Acetyl CoA is generated. While it was not possible to ascertain the enzyme responsible, it is likely this occurred via Acetate:Succinate CoA-transferase (ACST) observed in all other trypanosomatids and the closely related *T. brucei* (Van Hellemond *et al.*, 1998; Rivière *et al.*, 2004).

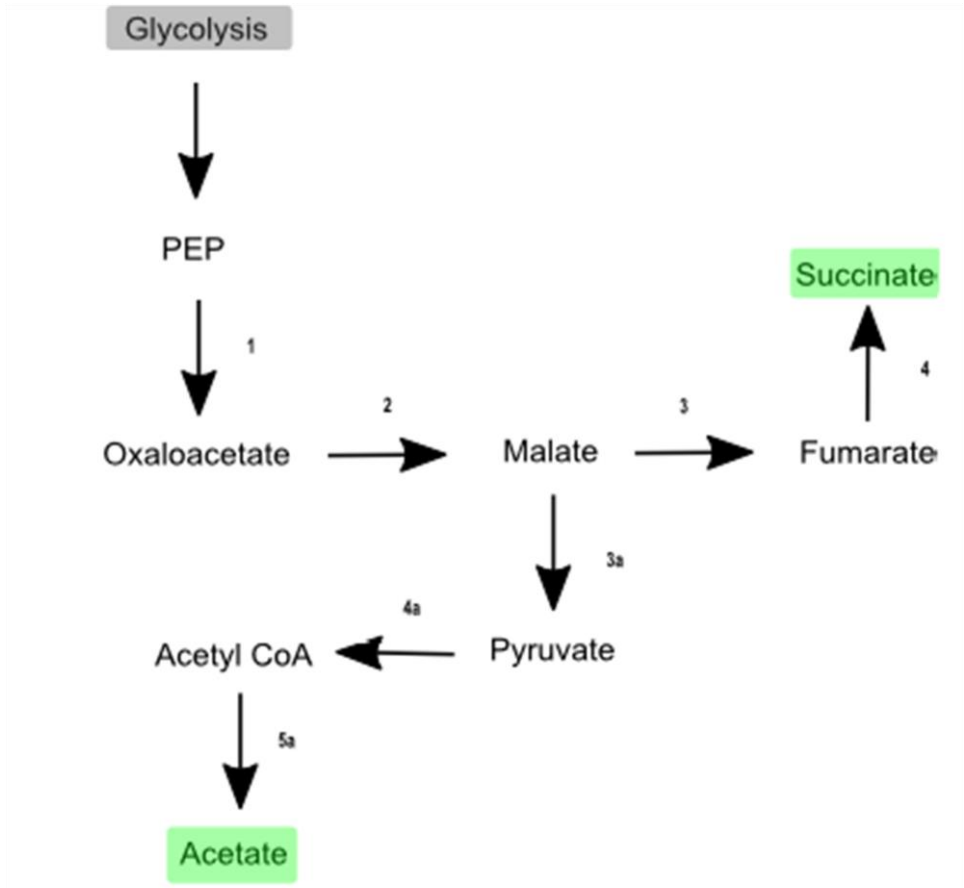


Figure 3 Simplified glycolysis pathway of *T. congolense* and the generation of partially oxidised end products. Phosphoenolpyruvic acid (PEP). Enzymes: 1 - PEP carboxykinase, 2 - malate dehydrogenase, 3 – fumarase, 3a - NADP-linked malic enzyme, 4 - fumarate dehydrogenase, 4a - Acetate:Succinate CoA-transferase, 5 – pyruvate dehydrogenase. **Adapted from Obungu et al. (1999a).**

As outlined above however glucose is unlikely to be present in significant amounts for very long following a bloodmeal (Balogun, 1974). Vickerman (1985) proposed that glucose within the bloodmeal may only be available for as short a time as 15 minutes after feeding and considering the frequency of tsetse feeding (Sharma *et al.*, 2009; Peacock *et al.*, 2012) these brief periods of high glucose availability will likely only occur once a day. To compensate for this dearth in glucose-based carbohydrate sources, the procyclic form of *T. congolense* shifts away from the glycolytic pathways of the glycosome. Instead the metabolism uses a well-developed mitochondrion with numerous cristae (Brun, 1982) to capitalise on the primarily amino acid based environment of the tsetse midgut (Vickerman, 1985; Obungu *et al.*, 1999b; Aksoy *et al.*, 2003; Peacock *et al.*, 2012).

The amino acid proline has received the most attention as a carbon source during this insect stage of *Trypanosoma congolense* (Evans and Brown, 1972; Bowman and Flynn, 1976; Vickerman *et al.*, 1988; Obungu *et al.*, 1999b; Mantilla *et al.*, 2017). Indeed *in vitro* Brun (1982) observed the substantial depletion of proline in his attempts to produce a semi-defined medium for *T. congolense* but regrettably offered little explanation of this clear preference. A metabolic pathway for proline oxidation has been outlined by Obungu *et al.* (1999b) and is briefly summarised below (Figure 4).

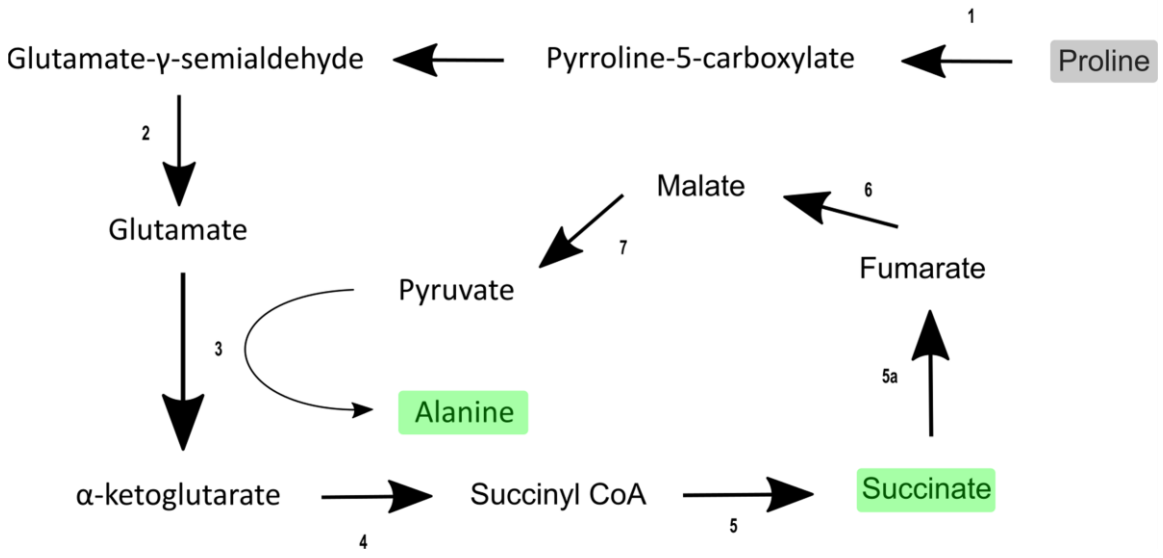


Figure 4 Simplified glycolysis pathway of *T. congolense* and the generation of partially oxidised end products. Phosphoenolpyruvic acid (PEP). Enzymes: 1 - proline dehydrogenase, 2 - pyrroline-5-dehydrogenase, 3 – alanine aminotransferase, 4 - a-ketoglutarate dehydrogenase, 5 – succinyl CoA synthetase, 5a – succinate dehydrogenase, 6 – fumarase, 7 - NADP-linked malic enzyme. **Adapted from Obungu et al. (1999b).**

It is of note that tsetse metabolism is also based upon proline, with levels reaching 60mM in tsetse haemolymph (Bursell, 1963, 1981; Cunningham and Slater, 1974) and represents the primary carbohydrate source for the generation of energy required for flight. Based upon the transient presence of glutamate during proline depletion it was ascertained that in tsetse proline is oxidised to glutamate, followed by oxidative deamination during the initial stages of flight, and finally yielding pyruvate for Acetyl CoA generation. During flight the transamination of pyruvate to form alanine and the resulting oxoglutarate enters the TCA cycle. The alanine produced via this pathway is transported via the haemolymph to the fat body and is converted in combination with lipid back to

proline, where it re-enters the haemolymph (Bursell, 1977). It is likely that trypanosomes are constrained by the same limits as their insect hosts in amino acid utilisation: chiefly the importance of nitrogen balance and the energetic costs of this process. Histidine and arginine only demonstrated minimal utilisation by procyclic *T. congolense* based upon Brun's observations (Brun, 1982) and tsetse similarly excrete these amino acids in large quantities, even when introduced directly to tsetse haemolymph (Moloo, 1977). Bursell (1963) asserts the high nitrogen content of these amino acids reduces the likelihood of their utilisation, as the net energy derived from the deamination process is severely hampered by the subsequent energetic costs required to dispose of the resultant nitrogen biproduct. KEGG pathways of *T. brucei* demonstrate a fully intact pathway whereby arginine is utilised to generate ornithine and subsequent conversion to putrescine to participate, in combination with methionine derived S-adenosylmethionine, in the generation of spermidine and the subsequent trypanothione pathway (Fairlamb *et al.*, 1987; Fairlamb and Cerami, 1992; Berger *et al.*, 1996). Ornithine is not present as a component in any CM derived media and to this authors knowledge any commonly used trypanosome media, however it is present in bovine plasma (Shimbayashi *et al.*, 1967) and from this it can be inferred that the quantity in serum combined with the surplus of methionine and arginine are sufficient to support *in vitro* culture requirements.

The amino acid threonine has been highlighted as an important metabolite (Cross *et al.*, 1975; Brun, 1982; Millerioux *et al.*, 2013). Initially observed by Cross *et al.* (1975) in their investigation of amino acid utilisation of procyclic *T. brucei*, threonine was found to be depleted in the semi-defined HX25 media (Cross and Manning, 1973) and later in SDM-79 medium (Lamour *et al.*, 2005). Cross *et al.* (1975) further elucidated its function as a source of energy generation and suggesting acetate as an essential component of lipid biosynthesis. This was later confirmed by investigations by Rivière *et al.* (2009), who identified the essentiality of the cytosolic enzyme acetyl-CoA synthetase (AceS) and transport of acetate to the cytosol from its initial site of synthesis in the mitochondrion, and the subsequent *de novo* synthesis of fatty acids. The threonine degradation pathway was later outlined in *T. brucei* by Millerioux *et al.* (2013) whereby the importance of threonine relative to glucose was

confirmed as it led to the production of 2.5 fold more acetate than glucose. In *T. congolense* the same observation of threonine depletion was observed by Brun (1982) and it is likely to fulfil the same functions as outlined above, however to date no investigations have confirmed this assumption.

Epimastigote Form

A proportion of the procyclic population cells that have successfully navigated from the tsetse midgut to the ectoperitrophic space, eventually migrate anteriorly to the hostile and highly alkaline environment of the proventriculus (Figure 2). The long and slender morphology of these cells is markedly different from the shorter, more rotund forms observed in the midgut (Evans *et al.*, 1979; Liniger *et al.*, 2003; Peacock *et al.*, 2012). At this juncture *T. congolense* and *T. brucei* diverge in their lifecycle: with *T. brucei* invading and establishing epimastigotes attached to the salivary gland epithelium (Vickerman, 1985; Vickerman *et al.*, 1988; Sharma *et al.*, 2009), while *T. congolense* occupies the cibarium and food canal; eventually attached cells shorten and proliferation is observed (Evans *et al.*, 1979; Peacock *et al.*, 2012). Attachment to the chitinous wall of the labrum occurs anteriorly via hemidesmosomes, with cells also commonly attached to the flagella of adjacent cells via desmosomes and subsequently form closely grouped rosettes above an electron dense plaque (Evans *et al.*, 1979; Beattie and Gull, 1997). A key benefit to the study of *T. congolense* is that the epimastigote form is available to culture *in vitro,* as initially demonstrated by Gray *et al.* (1981) whereby explanted infected tsetse proboscides could be transferred into medium and sub-cultured in the short-term. Hirumi and Hirumi (1991) obtained bloodstream form directly from infected mice and introduced these forms to a glucose-free variant of Eagle's Minimal Essential Medium designated TBM-5, whereby they rapidly transitioned to attached epimastigote clusters.

The proteins involved in this attachment process are not well characterised. The surface coat proteins of *T. congolense* epimastigotes include the previously discussed glutamine alanine rich protein (GARP) (Bayne *et al.*, 1993; Beecroft *et al.*, 1993) and indeed (Eyford *et al.*, 2011) observed a

greater level of expression of this protein in the epimastigote stage than in procyclics. Protease-resistant surface molecule (PRS) expression is also maintained in epimastigotes from the procyclic stage, but neither GARP nor PRS are expressed in the metacyclic or bloodstream stages (Sakurai *et al.*, 2008; Eyford *et al.*, 2011). Sakurai *et al.* (2008) later identified the congolense epimastigote-specific coat protein (CESP); as the name indicates, expression of this protein is not detectable in bloodstream, procyclic, or metacyclic stages but CESP is strongly expressed on the exterior of epimastigotes. This stage specific expression may indicate a role in the attachment mechanism of *T. congolense* epimastigotes or perhaps another equally essential process that enables the establishment and survival of *T. congolense* epimastigotes. This is only speculative however, and despite over a decade passing since it's discovery the role of CESP remains unclear.

The metabolism of *T. congolense* epimastigotes has thus far been overlooked aside from a single sentence regarding initial investigations of respiratory metabolism by Bienen *et al.* (1991), and the identification of the *T. congolense* haptoglobin-hemoglobin receptor (TcHpHbR) (Yamasaki *et al.*, 2016). *T. congolense* epimastigotes once acquired can be maintained in a variety of different media *in vitro* (Ross, 1987; Hendry and Vickerman, 1988; Hirumi and Hirumi, 1991; Coustou *et al.*, 2010; Eyford *et al.*, 2011; Yamasaki *et al.*, 2016), although initiating metacyclogenesis may prove problematic and represents a significant time investment depending on the strain used (Frame *et al.*, 1991). The ability to culture cells in these media offers little indication of the metabolic pathways being utilised however; each contains substantial quantities of glucose ranging anywhere from 5 to 25mM as well as a comprehensive repertoire of amino acids. The tsetse mouthparts will only periodically and briefly be exposed to mammalian blood that these media most closely resemble, and as such they are unlikely to be physiologically representative of conditions epimastigotes must endure and proliferate in for the majority of the infection. It is possible epimastigotes represent a hyper-efficient in response to this nutritional deficit, although one option is that tsetse saliva these mouthparts are continuously bathed in contains enough nutrients to maintain infection. A limited amount of work has been undertaken to assess and quantify the components of tsetse saliva. Patel

et al. (1981) painstakingly obtained sufficient quantities for biochemical investigation and highlighted the absence of free amino acids in uninfected *G. m. morsitans* saliva. Notably the presence of: alanine, asparagine, aspartic acid, cystine, cysteine, glutamate, glycine, histidine, isoleucine/leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine in hydrolysed saliva (Patel *et al.*, 1981), with glutamate, aspartate, and glycine as the major constituents. The presence of glycogen, glucose, galactosamine and inositol was also observed suggesting saliva may not be as nutrient poor as expected. Intriguing though these results may be, they only considered uninfected flies. A subsequent paper by Patel and colleagues rectified this and investigated the saliva of infected flies at differing timepoints post infection in the presence of epimastigotes or subsequent metacyclics (Patel *et al.*, 1982). A pH difference was observed of 7-7.5 for uninfected flies and 8 for infected, and levels of glucose were decreased, while inositol and triglyceride were absent entirely, and overall protein content decreased (Patel *et al.*, 1982). Regrettably no modern investigation has revisited this subject with more sensitive techniques. Considerable attention has been given to changes in transcriptomics in different life stages of *T. brucei* via high throughput RNA sequencing (RNA-seq), particularly comparing infected salivary glands to uninfected (Telleria *et al.*, 2014; Savage *et al.*, 2016). This technique has not been applied specifically to *T. congolense* and as epimastigotes are primarily observed in the labrum (Peacock *et al.*, 2012), not the salivary glands as in *T. brucei*, it is unclear how applicable these results may be. Metacyclogenesis occurs although a comprehensive understanding of this process remains elusive. Metacyclic forms of *T. congolense* have been obtained previously as stable cultures (Gray *et al.*, 1981, 1985; Bienen *et al.*, 1991) and successfully converted to bloodstream form (Hirumi and Hirumi, 1991). Some triggers have been highlighted such as the importance of glutamine and proline either singularly or in combination, although whether this triggers metacyclogenesis or simply supports metacyclics (Ross, 1987) is less clear. The difficulty in obtaining infective forms *in vitro* remains a challenge and earlier research indicates certain stocks of *T. congolense* may be particularly resistant to differentiation (Frame *et al.*, 1991).

