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The Impact of Mixed Species Infection on Trypanosome Virulence and Transmission

Frank Venter

A thesis submitted in partial fulfilment for the degree of

Doctor of Philosophy

The University of Edinburgh

January 2022
DECLARATION

I declare that the content of this thesis is my own work and that all contributions and collaborations have been explicitly acknowledged in the text. No material presented in this thesis has been submitted to any other university, or for any other degree.

Frank Venter

Date:

2022-01-28
LAY SUMMARY

Nagana is a devastating livestock disease, which is transmitted to animals through the bite of a tsetse fly. This disease not only causes illness and death in animals but also impacts heavily on the livelihoods of livestock-keeping communities in sub-Saharan Africa. The most important of these parasites, *Trypanosoma congolense*, has been found to represent different types and strains of the same species, which often differ in terms of their virulence – i.e. how easily they cause disease or kill the host that they infect. Trypanosomes, including *T. congolense*, can regulate their growth so as to not overwhelm their host, with differences in the genes and proteins that govern these processes being observed between strains.

Parasites can also be found in mixed infections with other parasites that cause nagana – a host can be infected with multiple species of parasite at the same time. Investigating field samples from Western-Kenya revealed that multiple species of trypanosome were indeed circulating in cattle, including *T. congolense*, *T. brucei* and *T. vivax*. Interestingly, parasites in mixed infections can compete, or cooperate, which have implications for these parasites, as well as the host. *Trypanosoma brucei*, for example, responds to the signals from *T. congolense*, impacting on parasite growth and transmission potential. Long-term coinfection between these two species indicated that competition avoidance may also be present, with only one species dominating an infection at a given time. Evidence also suggested that *T. brucei* was replicating in tissues outside of the host blood and then re-emerging in transmission-ready
forms, known as stumpies. To further investigate this phenomenon, we engineered a line of bioluminescent trypanosomes, which could be imaged within a live host, with the aim of monitoring tissue location during mixed trypanosome infections, and established an in vivo model for this purpose. Finally, my results indicated that *T. brucei* that was retrieved at the end of a long-term coinfection experiment, was more virulent – showing a depleted capacity to regulate its own growth.
ABSTRACT

Animal African trypanosomes are protozoan parasites that cause nagana, a devastating disease of livestock in sub-Saharan Africa. It is well documented that strains of one causative agent, *Trypanosoma congolense* (subtype: Savannah), exhibits strikingly different virulence profiles in the hosts that they infect – measured in terms of host survival - though the molecular basis of these different phenotypes are not known. This parasite is also known to be found in mixed infections with other African trypanosomes, namely *T. brucei* and *T. vivax*. Such mixed infections, or coinfections, affect both the host and parasite, and are modulated by a number of factors, including inter-strain or inter-species competition. Limited work has been done to study how such competition may impact upon parasite virulence, or transmission potential to a new host. To this end, we sought to generate genome and transcriptome data for different *T. congolense* field strains and identified differences in the predicted protein sequences between these lines. Transcriptome data also revealed differentially expressed transcripts between strains of high virulence and those of low virulence.

Next, we utilised field- and laboratory studies to investigate the interaction between coinfecting African trypanosomes. Surveys from the field indicated that *T. congolense*, *T. vivax*, and *T. brucei* indeed infect cattle in the same geographical area. This screen, using two different assays, was capable of detecting trypanosome coinfections, although mixed infections between African trypanosome species were not observed. In vivo experiments in mice
highlighted the interactions that occur between *T. congolense* and *T. brucei*. First, it was shown that a *T. congolense* field strain (MF1 CL1) could drive *T. brucei* EATRO 1125 PFR-Ty to become cell-cycle arrested, and pre-adapted for transmission. Secondly, we observed a dynamic cycling in parasitaemia between the two species within the same host over the course of a chronic infection – only one species peaked at a given time. Furthermore, *T. brucei* EATRO 1125 PFR-Ty that re-emerged after a peak of *T. congolense* IL3000 parasitaemia comprised of a high proportion of PAD1+ cells. Taken together, these data indicate competition avoidance between these conspecifics. Thirdly, a line of *T. brucei* that was retrieved from a chronic coinfection, exhibited enhanced virulence when compared to the original line. Mice infected with this chronic line of parasites showed significant weight-loss, and immunofluorescence assays highlighted a reduced capacity to form stumpy cells in this group. These data suggest selection for more virulent cells, which could enhance transmission.