Nuclear Magnetic Resonance Analysis

Over the last fifty years Nuclear Magnetic Resonance (NMR) analysis has remained at the forefront of biological research and, alongside mass spectrometry (MS), has fostered the development of metabolomics. Through NMR and MS it is now viable to expand our understanding of an organism's metabolism and begin to unpick these vastly intricate networks, as well as highlight key areas for subsequent therapeutic and preventative drug development. The principles of a pulsed NMR approach can be summarised relatively simply: the sample of interest is placed within a strong magnet and the atomic spins present within the sample subsequently align with the carefully homogenised or 'shimmed' magnetic field. A pulse of radio frequency energy is then generated, which surrounds the sample, and the nuclear spin is thereby moved out of alignment with the magnetic field. As the nuclear spins relax and return to their original alignment with the magnetic field the differences in the time this process takes are determined and generate a free induction decay NMR signal. These differences are indicative of the influences of nearby nuclei and electrons and after Fourier transformation give rise to a recognisable NMR spectrum. The signals are initially acquired in Hz and divided by the specific magnet field of the spectrometer (MHz) resulting in an impractically small number. For the sake of convenience this value is then multiplied by one million to produce a chemical shift locator axis in ppm. NMR resonance frequencies are not inherently characteristic as no two magnets will have precisely the same magnetic field. Instead a chemically inert reference substance, or the residual solvent peak of a suppressed aqueous sample, is utilised to reference the spectra at 0 ppm. Subsequent automated processing of spectral data occurs immediately after initial sample analysis is complete, this includes: zero-filling, apodization, Fourier transformation, phase correction, referencing, and spectral alignment. For the best results manual application of phase correction, baseline correction, and referencing typically follows, as well as careful peak integration prior to metabolite assignment and quantification. Integration and subsequent metabolite assignment can be achieved through a variety of automated programmes,

and while accurate in some niche scenarios, but often fall short of manual analysis for general purposes.

NMR analysis is carried out on a number of detectable nuclei including ^{13}C, ^{1}H, ^{15}N, and ^{31}P, although ^{13}C and ^{1}H have been most commonly applied in a biological context (Emwas, 2015; Dona *et al.*, 2016; Markley *et al.*, 2017). For instance ^{13}C NMR has seen significant use in the past in metabolomic investigations (Fry *et al.*, 1993; Besteiro *et al.*, 2002; Coustou *et al.*, 2005, 2006; Riviere *et al.*, 2009; Spitznagel *et al.*, 2009) and remains a relevant technique used in conjunction with mass spectrometry or other NMR analysis (Emwas, 2015). The primary benefit of ^{13}C NMR is the broad chemical shift range of 200 ppm, signals are considerably easier to resolve and interpret than other NMR approaches where the overlap of peaks is more common (Emwas, 2015; Markley *et al.*, 2017). The low natural abundance of ^{13}C (1.1%) also enables the tracking of specifically ^{13}C enriched metabolites, thereby elucidating their metabolic fates. This approach is limited, however, as the low sensitivity of ^{13}C NMR (Markley *et al.*, 2017) is combined with the costly enrichment process required in sample preparation for most biological molecules to be detectable. ^{13}C NMR is also a time-consuming process with experiments typically running overnight to generate well-resolved spectra.

Protons are naturally highly abundant in most biological molecules and therefore proton NMR represents a powerful metabolic tool that can be used to answer a wide variety of biological questions (Sanchez-Moreno *et al.*, 1995; Mazet *et al.*, 2013; Millerioux *et al.*, 2013; Bringaud *et al.*, 2015; Mantilla *et al.*, 2017). Utilising a one-dimensional proton (1-D ^{1}H) NMR approach allows an otherwise complex and inscrutable biological sample to be processed in minutes and later analysed both qualitatively and quantitatively. The sample itself requires little to no preparation beyond simple pH correction to control for the chemical shift sensitivities of certain types of molecules (Emwas, 2015; Dona *et al.*, 2016), thereby ensuring they can be effectively compared to spectra standards for identification and paired-samples contrasted accurately. While originally NMR was regarded an insensitive method, spectrometers have developed significantly. Modern 500 MHz

instruments utilising cryo-probes and advanced solvent peak suppression techniques can deliver high quality spectra capable of detecting molecules at the micromolar level. Several large databases such as the Biological Magnetic Resonance Bank and the Human Metabolome Database now exist that catalogue the standard spectra of known molecules. Utilising 3-(trimethylsilyl)propionic-2,2,3,3,d4 acid sodium salt (TMSP) internal standard defines the chemical shift of the spectra. Once referenced it is possible to match chemical shifts within the spectra with chemical reference spectra obtained from the BMRB and HMDB, enabling the rapid identification and qualitative assignment of metabolites within a complex biological sample. When analysing NMR spectra, it is also routine to quantitatively determine the concentrations of biological samples by the addition of a known quantity of TMSP internal standard to the sample. This utilises the principle that the integration area under a peak within a well-resolved NMR spectrum is directly proportional to the number of protons and therefore the concentration of a molecule. After this process the sample remains intact and can be subsequently re-run or stored and run again at a later stage, enabling a high degree of reproducibility. This represents a clear benefit of proton NMR analysis compared with the inherently destructive nature of mass spectrometry. ^{13}C enrichment can still be used to trace metabolic fates via a proton NMR approach as demonstrated by Millerioux *et al.*, (2013), however the overlap of signals due to the relatively constrained chemical shift and higher sensitivity of proton NMR, restricts this to less complex biological samples.

While NMR is clearly a viable technique the low sensitivity inherent to this analytical approach is the key area that most severely limits its application and accuracy in detecting low naturally abundant nuclei such as ^{13}C and ^{15}N, and to a lesser extent impacts ^{1}H analysis as well (Emwas, 2015; Ardenkjaer-Larsen *et al.*, 2015; Markley *et al.*, 2017). The first spectrometers to operate at beyond 1.1 GHz via high-temperature superconducting NMR magnets are on the horizon (Ardenkjaer-Larsen *et al.*, 2015; Markley *et al.*, 2017), with high permittivity coils having already been developed to complement these high-frequency investigations (Ardenkjaer-Larsen *et al.*, 2015). Both a reduction in size of the coil and further reduction in temperature of the probe will lead to greater increases in

sensitivity driving coil sizes to be smaller and smaller, and encouraging the development of techniques to cool probes beyond the liquid nitrogen-cooled probes achieved already (Emwas, 2015; Ardenkjaer-Larsen *et al.*, 2015).

Aims of this Investigation

The aim of this investigation is to refine the current media available for the cultivation of *T. congolense* procyclics, epimastigotes and bloodstream form via an empirically informed and quantitative ^{1}H NMR approach. The essentiality of media components and their relative importance will be determined, non-essential media components as determined by NMR spectroscopy will be experimentally removed and the viability of each media iteration verified by cell counts and comparative growth curves. Where possible the metabolism and biology of *T. congolense* and *T. brucei* will be compared directly and areas of interest for future research purposes will be highlighted.

Materials and Methods

Organisms and Reagents

The trypanosome strains used in this study are listed in Table 2.

Table.2 *T. congolense* experimental strains. All strains utilised in this investigation were of the savannah subtype and are viable in the tsetse host and undergo a complete life cycle.

T. congolense Strain	Animal Isolated	Location	Year	Reference
WG81	Domestic Goat	Matuga Region, Kenya	1981	(Baker and Godfrey, 1988)
1/148 FLY	Domestic Cow	River Donga Region, Nigeria	1960	(Baker and Godfrey, 1988)
IL3000 (Trans Mara I Derivative)	Domestic Cow	Trans Mara Region, Kenya	1966	(Wellde et al., 1974)

Bloodstream form medium was HMI-93 (Hirumi and Hirumi, 1991), which is derived from Iscove's Modified Dulbecco's Medium (IMDM) with 0.05 mM bathocuproine, 1.5 mM cysteine, 0.5 mM hypoxanthine, 0.12 mM 2-mercaptoethanol and 1 mM sodium pyruvate and supplemented with 20% goat serum (Gibco).

Procyclics and epimastigotes were grown in Cunningham's medium (Cunningham, 1977). CM was produced in 2L batches from individual components; the precise formulation is listed in the supplementary information. Before use, the medium was supplemented with 10 mg ml^{-1} gentamycin, 2.5 mg ml^{-1} hemin and 14.2% v/v foetal calf serum (FCS, Gibco); this complete medium is referred to as CM. Minimal media derivatives of Cunningham's medium (e.g. AHR-1) were obtained via omission of certain components or dilution as specified in the text; these media were supplemented with 10 mg ml-1 gentamycin, 2.5 mg ml-1 hemin and 14.2% v/v FCS like CM, unless otherwise stated in the text. All reagents were purchased from Sigma Aldrich, unless specified otherwise.

Passage Regimen

Bloodstream form were maintained in HMI-93 at 37°C in a humidified 5% CO_2 incubator in 1 ml wells in 24 well plates and were typically passaged 1:10 every three days into wells containing fresh medium. Procyclic and epimastigote cultures were maintained in CM and derivatives at 28°C in 1 ml wells in 24 well plates inside a humid chamber or in 5- 10 ml cultures in T25 flasks and typically passaged 1:10 every four days by replacement of spent with fresh medium.

Adaptation to minimal media

Adaptation to minimal media conditions was obtained by centrifugation, washing in PBS and resuspending a stationary culture at 1.6 x 10^7 cells ml^{-1} in minimal media overnight and then sub-passaging with no deleterious effects on growth, morphology, or motility observed. As previously described in the CM analysis 24 well plates of AHR-2 were inoculated with 1x 10^6 cells ml^{-1} 1/148 *T. congolense* procyclics and incubated at 24°C maintained in humid conditions to prevent evaporation and media only control wells were subjected to the same conditions.

Estimation of Trypanosome Numbers

Direct cell counts were obtained using an Improved Neubauer haemocytometer. Cell count data were subsequently transformed, and growth curves generated in Sigmaplot v11.

^{1}H Nuclear Magnetic Resonance Analysis

Samples for NMR were prepared by centrifugation (4000g / 10min / 4°C) to pellet trypanosomes and removal of the supernatant to a clean tube. All samples were pH corrected to 7.4 with NaOH and filter sterilised to ensure that no parasites were transported outside of the licensed lab facility. Medium only controls were prepared and processed in the same way. Samples were stored at -20 °C and chilled during transit to the Bath University Department of Pharmacy and Pharmacology NMR facilities for analysis. Upon arrival a known quantity of TMSP (3-(trimethylsilyl)propionic-2,2,3,3,d4 acid sodium salt) internal reference was added to each sample to enable quantitative estimates.

NMR spectra were obtained using a Bruker Avance III NMR spectrometer operating at 500.13 MHz for ^{1}H. The probe used was a BBFO+ with three channels. Unless otherwise specified samples were analysed at 25 °C using standard Bruker pulse sequences (Topspin 2.1). ^{1}H spectra were acquired with a SW of 20 ppm, and 16 transients. Spectra were referenced using the residual solvent signal, at 7.26 ppm for ^{1}H. Solvent suppression for samples analysed in proteo-solvents was achieved using a presaturation pulse sequence (noesygppr1d). Typical parameters used were; TD 65536 points, 64 transients, spectral width 20.66 ppm and acquisition time 3.17 s, D1 2. NMR analysis was provided by the Chemical Characterisation and Analysis Facility (CCAF) at the University of Bath.

A reference library was compiled from the Biological Magnetic Resonance Data Bank (BMRB) and the Human Metabolome Data Base (HMDB) for media component and predicted end product reference spectra. Mestrenova v11 was used to compile the reference library as well as analyse NMR data via manual phase correction, baseline correction, and integration of signals to facilitate the quantification of media components and metabolic end products via the formula below:

$$\text{Analyte Concentration} = \frac{Normalised\ Area\ Analyte \times Standard\ Concentration}{Normalised\ Area\ Standard}$$

Final spectra slices were assembled and annotated in Inkscape v0.92.

Procyclic Form in Glucose-rich Media

Introduction

Cunningham's medium (Cunningham, 1977) was selected for the initial cultivation of procyclic *T. congolense* to better understand the metabolism of *T. congolense* and to determine essential components for the design of a minimal medium to facilitate differentiation to epimastigote form. A well-established culture medium with a proven track record of effectiveness, CM is based upon tsetse haemolymph (Cunningham and Slater, 1974) and therefore has at least a tangential physiological relationship with the digested blood meal and initial conditions within the tsetse midgut. As preliminary investigations had indicated beta-alanine was not utilised by *T. congolense* and as this amino acid was not in a region contaminated by other signals, it was used in to derive semi-quantitative calculations of depletion of substrates and subsequent synthesis of end products.

Results and Discussion

Changes in medium components were determined from procyclic cultures of *T. congolense* 1/148 grown in CM over a 5-day period. On each day, cell counts and NMR samples were made from replicate wells; the growth curve is presented in Figure 5.

The initial NMR of Cunningham's Media (CM) yielded an impressively data-rich set of spectra (Figures 6A, 6B and 6C, as well as Figures 7A and 7B) that required careful attention to interpret. As described previously trypanosomes utilisation of glucose abundant in the initial tsetse bloodmeal is well characterised, and it was no surprise when the D-glucose signal at 5.25 was depleted fully. This result indicates the culture depleted the 3.89 mM over the course of the 5-day experiment, and that this depletion was almost complete by day 3 (Figure 7A) coinciding with the culture reaching peak density (Figure 4). Of note was the clear observation that the sucrose signal at 5.42 (Figure 6A) is not

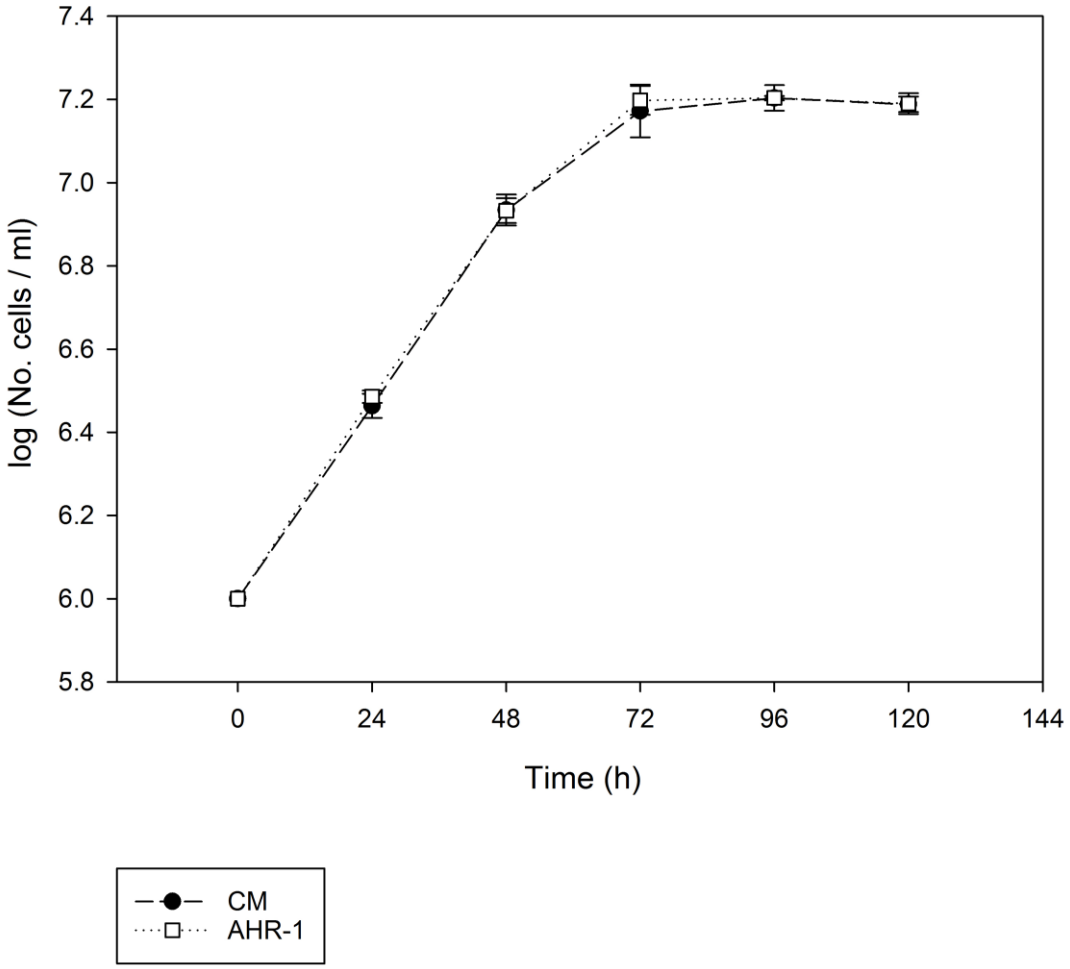


Figure 5. Comparison of growth of *T. congolense* procyclics in Cunningham's Medium and AHR-1 Minimal Medium

Cultures were initiated with 1 x 10^6 cells 1/148 *T. congolense* procyclic cells into 5 ml of CM or AHR-1 medium in T25 flasks at 28 ^oC. Cells grew exponentially for 48hrs and plateaued at 72hrs. The data presented here are counts obtained from five biological replicates.

utilised suggesting that, in common with *T. brucei* (Berriman *et al.*, 2005)*, T. congolense* is unable to hydrolyse disaccharides and this likely reflects the minimal *in vivo* concentrations of sucrose available within the tsetse host. Regrettably due to the significant overlap of signals it was not possible to accurately discern fructose within this initial investigation, although evidence for fructose metabolism is observable in the KEGG pathways of *T. brucei*. There is a full depletion in a signal at 3.56 (Figure 6B) that is likely fructose and this would indicate that *T. congolense* can utilise this

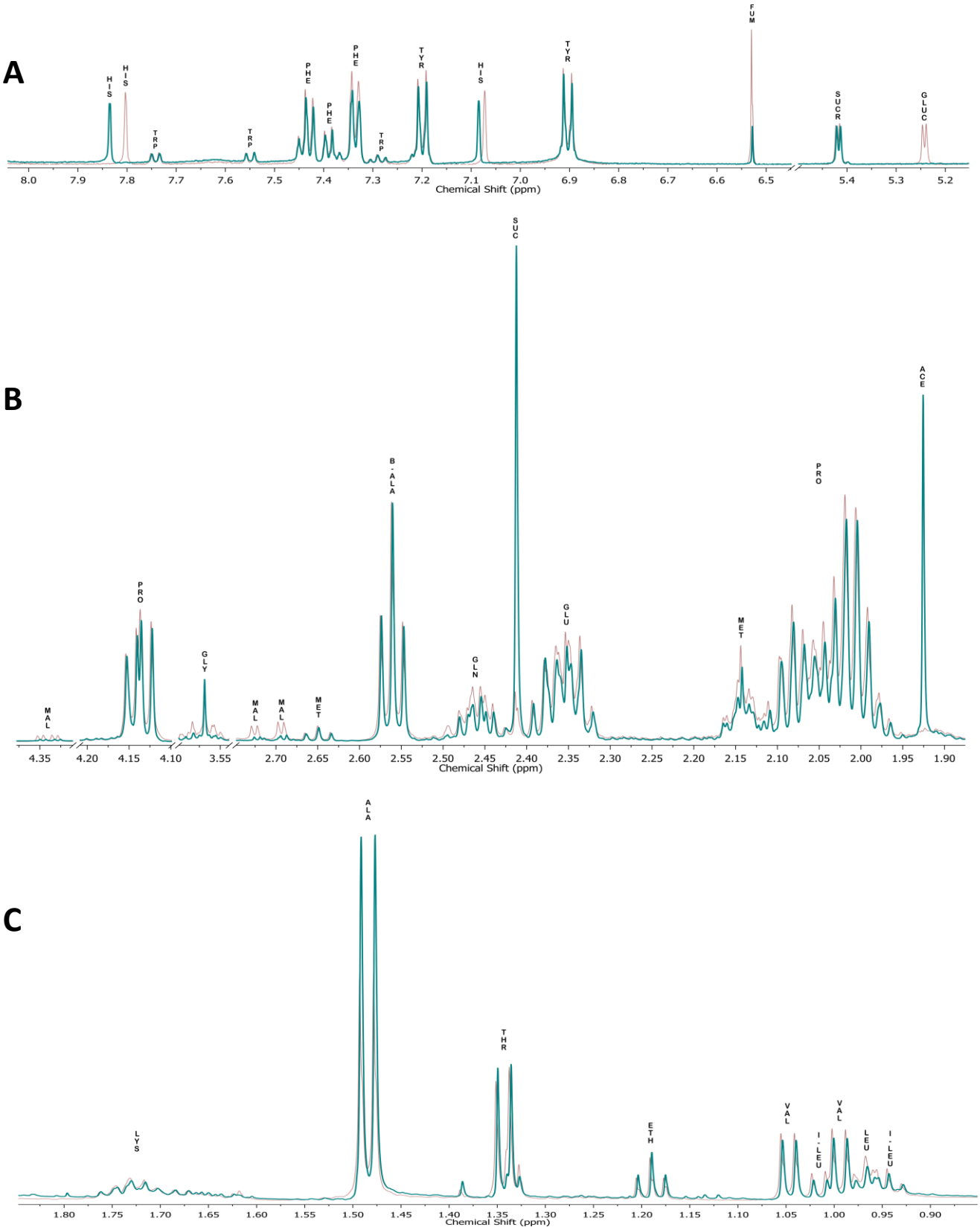


Figure 6 Cunningham's Media ^{1}H NMR Spectra

Proton NMR spectra representing spent medium after the growth of *T. congolense* procyclics for 5 days at 28°C in T25 flasks inoculated with 1 x 10^{6} cells ml^{-1} (**Blue**) superimposed on a medium only control (**Red**). **A** includes aromatic amino acids: L-Histidine (**HIS**), L-Tryptophan (**TRP**), L-Phenylalanine (**PHE**), L-Tyrosine (**TYR**); tricarboxylic acid Fumarate (**FUM**); disaccharide Sucrose (**SUCR**) and monosaccharide D-Glucose (**GLUC**). **B** includes tricarboxylic acids Malate (MAL) and Succinate (**SUC**); aliphatic amino acids: Proline (**PRO**) and Glyine (**GLY**); sulphur containing amino acids: L-Methionine (**MET),** acidic amino acid L-Glutamate (**GLU**); neutral amino acid L-Glutamine (**GLN**); organic acid Acetate (**ACE**). **C** includes the aliphatic amino acids: L-Leucine (**LEU**), L-Isoleucine (**I-LEU**), L- Valine (**VAL**), and L-Alanine (**ALA**); basic amino acid Lysine (**LYS**); and hydroxylic amino acid Threonine (**THR**).

These spectra correspond to a representative experiment from a set of five biological replicates.

Table 2. Summary of 1H NMR Cunninghams Medium Procyclic Form Results. Negative values represent the net depletion in a given media component, while a bold positive value represent the net production of a media component. Media components observed to change include: D-Glucose (**Gluc**), Malate (**Mal**), Fumarate (**Fum**), Succinate (**Suc**), L-Alanine (**Ala**), Acetate (**Ace**), L-Glutamine (**Gln**), Glycine (**Gly**), L-Methionine (**Met**), Proline (**Pro**), Threonine (**Thr**), and L-Valine (**Val**).

Media Components (mM)								
Gluc	**Mal**	**Fum**	**Suc**	**Ala**	**Ace**	**Gln**	**Gly**	**Met**
-3.890	-3.571	-0.240	8.875	3.81	8.239	-1.278	0.674	-0.091
Pro	**Thr**	**Val**						
-7.222	-0.035	-0.225						

hexose sugar in a similar manner to *T. brucei* (Fry *et al.*, 1993). Cunningham's media is unusual in its complement of tricarboxylic acid (TCA) intermediates with no other commonly used trypanosome media containing malate, fumarate, α-ketoglutarate, and succinate as standard. Succinate is the major end-product of *T. congolense* grown in glucose-rich conditions with 8.88 mM produced after 5 days as a clear singlet at 2.45 (Figure 6B), mirroring results observed in *T. brucei* (Lamour *et al.*, 2005; Michels *et al.*, 2006; Coustou *et al.*, 2008).

Fumarate is depleted at a similar percentage as malate with the signal at 6.56 (Figure 6A) decreasing by 75%; however, this represents only a total of 240 µM utilised. Considering the essentiality of fumarate as an electron acceptor in *T. brucei* (Coustou *et al.*, 2006) and the role in *de novo* biosynthesis of pyrimidines in *T. cruzi* (Takashima *et al.*, 2002), the direct uptake of extracellular fumarate is an expected result and indeed the generation of fumarate may be driving the uptake of malate. Any signal relating to α-ketoglutarate is not discernible due to excess levels of HEPES and glutamine at chemical shift 3.0 and 3.4 respectively (Figure 6B).

Cunningham's medium is unusual in its complement of tricarboxylic acid (TCA) intermediates; no other commonly used trypanosome media contain malate, fumarate, α-ketoglutarate, and succinate as standard. A unique malate signal at 4.34 (Figure 6B) is significantly depleted compared to the medium only control and indicates 3.57 mM (71.4% initial concentration) is consumed by the end of the experiment, suggesting that *T. congolense* is capable of direct uptake of malate. The precise fate

of this consumed malate is beyond the scope of this report, but based upon previous investigations it is likely it enters various metabolic pathways as in Obungu *et al.* (1999a) whereby malate derived from glycolysis is converted to pyruvate and subsequently oxidised to generate acetyl CoA and acetate. Alternatively, malate may be converted to fumarate via fumarases and subsequently excreted as succinate (Obungu *et al.*, 1999a; Bringaud *et al.*, 2015).

The amino acid utilisation observable in CM is modest when compared to the extensive utilisation of glucose, malate, and fumarate. Proline is the primary amino acid utilised as indicated by the signal at 4.14 (Figure 6B) with 7.22 mM depleted from the culture medium; this fits well with the pre-existing understanding of *T. congolense* metabolism and is within the same order of magnitude as the result obtained by Brun (1982). Interestingly proline the rate of depletion of proline did not significantly increase when glucose was depleted on day 3 (Figures 7A and 7B); however, this may reflect the residual utilisation of malate and fumarate.

Methionine was depleted by 91 µM, a similar, albeit lower quantity to that obtained by Brun (1982) and it is likely taken up via a similar transporter as that observed in *T. brucei* (Goldberg *et al.*, 2000). The lower quantity depleted in CM as compared to Brun's medium 109c may be due to the proximity of HEPES to this methionine signal, whereby HEPES locally increases the baseline via its broad signal around chemical shift values 2.75 – 2.95 (Figure 6B). The signal at 2.14 (Figure 6B) demonstrates a more significant decrease, but this result is partially obscured by the glutamine signal at the same shift. Methionine plays a vital role in the biosynthesis of trypanothione (Fairlamb *et al.*, 1987; Fairlamb and Cerami, 1992) and therefore a greater amount of methionine degradation might be expected considering the large number of cells cultured. However methionine recycling has been reported in trypanosomatids previously (Berger *et al.*, 1996) and therefore it may be that this comparatively small quantity is sufficient due to efficient intracellular recycling of methionine.

Unexpectedly considering previous results (Cross *et al.*, 1975; Brun, 1982) whereby threonine is depleted, in this investigation the threonine signal at 1.35 (Figure 6C) does not appreciably decrease.

While there is some modest overlap with the lactate signal at 1.32, this does not appear to be the cause of this discrepancy. This may be due to the large amount of exogenous malate present in CM, which is absent in Brun's 109c medium, whereby the pyruvate and subsequent production of acetyl CoA and acetate generated from malate, in combination with that derived from glucose and proline, is sufficient and metabolically favourable over the uptake of threonine. By comparison a valine signal at 1.05 (Figure 6C) decreases moderately by 224 μM and this is similar result to that obtained by Brun (1982). Valine does not feature in the literature surrounding *Trypanosoma* metabolism, but upon examination of the KEGG pathways for *T. brucei* it seems likely that *T. congolense* may be oxidising valine ultimately towards generating acetyl CoA. Leucine and Iso-leucine both demonstrably decrease (Figure 6C), but it is not possible to separate these signals in the present investigation due to a signal from valine at 0.95 (Figure 6C) and some overlap between these signals. Based upon examination of the *T. brucei* pathway it is likely these branched amino acids are oxidised to acetyl CoA in a similar fashion to valine; however these are not well characterised pathways and at present it is unclear whether branched amino acids have a role in sterol biosynthesis in trypanosomes (Carrero-Lérida *et al.*, 2009).

For the signals of the amino acids: tryptophan 7.54, phenylalanine 7.43 and tyrosine 0.12 (Figure 6A), and lysine 1.73 (Figure 6C) no discernible decreases are observed, suggesting these amino acids are not utilised or the amount utilised is lower than the detectability threshold of this method. Glutamine signals at chemical shift 2.46 decrease markedly (Figure 6B). This is an expected result as in related kinetoplastids the pathways for pyrimidine biosynthesis from glutamine are well characterised (Hammond and Gutteridge, 1982, 1984b). The KEGG pathways for *T. brucei* indicate the gene is present for the aptly named enzyme, glutamine hydrolysing carbomoyl phosphate synthase [EC:6.3.1.2]. Glutamine is therefore likely being hydrolysed to form carbomoyl phosphate, and a complete pathway is present for the generation of the first pyrimidine nucleotide uridine monophosphate in *T. brucei* from this stage. It is notable that while a pathway is present for the synthesis of glutamine via glutamate, this likely plays a prominent role in the procyclic lifestage

where proline is in greater abundance than glutamine (Aksoy *et al.*, 2003) and the proline-derived glutamate may be converted to glutamine, as well as to alpha-ketoglutarate and alanine.

Regarding the partially oxidised end-products of *T. congolense* metabolism: at the end of the 5 days succinate was the main product with a signal at 2.4 (Figure 6B and 7B) corresponding to an increase of 8.88 mM, closely followed by 8.24 mM of acetate produced and observable at 1.93 (Figure 6C and 7B). The alanine signal around 1.5 also increases substantially by 3.82 mM (Figure 6C), likely arising as the transamination product of pyruvate (Obungu *et al.*, 1999b; Lamour *et al.*, 2005; Coustou *et al.*, 2008). As in previous investigations, only trace amounts (720 µM) of glycine are produced based on the signal at 3.56 (Figure 6B). This likely reflects its role in the generation of ammonia via glycine cleavage in order to maintain nitrogen balance via the conversion of aspartate to asparagine (Loureiro *et al.*, 2013) or participation in the trypanothione generation pathway as outlined by Fairlamb and Cerami (1992) and observed in *T. brucei* by Millerioux *et al.* (2013). The amount of CO_2 produced as an end product of *T. congolense* metabolism was not quantified by NMR.

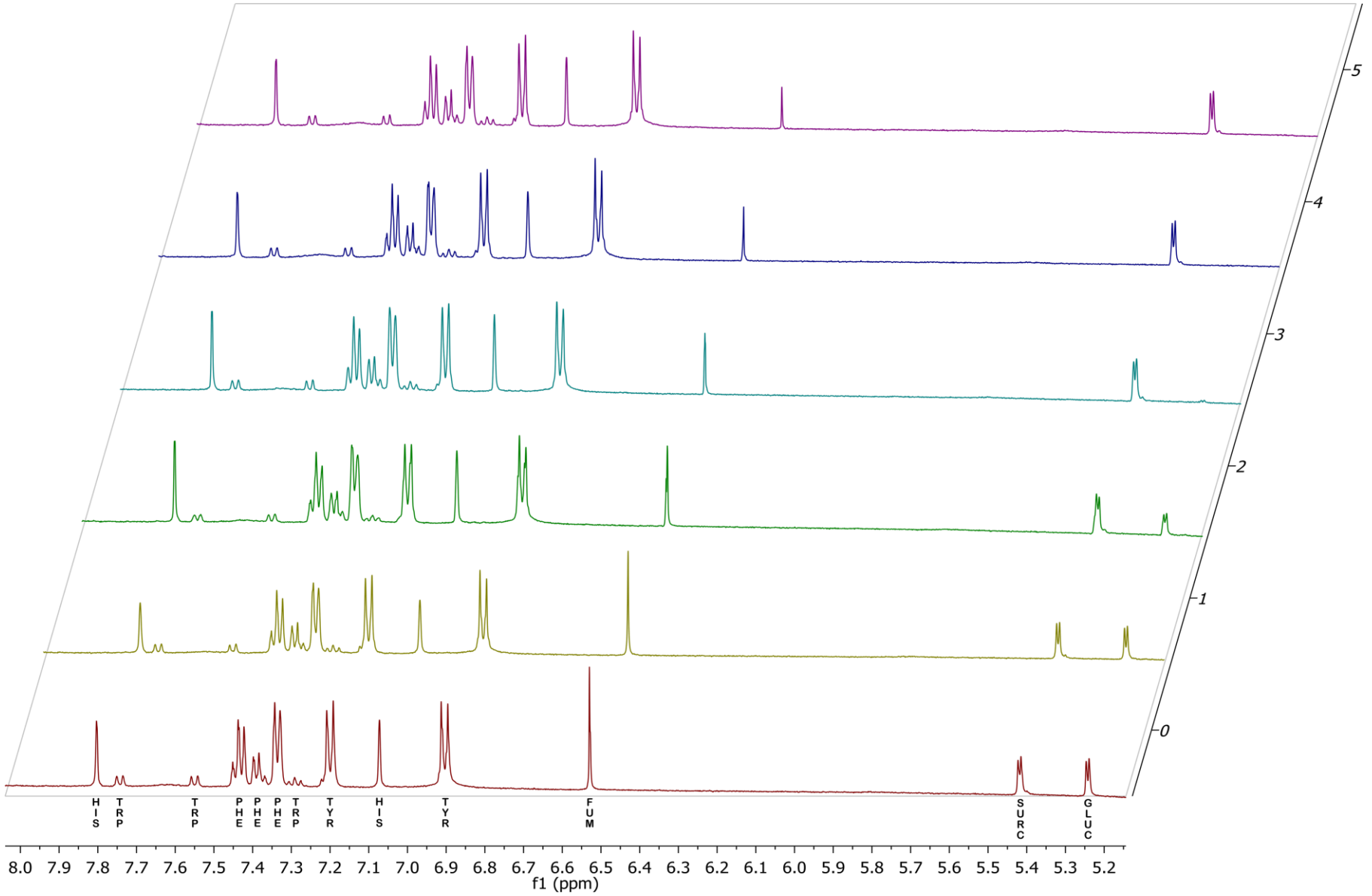


Figure 7A Cunningham's Medium ^{1}H NMR Spectra

Proton NMR spectra representing samples obtained over 5 days of growth of *T. congolense* 1/148 procyclics at 28°C in 24-well plates inoculated with 1 x 10^{6} cells ml^{-1} with each day colour coded as follows: Day 0 (**Red**), Day 1 (**Yellow**), Day 2 (**Green**), Day 3 (**Cyan**), Day 4 (**Blue**) and Day 5 (**Purple**). The region displayed includes the: aromatic amino acids: L-Histidine (**HIS**), L-Tryptophan (**TRP**), L-Phenylalanine (**PHE**), L-Tyrosine (**TYR**); tricarboxylic acid Fumarate (**FUM**); disaccharide Sucrose (**SUCR**) and monosaccharide D-Glucose (**GLUC**). These spectra correspond to a representative experiment from a set of five biological replicates.

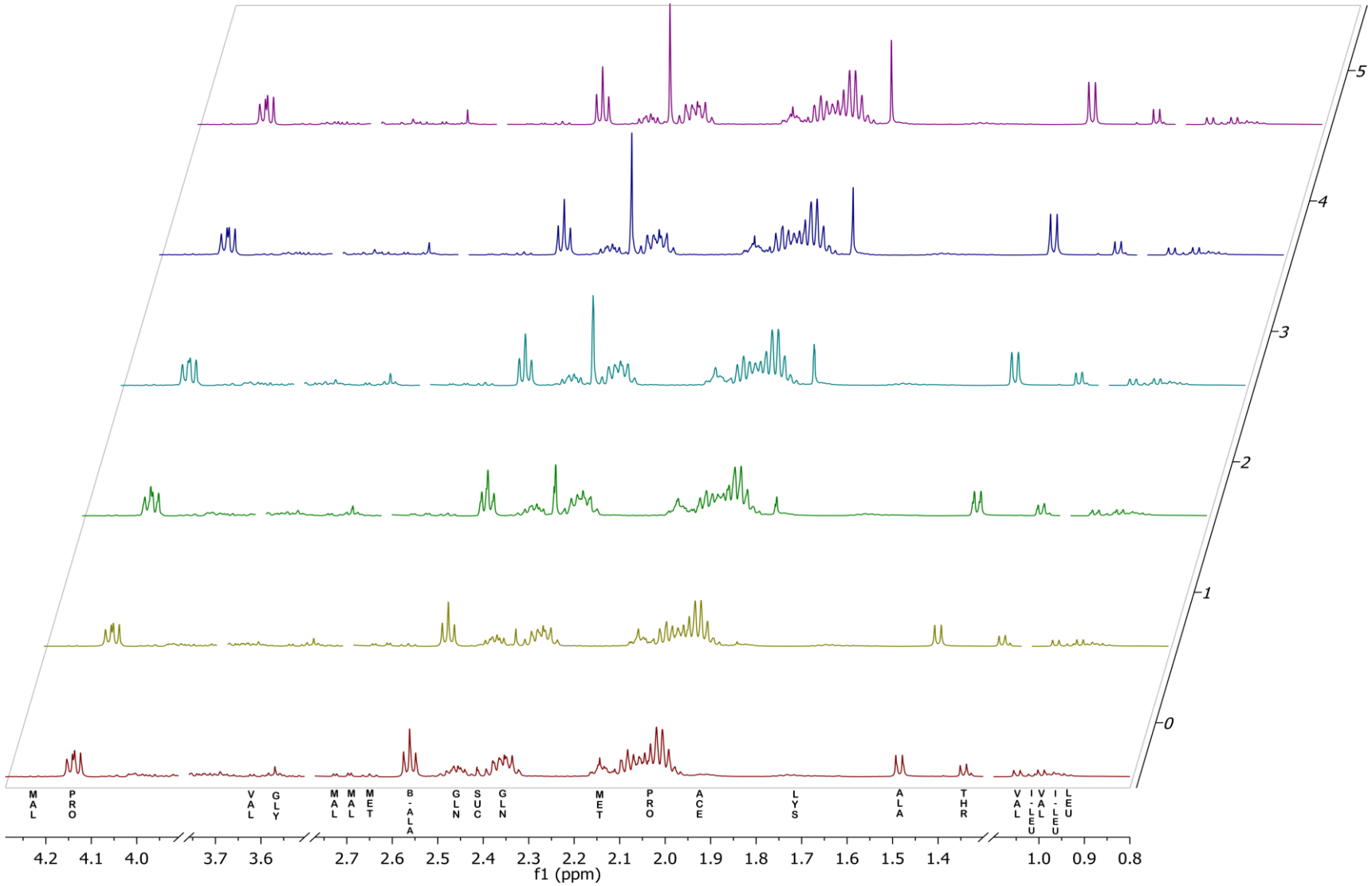


Figure 7B Cunningham's Media ^{1}H NMR Spectra

Proton NMR spectra representing samples obtained over 5 days of growth of *T. congolense* 1.148 procyclics at 28°C in 24-well plates inoculated with 1 x 10^{6} cells ml^{-1} with each day colour coded as follows: Day 0 (**Red**), Day 1 (**Yellow**), Day 2 (**Green**), Day 3 (**Cyan**), Day 4 (**Blue**) and Day 5 (**Purple**). The region displayed includes the: includes tricarboxylic acids Malate (**MAL**) and Succinate (**SUC**); aliphatic amino acids: Proline (**PRO**) and Glycine (**GLY**); sulphur containing amino acids: L-Methionine (**MET),** acidic amino acid L-Glutamate (**GLU**); amidic amino acid L-Glutamine (**GLN**); organic acid Acetate (**ACE**). **C** includes the aliphatic amino acids: L-Leucine (**LEU**), L-Isoleucine (**I-LEU**), L- Valine (**VAL**), and L-Alanine (**ALA**); basic amino acid Lysine (**LYS**); and hydroxylic amino acid Threonine (**THR**). These spectra correspond to a representative experiment from a set of five biological replicates.

Procyclic Minimal Media AHR-1 and AHR-2

Introduction

Based upon the CM results described above, new minimal media were designed to better reflect the low to zero glucose conditions procyclics experience in the tsetse midgut and within the ectoperitrophic space (Vickerman, 1985), enabling an assessment of *T. congolense* metabolism in glucose-poor, proline-rich minimal media. These media were designated AHR-1 and AHR-2.

In AHR-1 all sugars as well as all TCA intermediates were left out as, despite the clear utilisation demonstrated by the CM results, these substrates are unlikely to persist in the tsetse midgut for any significant period (Vickerman, 1985; Aksoy *et al.*, 2003). Glycine was also removed, as the CM results and previous literature demonstrated its role as an end-product of trypanosome metabolism (Figure 6B, Lamour *et al.*, 2005). Beta alanine was removed as preliminary results and the KEGG pathways indicated that it is a metabolically inert substrate neither increasing or decreasing regardless of parasite presence. Alanine was retained, despite being a product of metabolism, as it serves to aid the maintenance of osmolarity within the medium. Glutamine was omitted largely due to concerns regarding its lability giving false results and potential toxicity issues associated with the generation of ammonia, particularly as this reaction was favourable at room temperature and under acidic conditions. Initially it was expected that glutamine might prove important to trypanosomes as it is present in all other trypanosome media and is involved in the pyrimidine biosynthesis pathways as previously discussed (Hammond and Gutteridge, 1984b); however no impact on cell viability or growth rate was observable in procyclics cultured in AHR-1 (Figure 5). Glutamate was omitted also as it appeared to have little importance according to the literature (Brun, 1982; Lamour *et al.*, 2005) and is readily available within the proline degradation pathway. Due to the inconclusive nature of threonine results prior to this experiment (Figure 6C) and suggested importance of threonine within the literature (Cross *et al.*, 1975; Brun, 1982; Millerioux *et al.*, 2013; Ong *et al.*, 2015) the

supplementation of this amino acid was increased to 4.5 mM to ensure it was available in excess quantities and to minimise the impact of the overlapping lactate signal.

As many media components still appeared to be in excess, a dilution series was undertaken whereby AHR-1 was diluted by 50%, 25% and 10% into a CM salts solution, and cell counts over 5 days were obtained to determine the impact on cell viability and growth rate. Notably 50% and 25% proved satisfactory and in fact were indistinguishable from cultures grown in stock AHR-1 (Supplementary Information), whereas the 10% dilution demonstrated a marked decrease in growth rate (Data Not Shown). The 25% was therefore selected as the final minimal medium named AHR-2 and subsequent NMR analysis was carried out on this medium to further our understanding of *T. congolense* metabolism in proline-based media, and potentially develop a minimal medium conducive to differentiation to epimastigote form.

Changes in medium components were determined from procyclic cultures of *T. congolense* 1/148 grown in AHR-1 or AHR-2 over a 5-day period. On each day, cell counts, and NMR samples were made from replicate wells; the growth curves are presented in Figure 8. It can be seen that AHR-1 and AHR-2 support equivalent rates of growth and final density to each other and to CM (Figure 5). As AHR-2 was a more minimal medium than AHR-1, this was taken forward for NMR analysis (Figures 9 and 10).

Results and Discussion

The minimal aspect of AHR-2 yielded a far simpler set of spectra (Figures 9 and 10) to interpret than those obtained in the richer CM. While no additional sugars or purines were components of AHR-2, FCS contributed micromolar amounts of these substances to the medium. The trace amounts of glucose and hypoxanthine were detectable and are labelled at chemical shifts 5.25 and 8.2 (Figure 9A) respectively, and it is significant that both components were depleted within one day of the experiment commencing (Figure 10A).

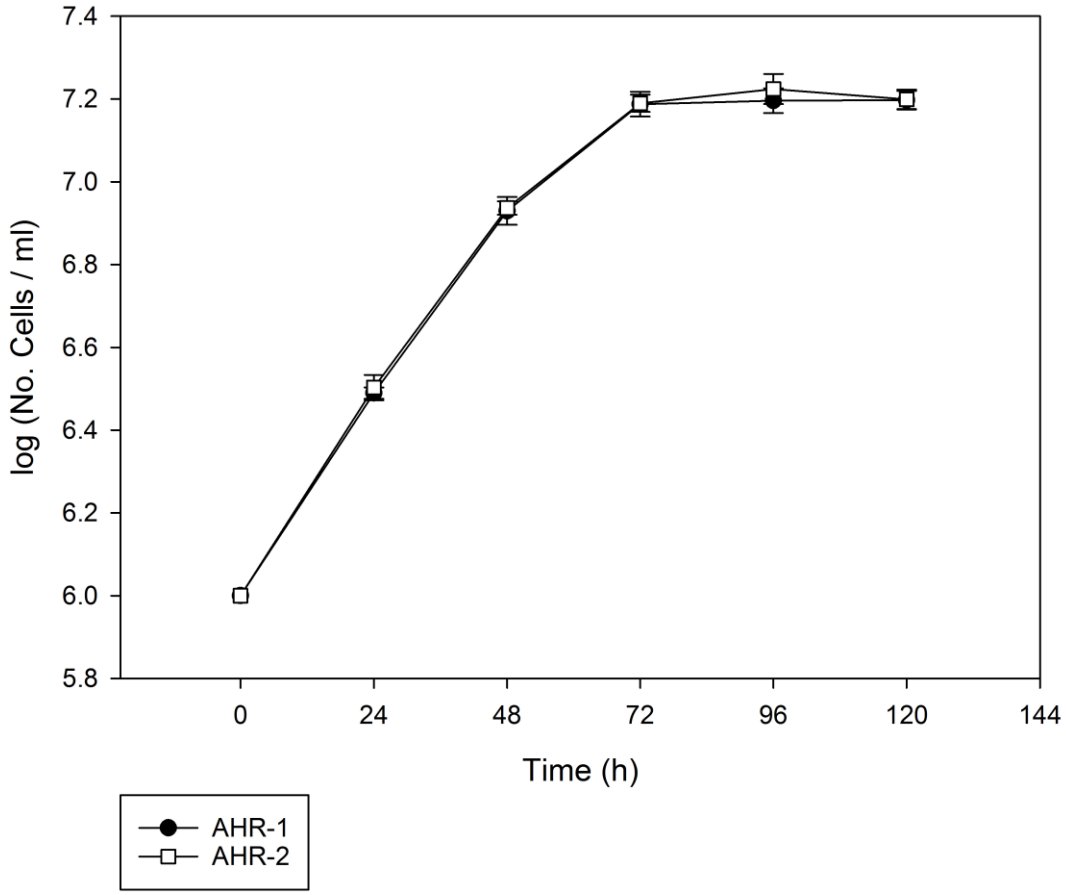


Figure 8. Growth of *T. congolense* Procyclics in AHR-1 and AHR-2 Minimal Media

Cultures were initiated with 1 x 10^6 cells ml^{-1} 1/148 *T. congolense* procyclic cells into 5 ml of AHR-1 and AHR-2 media in T25 flasks or 24-well plates at 28 ^oC.

Cells grew exponentially for 48hrs with a final increase in cell number observed after 72hrs. Having reached peak density at 72 hrs cell number remained stationary until 120hrs and steadily declined from that point onwards. The growth of *T. congolense* procyclics in minimal medium AHR-2 is equivalent to that achievable in AHR-1 medium. The data presented here are counts obtained from four biological replicates.

As observed in *T. congolense* procyclics grown in glucose-rich conditions (Figure 6B), proline was the amino acid primarily utilised by procyclics with the proline signal at 4.15 (Figure 9B) indicating that 6.75 mM was consumed by the end of the experiment.

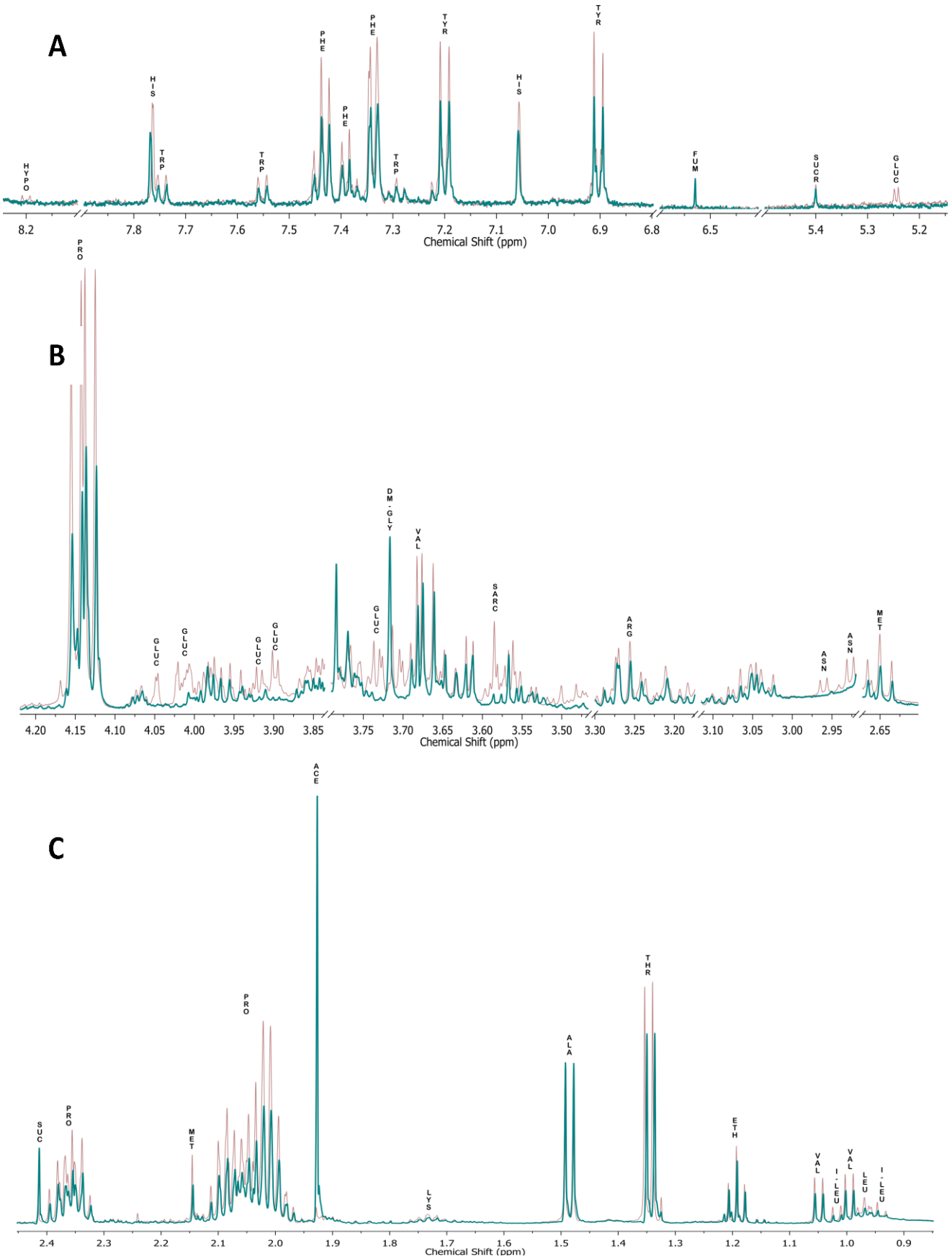


Figure 9 AHR-2 Media ^{1}H NMR Spectra

Proton NMR spectra representing spent medium after the growth of *T. congolense* procyclics for 5 days at 28°C and in T25 flasks inoculated with 1 x 10^{6} cells ml^{-1} (**Blue**) alongside a media only control (**Red**). **A** includes aromatic amino acids: L-Histidine (**HIS**), L-Tryptophan (**TRP**), L-Phenylalanine (**PHE**), L-Tyrosine (**TYR**); tricarboxylic acid Fumarate (**FUM**); disaccharide Sucrose (**SUCR**) and monosaccharide D-Glucose (**GLUC**). **B** includes tricarboxylic acid Succinate (**SUC**); aliphatic amino acids: Proline (**PRO**) and Glycine (**GLY**); basic amino acid arginine (**ARG**); amidic amino acid Asparagine (**ASN**) sulphur containing amino acids: L-Methionine (**MET**); organic acid Acetate (**ACE**). **C** includes the aliphatic amino acids: L-Leucine (**LEU**), L-Isoleucine (**I-LEU**), L- Valine (**VAL**), and L-Alanine (**ALA**); basic amino acid Lysine (**LYS**); and hydroxylic amino acid Threonine (**THR**).

These spectra correspond to a representative experiment from a set of four biological replicates.

Table 3. Summary of 1H NMR AHR-2 Medium Procyclic Form Results. Negative values represent the net depletion in a given media component, while a bold positive value represents the net production of a media component. Media components observed to change include: Fumarate (**Fum**), Succinate (**Suc**), L-Alanine (**Ala**), Acetate (**Ace**), Arginine (**Arg**), Asparagine (**Asn**), Glycine (**Gly**), L-Histidine (**His**), L-Isoleucine (**I-Leu**), L-Leucine (**Leu**), L-Methionine (**Met**), L-Phenylalanine (**Phe**), Proline (**Pro**), Threonine (**Thr**), L-Tryptophan (**Trp**), L-Tyrosine (**Tyr**), and L-Valine (**Val**).

Media Components (mM)								
Fum	**Suc**	**Ala**	**Ace**	**Asn**	**Arg**	**Gly**	**His**	**I-Leu**
0.025	**0.375**	**1.400**	**3.43**	-0.240	-0.200	**0.900**	-0.150	-0.283
Leu	**Met**	**Phe**	**Pro**	**Thr**	**Trp**	**Tyr**	**Val**	
-0.250	-0.220	-0.240	-6.750	-0.967	-0.150	-0.225	-0.483	

Methionine depletion was far easier to isolate and quantify in minimal medium and the signal at 2.15 (Figure 9C) decreased substantially by 220 µM, and this result is concordant with that obtained by Brun (1982). Within this investigation it is not possible to state whether methionine metabolism is altered by carbohydrate source as the methionine results of the CM spectra (Figure 6C) were difficult to separate from the surrounding glutamate signals. If the wider literature is considered however, the results obtained by Brun (1982) in the glucose rich 109c medium (210 µM) were similar to those obtained in this investigation and it is likely that methionine metabolism is not linked to the carbohydrate source. As mentioned previously, methionine is important in the generation of trypanothione, but also important in this pathway is the amino acid arginine and the conversion of arginine to ornithine via an arginase [EC:3.5.3.1]. A complete pathway for arginine metabolism to generate trypanothione is outlined when examining the *T. brucei* KEGG pathway for arginine and proline metabolism, although subsequent liquid chromatography and mass spectrometry conducted by Hai *et al.* (2015) indicate this pathway was not functional in bloodstream form. In other trypanosomatids such as *Leishmania,* arginase activity has been observed (Roberts *et al.*, 2004). Considering the differences already observed between *T. congolense* and *T. brucei* arginine metabolism this likely requires further investigation. Within the previous CM investigation, it was not possible to observe any arginine signals due to the broad HEPES signals. In AHR-2 spectra a signal at 3.25 (Figure 9B) is observable as well as a moderate depletion of 200 µM, although despite careful

integration of this signal, there is possible contamination by a nearby glucose multiplet at 3.21 and it may represent an overestimation. Further investigation of this amino acid is therefore required, particularly as Brun (1982) also observed inconsistent results.

It was also possible to observe asparagine signals at 2.92 and 2.96 (Figure 9B) and while it would appear at first glance that the signals are fully depleted, this result is difficult to interpret due to the broad HEPES signal in this region resulting in a highly erratic baseline. It is therefore difficult to confidently state whether the signal was fully depleted, but it can be concluded that a substantial amount of this amino acid has been utilised. This result mirrors those obtained by Brun (1982) whereby all the asparagine was utilised in glucose rich conditions, although this result was curiously absent from their discussion. The role of asparagine remains poorly understood, although it has been suggested it may be necessary for the maintenance of nitrogen balance and protein synthesis as demonstrated by Loureiro *et al.* (2013). In that study, knockdown of the enzyme responsible for the generation of asparagine from aspartate, asparagine synthetase (AS-A), hampered trypanosome growth under experimental conditions. This reduced growth phenotype was subsequently alleviated by addition of exogenous asparagine to the culture media (Loureiro *et al.*, 2013). Within this investigation signals for aspartate were partially occluded by HEPES signals and therefore it was not possible to verify if this process was occurring in *T. congolense*. AS-A is however also present *T. cruzi* (Loureiro *et al.*, 2013) and in *Leishmania infantum* (Faria *et al.*, 2016), suggesting its possible presence in *T. congolense.* Notably in the KEGG pathway for *T. brucei* the enzyme responsible for the inverse process to convert asparagine to aspartate is not present, suggesting that nitrogen balance phenotypes may well be highly conserved in *Trypanosoma spp.* Time series data indicate that asparagine is depleted by day 3 (Figure 11A), coinciding with peak density, and signals that are likely aspartate at 2.66 continue to diminish past this point; however, the effect of HEPES is both pervasive and pernicious.

While a nebulous result was obtained in the previous CM NMR analysis for threonine, a clear threonine result was obtained in AHR-2 medium whereby the signal at 1.34 (Figure 9C) decreased substantially by 967 µM. This represents a greater decrease than the 510 µM observed previously (Brun, 1982); however in 109c medium that figure represented a complete depletion of the available threonine. The value obtained within this investigation may represent a closer approximation of the saturating concentration of threonine. Similarly, valine decreases by a greater degree in AHR-2 than in CM with the signal at 1.05 (Figure 9C) indicating 483 µM was depleted. Isoleucine and leucine signals at shifts 1.04 and 0.97 (Figure 9C) also decreased and in AHR-2 it was far easier to resolve the individual signals from baseline noise; with the minimal overlap present it was also possible to estimate decreases of 283 µM and 250 µM respectively. Unpicking the precise metabolic fates of these individual amino acids is beyond the scope of this report and will require further investigation. However, as described previously and with reference to the KEGG pathways of *T. brucei*, it is likely that procyclic *T. congolense* are metabolising threonine, valine, isoleucine and leucine ultimately towards generating acetyl CoA, acetate, and feeding the lipid/sterol biosynthesis pathways (Cross *et al.*, 1975; Millerioux *et al.*, 2013; Ong *et al.*, 2015). It is of note that threonine depletion (967 µM) is almost 2-fold greater than valine depletion (483 µM) and more than 3-fold greater than isoleucine and leucine, suggesting a clear preference, perhaps due to greater transport capability or potentially more efficient pathways for threonine than for the breakdown of the branched-chain amino acids. Time series data (Figure 11B) suggest these amino acids are being taken up simultaneously and not with any sequential preference.

The aromatic amino acids tryptophan, tyrosine and phenylalanine with signals: 7.55, 7.4, and 6.9 respectively (Figure 9A) all decrease substantially by approximately 225 µM and 238 µM for tyrosine and phenylalanine, and modestly by 150 µM for tryptophan. The role of aromatic amino acids in the metabolism of *Trypanosoma* spp. is strongly linked to transamination activities (Stibbs and Seed, 1975a; b; Nowicki and Cazzulo, 2008). In *T. congolense* it is likely important in the methionine recycling pathway, whereby the methylthioadenosine generated in the essential polyamine and

trypanothione biosynthesis pathways can be salvaged via a-ketomethiobutyrate and transamination of tryptophan, tyrosine and phenylalanine to produce methionine (Berger *et al.*, 1996). Hydroxyphenylpyruvate, phenylpyruvate, indolepyruvate are also produced as a result of this pathway and while the possible effects of these end products of transamination are still an area of interest they were not detectable in this investigation. It would be beneficial to elucidate this pathway in *T. congolense* as there appear to be significant differences observed in *T. cruzi* and other trypanosomatids (Nowicki and Cazzulo, 2008; Marciano *et al.*, 2009).

The partially oxidised end products of *T. congolense* change substantially in procyclics grown in minimal medium, not in the type of product produced, but in the ratios observed. Acetate signal at 1.94 is now the primary product with 3.43 mM produced, followed by alanine (1.5) with 1.4 mM generated and only a minor product 375 µM of succinate (Figure 9C). This indicates a far more efficient metabolism with lower amounts of product generated overall and the time series data (Figure 11B) indicate that these products may in fact be reincorporated as the levels of acetate and alanine peak on day 3 and fluctuate on days 4 and 5. Further investigation of this result is required however, and the uptake of alanine as an osmolyte would not be remarkable. An entirely new product not observable in the CM spectra and therefore of particular interest, is the production of a signal at 6.5 (Figure 9A). This signal is putatively annotated as fumarate as it matches no other molecule from the reference library compiled for this investigation except fumarate, but subsequent 2D analysis would be required to confirm this result. Time series data (Figure 11A) indicate that fumarate is gradually produced over the experiment period and a distinct peak is observable from day 1 of the experiment. Glycine is no longer a product and instead dimethylglycine is produced at 3.71 (Figure 9B) and while it is possible the glycine signal may have shifted, this seems unlikely as no other signal has significantly changed in chemical shift and all the samples analysed were prepared carefully to ensure chemical shifts were consistent between experiments.

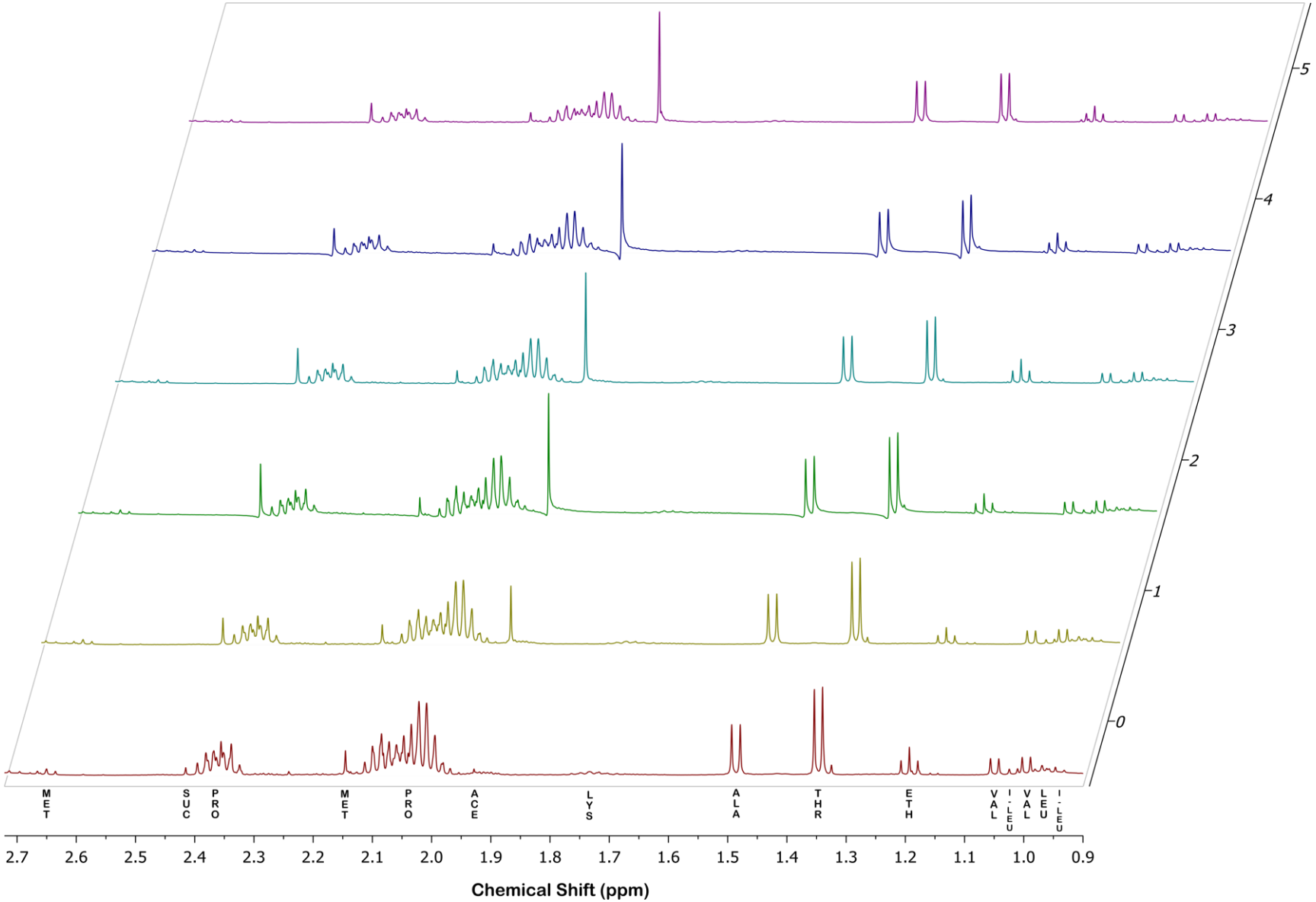


Figure 10A AHR-2 ^{1}H NMR Spectra

Proton NMR spectra representing samples obtained over 5 days of growth of *T. congolense* 1.148 procyclics at 28°C in 24-well plates inoculated with 1 x 10^{6} cells ml^{-1} with each day colour coded as follows: Day 0 (**Red**), Day 1 (**Yellow**), Day 2 (**Green**), Day 3 (**Cyan**), Day 4 (**Blue**) and Day 5 (**Purple**). The region displayed includes the: aromatic amino acids: L-Histidine (**HIS**), L-Tryptophan (**TRP**), L-Phenylalanine (**PHE**), L-Tyrosine (**TYR**); tricarboxylic acid Fumarate (**FUM**); disaccharide Sucrose (**SUCR**) and monosaccharide D-Glucose (**GLUC**).

These spectra correspond to a representative experiment from a set of five biological replicates.

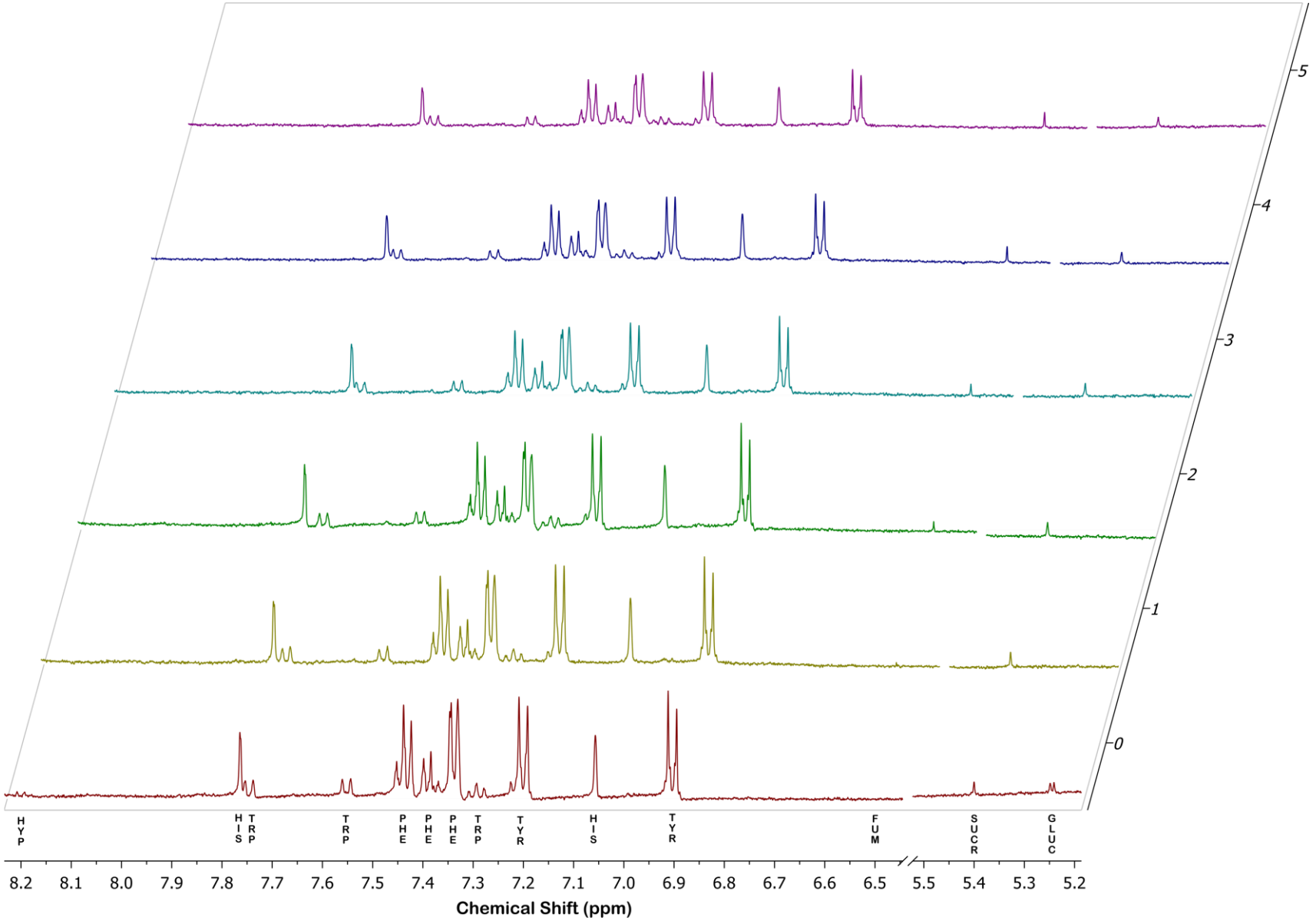


Figure 10B AHR-2 ^{1}H NMR Spectra

Proton NMR spectra representing samples obtained over 5 days of growth of *T. congolense* 1.148 procyclics at 28°C in 24-well plates inoculated with 1 x 10^{6} cells ml^{-1} with each day colour coded as follows: Day 0 (**Red**), Day 1 (**Yellow**), Day 2 (**Green**), Day 3 (**Cyan**), Day 4 (**Blue**) and Day 5 (**Purple**). The region displayed includes the: sulphur containing amino acid: L-Methionine (**MET),** organic acid Acetate (**ACE**); the aliphatic amino acids: Proline (**PRO**), Glycine (**GLY**), (L-Leucine (**LEU**), L-Isoleucine (**I-LEU**), L- Valine (**VAL**), and L-Alanine (**ALA**); basic amino acid Lysine (**LYS**); and hydroxylic amino acid Threonine (**THR**). These spectra correspond to a representative experiment from a set of four biological replicates.

Epimastigote Form

Introduction

To better understand the culture requirements of epimastigotes and further our understanding of the possible metabolic differences and peculiarities present in *T. congolense*, Cunningham's media (Cunningham, 1977) was once again utilised. As epimastigote metabolism is poorly characterised at present it was deemed prudent to provide a rich culture medium, and thereby providing the greatest variety of substrates, to determine any initial preferences. Initial culture conditions were assessed and are briefly summarised here: T25 flasks were seeded with ca. 2 x 10^6 cell ml^{-1} of *T. congolense* WG81 epimastigotes in five millilitres of pH 7.4 Cunningham's medium and transferred to a 24 ^oC incubator. Attachment occurs as a continuous layer of anteriorly attached cells forming proliferative rosettes across the surface of the flask eventually reaching a macroscopic level after three days, at this stage the pH has decreased to ca. 6 - 6.4 and there are few to no unattached cells present in the column. Flasks were kept for a further three days with no change in media and after this period the contents of the flask was centrifuged, and the supernatant retained for NMR analysis as described previously. TMSP was utilised only as a chemical shift reference and was not added to the samples as a quantitative internal standard. As preliminary investigations had indicated beta-alanine was not utilised by *T. congolense* and as this amino acid was not in a region contaminated by other signals, it was used in to derive semi-quantitative calculations of depletion of substrates and subsequent synthesis of end products.

Results and Discussion

Changes in medium components were determined from epimastigote cultures of *T. congolense* WG81 grown in CM over a 5-day period. It was not possible to perform cell counts, as the cells were firmly attached to the surface of the flask.

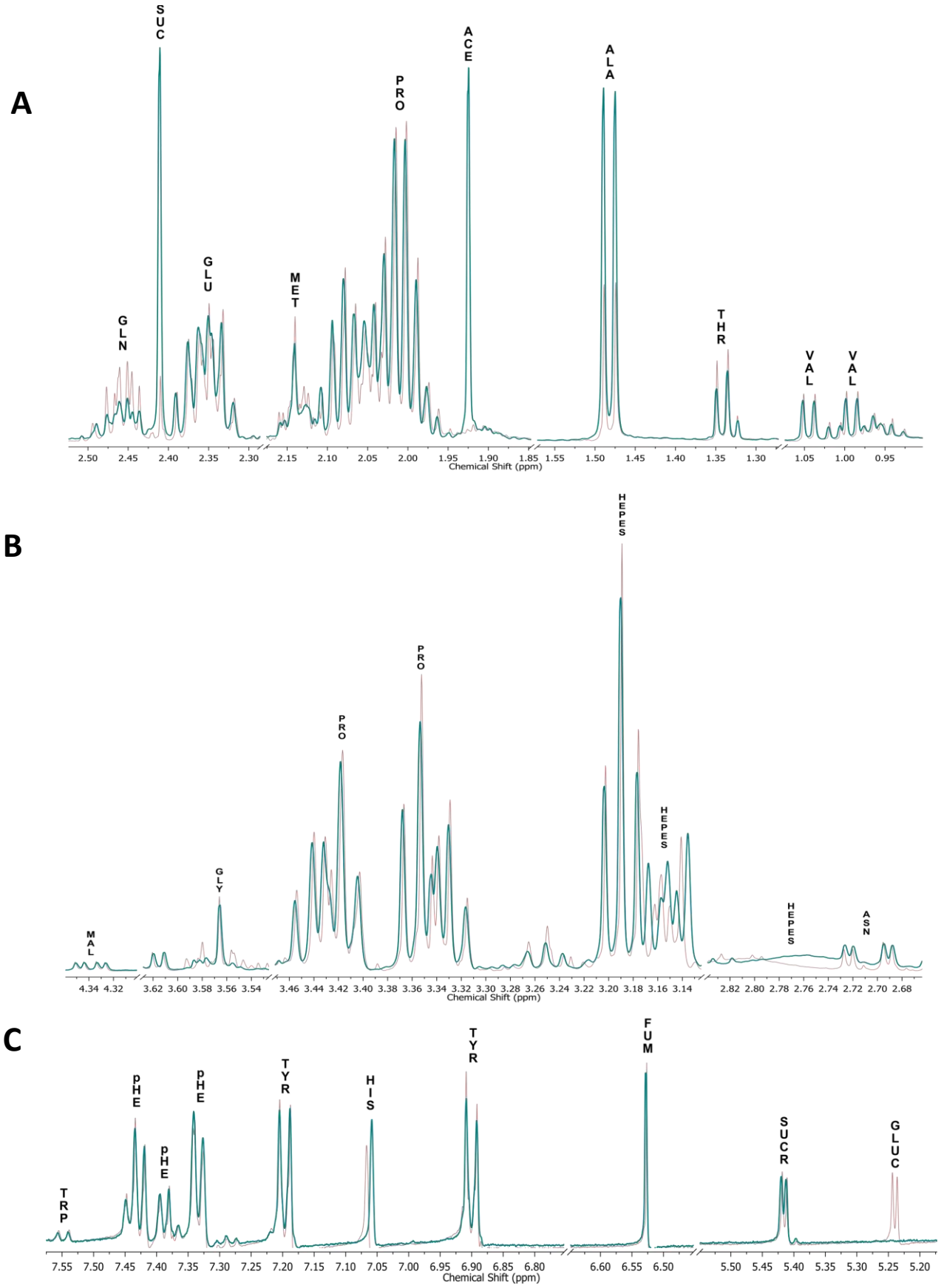


Figure 11 Cunningham's Media ^{1}H NMR Spectra

Proton NMR spectra representing spent media after the growth of *T. congolense* epimastigote form for 6 days at 24°C T-25 flasks inoculated with seeded with ca. 2 x 10^{6} cell ml^{-1} (**Blue**) alongside a media only control (**Red**). **A** includes aromatic amino acids: L-Histidine (**HIS**), L-Tryptophan (**TRP**), L-Phenylalanine (**PHE**), and L-Tyrosine (**TYR**); tricarboxylic acid Fumarate (**FUM**); disaccharide Sucrose (**SUCR**) and monosaccharide D-Glucose (**GLUC**). **B** includes tricarboxylic acid Succinate (**SUC**); aliphatic amino acids: Proline (**PRO**) and Glycine (**GLY**); amidic amino acid aspartate (**ASN**) sulphur containing amino acids: L-Methionine (**MET**); organic acid Acetate (**ACE**). **C** includes the aliphatic amino acids: L- Valine (**VAL**), and L-Alanine (**ALA**); basic amino acid Lysine (**LYS**); and hydroxylic amino acid Threonine (**THR**).

These spectra correspond to a representative experiment from a set of three biological replicates.

Table 4. Summary of 1H NMR Cunningham's Medium Epimastigote Form Results. Negative values represent the net depletion in a given media component, while a bold positive value represent the net production of a media component. Media components observed to change include: D-Glucose (**Gluc**), Malate (**Mal**), Fumarate (**Fum**), Succinate (**Suc**), L-Alanine (**Ala**), Acetate (**Ace**), L-Glutamine (**Gln**), L-Methionine (**Met**), Proline (**Pro**), Threonine (**Thr**), L-Tryptophan (**Trp**), and L-Tyrosine (**Tyr**).

Media Components (mM)								
Gluc	**Mal**	**Fum**	**Suc**	**Ala**	**Ace**	**Gln**	**Met**	**Pro**
-3.890	-0.5	-0.23	**5.62**	**10.26**	**7.49**	-6.29	-0.34	-7.53
Thr	**Trp**	**Tyr**						
-0.21	-0.21	-0.11						

at 5.56 (Figure 11C) epimastigotes clearly retain the glycolytic pathways present in the previous procyclic, likely scavenging the transient presence of glucose in the tsetse bloodmeal before it passes into the insect's crop. Upon initial inspection the spectra generated by epimastigote cultures grown in glucose-rich Cunningham's media (Figures 11A, 11B and 11C) bear a striking resemblance to those obtained for procyclics grown under the same conditions. As demonstrated by the depletion of the glucose signal seems unlikely that significant amounts of blood remain in the tsetse mouthparts, with any residue most likely dislodged by the flow of saliva; however, to this authors knowledge this has not been investigated.

Unusually TCAs are not utilised as much as in procyclics with the malate signal at 4.34 only decreases moderately by 500 µM (Figure 11B) rather than the 3.57 mM observed in procyclics (Figure 11B), however fumarate signal at 6.56 decreases by a similar amount of 224 µM (Figure 11B). The most intriguing difference in the TCAs results however is in the succinate signal at 2.41 (Figure 11C). While there is still a substantial increase of 5.61 mM indicating its role as a significant metabolic end-product, the increase is considerably less than in procyclics (8.88 mM, Figure 6C). This likely represents the lack of malate utilisation, and combined with that result perhaps suggests that either malate is less efficiently transported at this lifecycle stage, or possibly that malate may represent a preferred substrate of metacyclics which epimastigotes do not compete for.

The amino acid utilisation of epimastigotes may also appear superficially similar to that of procyclics. Once again proline is the primary amino acid utilised and the values obtained for the depletion of proline based on the signal observable at 4.15 (Figure 11B and 11B) are similar for epimastigotes and procyclics at 7.52 mM and 7.22 respectively. It seems the proline metabolic pathway is well conserved in the insect forms of *T. congolense* as predicted by the literature. Whereas the amino acid glutamine decreased substantially more in epimastigotes than procyclics based on the signal at 2.54 (Figure 11C and 11C) with 6.29 mM depleted by epimastigotes compared to 1.27 mM in procyclics. As previously discussed glutamine plays a key role in pyrimidine biosynthesis (Hammond and Gutteridge, 1982, 1984a) and is likely fulfilling the same role here. Transcriptomic (Helm *et al.*, 2009) and proteomic (Eyford *et al.*, 2011) investigations of *T. congolense* have determined GARP (Glutamine Alanine Rich Protein) is expressed at considerably higher rate in epimastigotes than procyclics, and this may account for some of the increased uptake.

Curiously no discernible amounts of glycine or glycerol are produced, and the major product of epimastigotes is not succinate as it was in procyclics (Figure 6C) but is instead the amino acid alanine. The signal at 1.49 indicated 10.26 mM (Figure 11C) of alanine is produced compared to the 3.82 mM generated by procyclics under the same conditions (Figure 6C). These results may indicate the presence and greater flux towards an as yet unidentified glutamine aminotransferase similar to that observed in *T. brucei* procyclics (Marciano *et al.*, 2009) utilising glutamine as a substrate to form the TCA alpha-ketoglutarate with the subsequent generation of ATP. This greater flux once again likely reflects the relative abundance of free glutamine in the bloodmeal as it passes through the mouthparts, rather than the higher levels of proline observed in the digested bloodmeal available within the tsetse midgut. Intriguingly the *T. brucei* glutamine aminotransferase also has a high affinity for cysteine and therefore may be important in the redox balance in epimastigotes. Regrettably cysteine was not observable in this investigation thus it remains unclear whether this occurs in *T. congolense*. It is also possible that glutamine may form the aminosugar D-glucosamine-5-Phosphate, via glucosamine-fructose-6-phosphate aminotransferase [EC:2.6.1.16], entering

glycolysis or forming the sugar nucleotide UDP-N-acetylglucosamine, via UDP-N-acetylglucosamine/UDP-N-acetylgalactosamine diphosphorylase [EC:2.7.7.23 2.7.7.83].

A signal for aspartate is observable at 2.69 and 2.72 (Figure 11C), but due to the surrounding HEPES signal it isn't possible to determine any changes. Based on the quantitative analysis of the data the differences in signal for tyrosine and histidine are not genuine decreases, and this may be due to changes in protein content of the samples interacting with the internal standard.

Bloodstream Form

Introduction

Based upon Iscove's Modified Dulbecco's Medium (IMDM), HMI-93 has a proven track record as a reliable medium for the culture of *T. congolense* bloodstream form. The design of this medium was the result of the admirable work of Hirumi and Hirumi (1991), who carried out a systematic and meticulous investigation to identify essential components and attain stable, continuous culture. Key to the success of this medium was the capability it offered to dispense with the requirement for co-incubation of trypanosomes with bovine arterial endothelial cells. Instead bathocuproine was used to prevent autoxidation of cysteine with mercaptoethanol functioning as a reducing agent as demonstrated by Duszenko *et al.*, 1992 in *T. brucei*, and the importance of goat serum in *T. congolense* bloodstream form culture was highlighted (Hirumi and Hirumi, 1991).

Results and Discussion

Preliminary Results

In the initial stages of the investigation, the tolerance of IL3000 bloodstream form to different levels and types of serum was assessed. FCS even when supplemented at levels of 25% was found to produce abnormal forms and while these cells were capable of division, only low peak densities of 2 x 10^6 cells ml^{-1} were achievable. Goat serum however was highly effective even when supplemented at the comparatively low level of 15% and peak densities of well-formed, rapidly dividing cells were observable and typically reached peak densities of 5-6.5 x 10^6 cells ml^{-1} (Figure 12).

Once the preliminary investigations were complete an NMR-based approach was undertaken to determine the essentiality of the components of HMI-93, as well as probe the metabolic pathways of *T. congolense* bloodstream form via the patterns of component depletion and the generation of

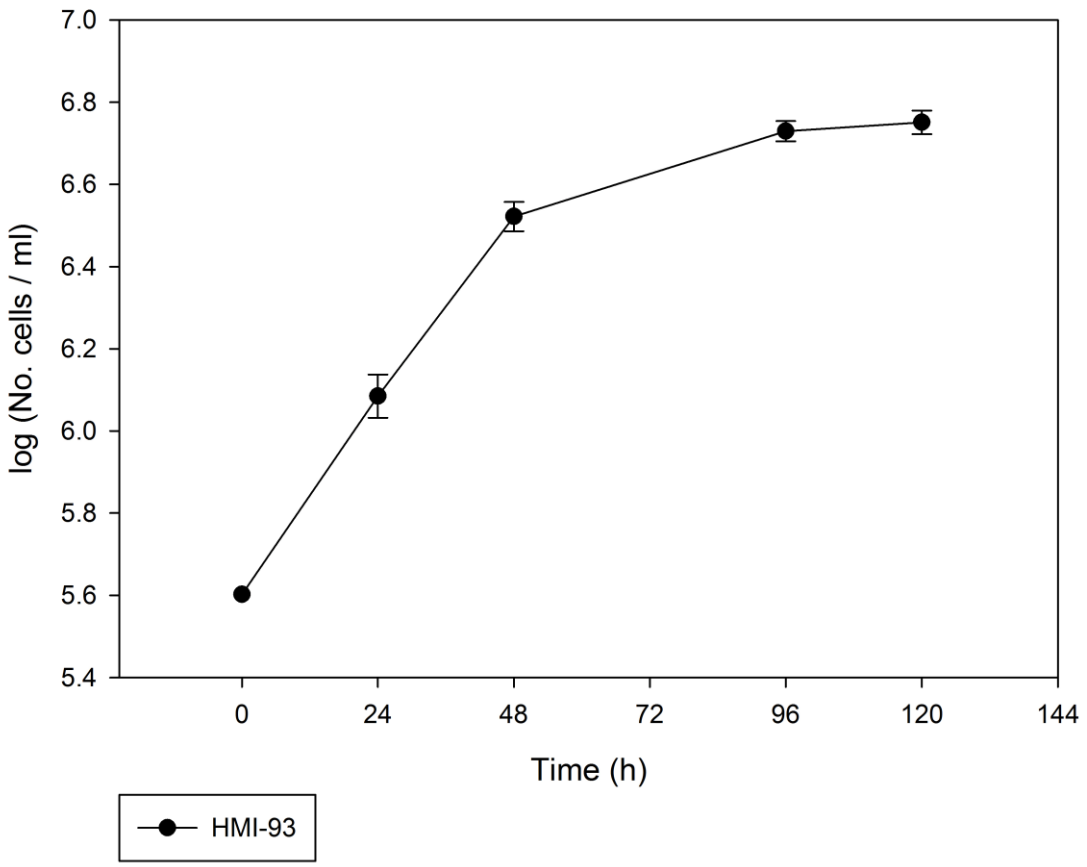


Figure 12 Growth Curve of Bloodstream Form *T. congolense* Cultured in HMI-93

Cultures were initiated with 4 x 10^5 cells ml^{-1} IL3000 *T. congolense* bloodstream form cells into 1.8 ml of HMI-93.

Cells grew exponentially from Time zero to 48hrs with a final increase in cell number observed at 96hrs. Cell number remained stationary until 120 hrs.

metabolic end products, with these results then compared those obtained in other trypanosomatids. The increase of goat serum supplementation to 25% did not increase the peak densities or alter that rate of growth indicating 15% was sufficient supplementation of the culture of IL3000 bloodstream form. Supplementary purines from freshly prepared stock solutions were tested in combinations and in isolation at amounts equimolar to the hypoxanthine provision already present in HMI-93. This supplementation however had either no effect on growth rate when compared to controls, as in the case of guanosine, or an inhibitory effect in the case of supplementary adenosine (data not shown). Indeed in the adenosine treatment the majority of the cultured cells appeared malformed and non-

uniform and this was the case in a separate biological replicate. No supplementary purines were therefore added to the final medium used for NMR analysis.

Considering the marked differences in efficacy of the supplementation of goat serum when compared to FCS, an NMR comparison of these sera was conducted to determine whether any significant difference could be identified. The results from this analysis were inconclusive however, as the, goat serum contained fewer components overall as was observed by Baker *et al.* (1988), possibly indicating that something in FCS is deleterious, or perhaps more likely reflecting that the key components of goat serum are not detectable via the present NMR methodology, e.g. lipids.

1H NMR of HMI-93 Results

Clear changes were present in the NMR spectra of the partially spent HMI-93 medium when compared to the spectra of medium only controls (Figures 14A and 14B). As expected the largest observable decrease was glucose as demonstrated by the substantial decrease at chemical shift 5.25 (Figure 13A) corresponding to a decrease of 9.96 mM. This decrease is consistent with previous *T. congolense* bloodstream form results obtained by Agosin and Von Brand (1954); however it also indicates that the amount of glucose present in HMI-93 is in vast excess of what is necessary for the routine culture of *T. congolense*.

A glutamine signal at 2.46 indicates a marked decrease in glutamine of 0.93 mM similar to the decreases observed by Creek *et al.* (2013) in *T. brucei* bloodstream form. In related kinetoplastids the pathways for pyrimidine biosynthesis from glutamine are well characterised (Hammond and Gutteridge, 1982, 1984b). It is notable that while a pathway is present for the biosynthesis of glutamine via glutamate, this pathway should not be essential as it would be expected that extracellular levels of glutamine in the host plasma would be largely homeostatic.

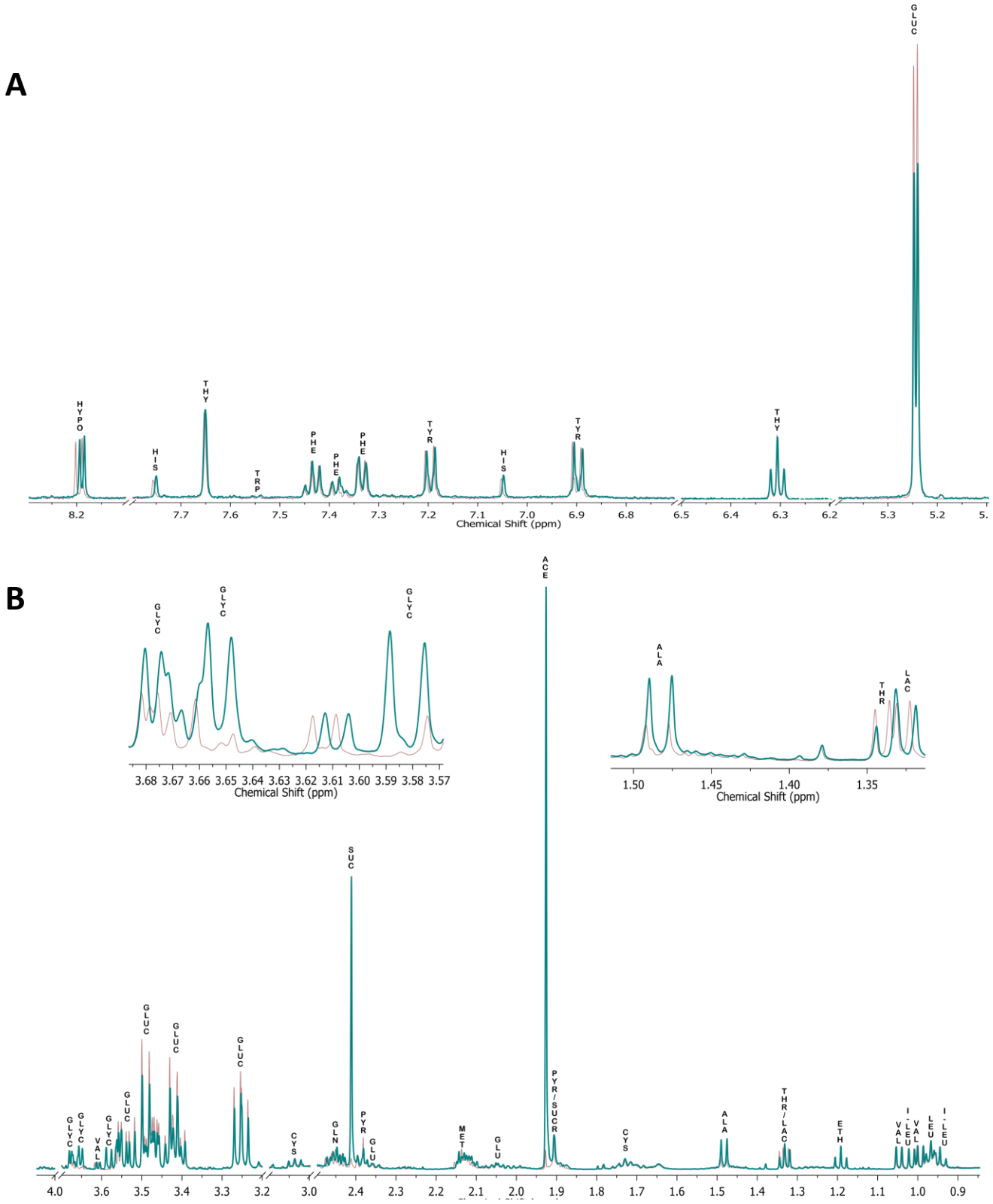


Figure 13 HMI-93 Media ^{1}H NMR Spectra

Proton NMR spectra representing spent media after the growth of *T. congolense* bloodstream form for 4 days at 37°C and 5% CO_2 in 24-well plates inoculated with 4 x 10^5 cells ml^{-1} (**Blue**) alongside a media only control (**Red**). **A** includes aromatic amino acids: L-Histidine (**HIS**), L-Tryptophan (**TRP**), L-Phenylalanine (**PHE**), L-Tyrosine (**TYR**); purine derivative Hypoxanthine (**HYPO**); pyrimidine deoxynucleoside Thymidine (**THY**); and monosaccharide D-Glucose (**GLUC**). **B** includes aliphatic amino acids: L-Leucine (**LEU**), L-Isoleucine (**I-LEU**), L- Valine (**VAL**), and L-Alanine product (**ALA**); sulphur containing amino acids: L-Methionine (**MET**) and L-Cysteine (**CYS**); acidic amino acid L-Glutamate (GLU); amidic amino acid L-Glutamine (**GLN**); hydroxylic amino acid Threonine (**THR**); and the organic acids: Pyruvate (**PYR**) and Succinate (**SUC**), and Acetate (**ACE**).

These spectra correspond to a representative experiment from a set of three biological replicates.

Table 5. Summary of 1H NMR HMI-93 Medium Bloodstream Form Results. Negative values represent the net depletion in a given media component, while a bold positive value represents the net production of a media component. Media components observed to change include: D-Glucose (**Gluc**), Pyruvate (**Pyr**) Succinate (**Suc**), L-Alanine (**Ala**), Acetate (**Ace**), L-Glutamine (**Gln**), Threonine (**Thr**), and Hypoxanthine (**Hyp**).

Media Components (mM)								
Gluc	**Pyr**	**Suc**	**Ala**	**Ace**	**Gln**	**Gly**	**Thr**	**Hyp**
-9.960	-0.250	**6.432**	**0.722**	**14.47**	-0.925	**1.140**	-0.563	**0.028**

The conversion of glutamate to glutamine therefore may be conserved and play a more prominent role in the procyclic lifestage where proline is in greater abundance than glutamine (Aksoy *et al.*, 2003) as previously described. The thymidine signal at 6.3 (Figure 13A) indicates no change after 5-days and from this we can infer that, either the *de novo* biosynthesis pathways are sufficient to support growth in *T. congolense*, or that the utilisation is lower than the sensitivity of this method. A putative methionine signal has been labelled at 2.14 (Figure 13B) where a small depletion is notable, however it is not possible to discern this signal from the surrounding glutamine signal. Regrettably another methionine signal that should be observable is instead obscured by a broad signal due to HEPES buffer at 2.55 – 3.22 (Figure 13B).

A threonine signal is also observable at 1.35 (Figure 9B) and indicates substantial depletion and appears to be the only amino acid apart from glutamine that is utilised; however, this result must be considered prudently as there is also considerable overlap with the neighbouring lactate signal. Careful integration of this signal would indicate depletion of approximately 563 µM, however further investigation is required with greater concentrations of threonine to better separate these signals. Threonine degradation has been observed in the bloodstream form of *T. brucei* and is likely involved in the biosynthesis of fatty acids via acetate (Linstead *et al.*, 1977; Lee *et al.*, 2006; Hee Lee *et al.*, 2007) to supplement the salvaged lipids and free fatty acids salvaged by bloodstream form *in vivo* and likely utilised from the serum *in vitro* (Coppens and Courtoy, 2000). All other amino acids present in the medium at detectable levels were utilised to a lesser degree than the sensitivity of this method (50 to 100 µM) as in the case of: valine, isoleucine, leucine, glutamate, or lysine (Figure

9B) and tryptophan, phenylalanine or tyrosine (Figure 13A). The amino acids not directly observable in this experiment due to the substantial glucose and HEPES signals produced were: serine, asparagine, arginine, and aspartate. These components therefore require further investigation. Insufficient proline was present in the medium to generate a clearly identified signal.

A surprising result was the moderate depletion of a pyruvate signal at 2.37 (Figure 13B) indicating a decrease which has not been observed previously; this indicates *T. congolense* may be capable of direct uptake of exogenous pyruvate, simultaneous to the uptake of glucose. This result highlights a clear difference in the metabolism of *T. congolense* and *T. brucei*, whereby pyruvate is the major end product of *T. brucei* glycolytic pathways. While Mazet *et al.* (2013) further outlined the role and highlighted the importance of an acetate generating pathway in *T. brucei* and demonstrated moderate flux towards acetate formation in the mitochondrion as a minor product with transamination of pyruvate to form the end product alanine, the majority of the pyruvate is still excreted from the cell (Mazet *et al.*, 2013). Instead in *T. congolense* the pattern of products formed is substantially different from that in *T. brucei* both in this investigation and in previous work (Agosin and Von Brand, 1954; Bowman and Flynn, 1976).

Pyruvate instead appears to have been degraded by pyruvate dehydrogenase to acetate as the major end product for *T. congolense* metabolism, as demonstrated by the considerable acetate signal at 1.93 (Figure 13B) indicating approximately 14.46 mM is produced by the end of the 5-day experiment. Most of this acetate will be derived from the glucose, pyruvate, and threonine depletions observed above, but it would appear likely other serum components that are not detectable in the medium-only control are also being utilised. The presence of alanine as a product at 1.49 (Figure 13B) also indicates transamination of pyruvate to alanine via alanine aminotransferase; however only a moderate amount (722 μM) was detected by the end of the experiment.

No lactate is produced in *T. congolense* but instead 1.42 mM glycerol is produced demonstrated by the signals at 3.58 and 3.65 (Figure 13B) and 6.43 mM succinate as indicated by the signal at 2.4 (Figure 13B). Agosin and Von Brand (1954) only observed traces of succinate and no glycerol compared to the high levels observed in this experiment; however, their experiments were undertaken in the absence of CO_2 and utilised whole blood or rabbit serum. The results obtained by (Ryley, 1956) are more harmonious with the results presented here and were conducted in the presence of CO_2. Hypoxanthine signals at 8.2 (Figure 13B) show a very minor increase of 28 µM; however considering the sensitivity of 1H NMR this may be a false result, and if genuine may be the result of spontaneous deamination of adenine present in serum (Wang and Hu, 2016), rather than representing any metabolic activity. It would appear from this result that all the purines required for *T. congolense* growth are satisfactorily provided by the goat serum, and signals corresponding to adenosine are just barely detectable in the 1H spectra obtained when it is scrutinised carefully.

Conclusion

With only minimal sample preparation the simple quantitative ^{1}H NMR approach used in this study, in combination with the extensive and open-source spectral reference libraries available, has proven capable of distinguishing the components of two widely used trypanosome growth mediums. These results have subsequently guided the generation of minimal media that has been clearly shown to be equal in efficacy to its precursors, and demonstrated the redundancy of many media components presumed essential. When operated competently ^{1}H NMR is capable of high throughput sample analysis with complex samples converted to spectra ready for interpretation in a matter of minutes. Assignment of the spectra remains a largely manual process, however automation in spectral assignment is an active area of research and in experiments with less complex samples is already capable of accurately determining and quantifying as well as an experienced human operator. This area must see further improvement, however, as the sensitivity of ^{1}H NMR increases the complexity of the spectra generated will increase also. Particular care must be taken to ensure that essential components such as buffering systems or reducing agents are selected which have minimal impact on areas of particular interest in the spectra. Use of electronic internal references such as the ERETIC method (Akoka *et al.*, 1999) may also be necessary in samples with higher protein content than those presented in this study. This is due to observations of TMSP binding to proteins, altering interactions in the sample and possibly leading to a loss in accuracy in the quantification process. The sheer scale of NMR spectrometers and their necessary maintenance is also a drawback to this method of analysis, necessitating the use of specialised facilities managed by expert operators, and this is unlikely to change in the future.

The present results indicate that *T. congolense* procyclic metabolism is broadly similar to that of procyclic *T. brucei* in glucose-rich conditions with only minimal quantities of amino acids utilised. However similarly to the results obtained in bloodstream form, there are substantial differences in the partially oxidised end products of these metabolic pathways. These results differ from the initial

work carried out by von Brand and Tobie (1959) where it was asserted that pyruvate, acetate, and succinate are produced by *T. congolense* procyclics. Brun (1982) detected small amounts of pyruvate while in this investigation no pyruvate was observed. The possibility that any pyruvate produced may oxidise too rapidly to be detected via this methodology is difficult to rule out, and future time sensitive analysis can be carried out to verify this result. Interestingly the pattern of these products remained relatively constant throughout the 5-days except for day 4 and 5 whereby the succinate peak increased only moderately, and the acetate peak increased substantially more. From this it is possible to infer that the succinate produced on previous days was due to glycolysis occurring within the glycosome and that the malate in the medium was primarily being converted to pyruvate and subsequently acetate to generate acetyl CoA as described previously (Obungu *et al.*, 1999a). The clear preference demonstrated for glucose as the sole carbohydrate source in procyclic *T. congolense* highlights how the metabolism of trypanosomes remains flexible despite the substantial shift in lifecycle stage and host environment. Glucose-rich conditions however provide a poor representation of the physiological conditions procyclic *T. congolense* are likely to experience in the tsetse fly.

T. congolense procyclic metabolism in proline-based culture media is markedly similar to that of procyclic *T. brucei* in amino acids preference, but once again there are substantial differences in the partially oxidised end products generated. *T. brucei* procyclic end products in proline-based minimal media are well characterised as primarily alanine, with small amounts of acetate, succinate, aspartate and lactate (Besteiro *et al.*, 2002; Lamour *et al.*, 2005; Coustou *et al.*, 2008). No lactate or aspartate was observed to be produced in this investigation and greater amounts of acetate and succinate were generated by *T. congolense* than expected. This indicates that succinate derived from proline degradation was further converted to pyruvate and acetyl CoA to a far greater degree than observed in *T. brucei*, even when the contributions from threonine, and the branched amino acids valine, leucine and isoleucine are accounted for. It is compelling that while *T. congolense* and the tsetse fly host will be in direct competition for many of these amino acids, alanine is utilised by

tsetse in the biosynthesis of proline. Therefore, the production of alanine by procyclic trypanosomes while not a symbiotic mechanism may at least ameliorate the effects of proline deprivation caused by their presence.

The decrease in methionine highlights the important role this amino acid has in the generation of ornithine and subsequent production of trypanothione. It is of note that significant decreases were not observed until a few days into the experiment, indicating that it is at this point the ornithine present in serum may prove insufficient. While Creek *et al.* (2013) suggested a methionine salvage pathway was unlikely to be present in *T. brucei* this may not be the case in *T. congolense*. The moderate depletion methionine is suggestive that an efficient methionine salvage pathway may be present, and this certainly warrants further investigation. The potential depletion of asparagine is also significant and the nitrogen balancing mechanisms of *T. congolense* should also be elucidated to confirm the presence of AS-A or if alternate enzymes are utilised.

Novel observations of the continuous net generation of the TCA intermediate fumarate offers a further line of enquiry, particularly when considering the differentiation step of bloodstream form to procyclic is facilitated by the presence of TCA intermediates. While in *T. brucei* cis-aconitate is most commonly utilised for this step it is generally not regarded as representative of the *in vivo* condition (Overath *et al.*, 1986; Sbicego *et al.*, 1999). The differentiation process carried out in this investigation (results not shown) was more successful than that undertaken in 109c medium as described by Brun (1982), however these may represent differences in the capabilities of the respective strains to adapt to culture. Or possibly that the *in vitro* obtained IL3000 bloodstream form utilised in this investigation was pre-adapted for *in vitro* conditions. Further investigation shall be required to investigation the possible role that the TCA intermediates present in CM have in the differentiation process. Preliminary results indicate their presence is essential as supplementation of glucose to minimal media is insufficient, and greater success is obtained in combination with malate, α-ketoglutarate, or fumarate. It is notable that the differentiation process is not fatal when only

glucose is utilised and indeed single, healthy appearing procyclic cells are observed even after 24hrs, however these cells are non-proliferative, and the culture failure is more attributable to extreme dilution, perhaps combined with the decrease in pH. Cunningham's medium therefore represents an adequate differentiation medium of bloodstream form and the use of citrate or cis-aconitate for *T. congolense* is likely dispensable. These results when combined with the observed generation of fumarate by procyclics *in vitro* are especially intriguing. If procyclics *in vivo* produce fumarate as a product in the absence of glucose it could function as a signal producing a positive feedback loop of differentiation.

The epimastigote NMR results are remarkable in their similarity to procyclic results obtained in CM, but the different pattern of product formation indicates greater flux towards alanine generating metabolic pathways. This suggests that *T. congolense* metabolism at the gross scale does not differ substantially as it transitions from the very different environments in the insect host. Instead it would appear that many metabolic pathways are maintained throughout the lifecycle with no pathway or organelle ever fully deactivated. This flexibility is at odds with the clear specialisation required by *T. brucei* epimastigotes to invade and establish in the salivary glands in insects or the invasion of the brain in mammals. It is possible *T. congolense* epimastigotes are not fully committed even once attached as proliferative rosettes, and retain the metabolic flexibility to revert to procyclics whereby they can be dislodged and return to the midgut without loss of cell viability. Curiously epimastigotes can be maintained in highly acidic media for several weeks, with no effect on cell viability. Rosette formations are intact, though elongated, and if seeded to a new flask in fresh media will rapidly shorten, re-attach, and resume division. This is at odds with procyclics which under acidic conditions deteriorate rapidly. When tied with the stronger preference for glutamine in epimastigotes it is possible that glutamine may be degraded to ammonia more rapidly under these acidic conditions and epimastigotes are more resistant to this toxicity or utilise this ammonia more efficiently into pyrimidine biosynthesis. Considering the harsh conditions of the proventriculus and tsetse mouthparts it is unsurprising this lifecycle stage should appear so hardy. Regrettably as it is

not possible to reliably culture *T. brucei* epimastigotes it is not possible to identify differences in their metabolism in relation to the results obtained here.

Despite the methodology outlined by (Coustou *et al.*, 2010) metacyclogenesis in this investigation remained elusive and in no instance were metacyclics obtained, despite epimastigotes being maintained in a variety of conditions for over 6 months. Yet when introduced into the *in vivo* conditions of the tsetse fly these parasites readily completed their lifecycle within weeks. This is of considerable concern and if widespread represents a significant hole in the otherwise well categorised *in vitro* culture of *T. congolense*. The triggering of all lifecycle stages is still an inexact process and requires considerably more work to achieve a full understanding of how current successful methods function and particularly how quorum sensing may play a key role (Silvester *et al.*, 2017)

While HMI-93 appears to be a highly efficacious and well-designed media as demonstrated by the growth curves obtained and the continuous culture of this form for ca. 90 days, the results as presented indicate it is ultimately too generous in its provision of all the components described. With no depletion of any one component suggesting further supplementation would be required the best recommendation would be to reduce the provisioning overall to determine a baseline media composition that is equal to or better than the current capabilities. Components that demonstrated no detectable depletion could then be systematically removed to refine this medium, as was done with procyclic media and preliminary thoughts and suggestions on this process are outlined below.

Only five components are supplemented to Iscove's Modified Dulbecco's Medium (IMDM) to form HMI-93: thymidine, hypoxanthine, cysteine, bathocuproine and mercaptoethanol. Based upon the results obtained here the provision of hypoxanthine and thymidine appears entirely dispensable and it is curious that, considering the lengthy and meticulous process conducted by Hirumi and Hirumi (1991), these components were retained. As it was not possible to obtain metacyclics it may be the case that these components would be essential to successfully transition these forms to

bloodstream form culture, and this is an area worth revisiting once metacyclics are more readily available. While these components were not removed as part of the present investigation it would be of interest to determine their essentiality. The synergistic activity of bathocuproine sulphonate blocking cysteine autoxidation and mercaptoethanol and cysteine thereby functioning together as reducing agents should be retained. Preliminary investigations in the absence of one or more of these components were not compatible with continuous culture.

Glutamine should be more carefully monitored considering its key role in pyrimidine biosynthesis (Hammond and Gutteridge, 1982, 1984b). While it has been demonstrated that *de novo* biosynthesis of pyrimidines is not essential *in vitro* (Ali *et al.*, 2013; Ong *et al.*, 2013) the presence of glutamine more closely represents the *in vivo* condition and therefore glutamine should be retained in future bloodstream form media. KEGG pathways for *T. brucei* indicate a well-supported role for aspartate by aspartate carbamoyltransferase [EC:2.1.3.2] to N-Carbomyl-L-Aspartate in pyrimidine biosynthesis and would likely fulfil a similar role in *T. congolense* (Hammond and Gutteridge, 1984a). Aspartate therefore warrants further investigation, preferably with an alternate buffer with fewer pervasive NMR signals than HEPEs, but with a similar pH optimum such as bicine. This would also likely allow the elucidation of the secondary methionine signal that was absent in this investigation, as well as asparagine and arginine. Thereby facilitating the determination of the utilisation of these amino acids in *T. congolense* metabolism, particularly regarding the generation of pyrimidines, trypanothione and nitrogen balance within the organism.

The lack of excreted pyruvate in *T. congolense* bloodstream form marks a clear difference from *T. brucei* metabolism and should be investigated further to identify precisely how this is achieved, and particularly where this occurs in the cell. Should it occur within the mitochondrion this would potentially explain the activity Vickerman (1965) observed so many years ago.

Concluding Remarks

The NMR analysis carried out in this investigation represents a step beyond the oft mysterious and at times alchemical media improvised in the past and is an important step in highlighting the myriad of differences between *T. brucei* culture and that of *T. congolense.* These results further underline the importance of refocusing research on clinically relevant AAT species such as *T. congolense*, as results obtained from *T. brucei* are clearly not generalisable to all African trypanosomes. It is my hope that the methods outlined here could yield further results in the study of another neglected trypanosome *T. vivax* as it's lifecycle is even further removed from *T. brucei* than that of *T. congolense,* and stable culture remains elusive using current methodology. The minimal medium generated from the analysis of *T. congolense* procyclics clearly demonstrates how many of the components presumed to be important are in fact dispensable and represent real savings that could remove another barrier to frontline research. The analysis of procyclics and epimastigotes in glucose-rich media and bloodstream form in HMI-93 has facilitated a better understanding of the underlying metabolic pathways of *T. congolense* and highlighted key areas for future investigation.

The media outlined in this report can and will be further improved, and it is my strongly held belief a truly defined media for *T. congolense* culture for all lifecycle stages will be achieved and provide the unifying baseline for future research.

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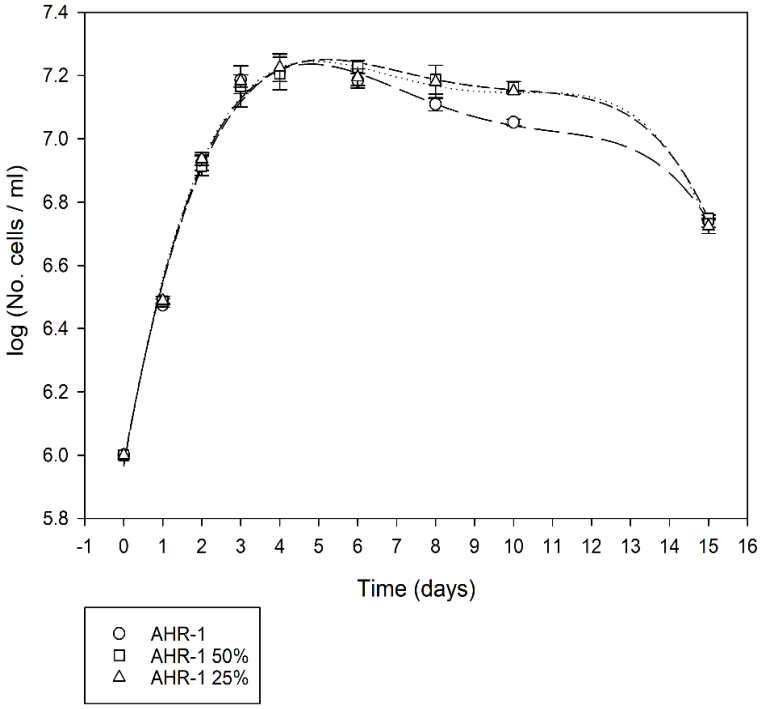
Supplementary Information

Experimental Media Comparison

Medium	Components (mM)										
	Gluc	Fruc	Sucr	Mal	Fum	α-keto	Suc	Pyr	Ala	β-Ala	Arg
CM	3.89	2.22	1.17	5	0.32	2.53	0.37	0	6.18	22.47	2.53
AHR	0	0	0	0	0	0	0	0	6.18	0	2.53
AHR-2	0	0	0	0	0	0	0	0	1.55	0	0.63
HMI-93	25	0	0	0	0	0	0	1.04	0.28	0	0.48
	Asn	Asp	Cys	Cystine	Gln	Glu	Gly	His	Ile	Leu	Lys
CM	1.82	0.83	0.57	0.13	11.23	1.7	1.6	1.03	0.69	0.69	1.03
AHR	1.82	0.83	0.57	0.13	0	0	0	1.03	0.69	0.69	1.03
AHR-2	0.46	0.21	0.1425	0.0325	0	0	0	0.26	0.17	0.17	0.26
HMI-93	0.19	0.23	1.5	0.38	4	0.51	0.4	0.2	0.8	0.8	1
	Met	Phe	Pro	Ser	Tau	Thr	Trp	Tyr	Val	Bath	Mer
CM	1.34	1.21	60	1.9	2.16	0.84	0.49	1.1	1.79	0	0
AHR	1.34	1.21	60	1.9	0	4.5	0.49	1.1	1.79	0	0
AHR-2	0.34	0.30	15	0.48	0	1.125	0.12	0.28	0.45	0	0
HMI-93	0.2	0.4	0.35	0.4	0	0.8	0.08	0.57	0.8	0.05	0.12
	FCS (%)	GS (%)	HEPES								
CM	14.2	0	19.75								
AHR	14.2	0	19.75								
AHR-2	14.2	0	4.94								
HMI-93	0	15	25.04								

The effect of Dilution of Mininal Media on *T. congolense Growth*



The effect of Varying Levels of Foetal Calf Serum on *T. congolense Growth*