Finally, we generated a luciferin-reporter line of *T. brucei* (AMLuc 4.2), to investigate competition avoidance between this species, and *T. congolense* IL3000. This newly-generated line of parasites was then used to establish a model for in vivo imaging of trypanosomes in a live host, and the ex vivo imaging of infected organs.
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Thank you to Dr Vincent Delespaux (Vrije Universiteit Brussel) for providing stabilates of *T. congolense* field strains and Professor Mark Carrington (University of Cambridge) for providing primers for trypanosome species-identification. I would also like to thank everyone at the International Livestock Research Institute (ILRI), Nairobi, for being welcoming hosts. Field samples, and support, were kindly provided by Dr Mark De Clare Bronvoort, Dr Phil Toye, Dr Johanneke Hemmink, Benedict Karani, and Dr Erhan Yalcindag at the Roslin Institute, Edinburgh and ILRI, Nairobi. I would also like to thank Dr Martin Taylor (LSHTM), for kindly providing advice and the plasmid construct that was used to generate bioluminescent parasites. Thanks to Dr
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Finally, I would like to thank my mother, Doreen, for steadfastly supporting our family, and for always encouraging me on my academic journey.
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>8-CPT-cAMP</td>
<td>8-(4-chlorophenylthio)-cAMP</td>
</tr>
<tr>
<td>AAT</td>
<td>Animal African trypanosomiasis</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AdSS</td>
<td>Adenylosuccinate synthetase</td>
</tr>
<tr>
<td>bp</td>
<td>Base-pairs</td>
</tr>
<tr>
<td>BES</td>
<td>Bloodstream expression site</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CCA</td>
<td>cis-aconitate</td>
</tr>
<tr>
<td>CP</td>
<td>Cysteine protease</td>
</tr>
<tr>
<td>DABCO</td>
<td>1, 4-diazabicyclo[2.2.2]octane</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEAE-C</td>
<td>Diethylaminoethyl-cellulose</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EATRO</td>
<td>East African Trypanosomiasis Research Organisation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ES</td>
<td>Expression site</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>ESAG</td>
<td>Expression site associated gene</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HAT</td>
<td>Human African trypanosomiasis</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IMP</td>
<td>Inosine monophosphate</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>I-TASSER</td>
<td>Iterative-Threading Assessment Refinement</td>
</tr>
<tr>
<td>ITS1</td>
<td>Internal transcribed spacer region 1</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>PAD</td>
<td>Protein associated with differentiation</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pCPT-cAMP</td>
<td>8-(4-chlorophenylthio)-cAMP</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>PFR</td>
<td>Paraflagellar rod</td>
</tr>
<tr>
<td>p/s</td>
<td>Photons per second</td>
</tr>
</tbody>
</table>
PSG  Phosphate saline glucose
qPCR  Quantitative polymerase chain reaction
QS   Quorum-sensing
QTL  Quantitative trait loci
RAT  Relative abundance to alpha-tubulin
RBC  Red blood cell
RLU  Relative light units
RNA  Ribonucleic acid
RNAi RNA interference
RNA-seq RNA sequencing
RPK Reads per kilobase
SA   Sialidases
SIF  Stumpy induction factor
TAE  Tris-acetate-EDTA
TfR  Transferrin receptor
TS   Trans-sialidases
TSIF Trypanosome-suppressive immunomodulating factor
UTR Untranslated region
VSG Variant surface glycoprotein
WGS  Whole-genome sequencing
Toegewy aan my pa, Frank Sr
# CONTENTS

## 1 GENERAL INTRODUCTION

1.1 BACKGROUND ........................................................................................................... 2

1.1.1 Animal African Trypanosomiasis ................................................................. 2

1.1.2 Burden and control ......................................................................................... 4

1.1.3 Morphology and cell biology ......................................................................... 6

1.2 ANTIGENIC VARIATION ....................................................................................... 10

1.3 THE TRYPANOSOME LIFE CYCLE ....................................................................... 12

1.3.1 Host stages ....................................................................................................... 12

1.3.2 Slender to stumpy formation ........................................................................... 15

1.3.3 The QS pathway and gene expression changes in T. brucei ..................... 19

1.3.4 Tsetse stages of T. brucei ............................................................................... 22

1.3.5 Tsetse vectorial capacity ............................................................................... 23

1.4 STRAIN VIRULENCE ............................................................................................ 25

1.4.1 Virulence factors in African trypanosomes .................................................. 26

1.4.2 The consequences and drivers of altered virulence ..................................... 29

1.5 COINFECTION ..................................................................................................... 30

1.5.1 Factors which shape the coinfecting community ......................................... 30

1.5.2 Microbial interaction and the implications for host and parasite ............ 33

1.6 TRYPANOSOME-TRYPANOSOME COINFECTIONS .................................... 35

1.7 AIMS ..................................................................................................................... 38

## 2 MATERIALS AND METHODS

2.1 TRYPANOSOME STRAINS .................................................................................. 41

2.2 TRYPANOSOME CULTURES .............................................................................. 42

2.3 TRYPANOSOME INFECTIONS IN MICE .......................................................... 44

2.4 EXTRACTION OF GENETIC MATERIAL .......................................................... 46

2.5 WHOLE-GENOME SEQUENCING .................................................................. 47

2.6 RNA-SEQ ANALYSIS .......................................................................................... 48

2.7 RAT-ANALYSES .................................................................................................. 50

2.8 FIELD DATA ........................................................................................................ 50

2.9 QUANTITATIVE PCR .......................................................................................... 53

2.10 IMMUNOFLUORESCENCE MICROSCOPY ..................................................... 55

2.11 TRYPANOSOME COINFECTION DYNAMICS ................................................. 57
4.2.2 **Towards estimating changes in parasite dynamics infecting individual animals, over time** .......................................................... 104

4.3 **Selection of strains for coinfections studies** ................................................. 107

4.4 **T. congoense field strains induce stumpy-formation in T. brucei in a short-term co-infection** ........................................................................ 111

4.5 **Long-term in vivo trypanosome co-infection** ...................................................... 114

4.5.1 **Coinfections reduce disease severity** .............................................................. 116

4.5.2 **Altered growth dynamics and increased transmission potential during co-infections** ........................................................................ 117

4.6 **The effect of coinfection on parasite virulence** .................................................. 125

4.6.1 **Coinfection selects for increased parasite virulence** ....................................... 127

4.7 **Discussion** ........................................................................................................... 131

5 **Within-host localisation and competition avoidance between co-infecting trypanosomes** ................................................................. 137

5.1 **Introduction** ......................................................................................................... 138

5.2 **Generating a luciferin-reporting line of T. brucei** ................................................. 139

5.2.1 **Clone selection and in vitro validation** .............................................................. 140

5.3 **In vivo validation** ..................................................................................................... 145

5.3.1 **Imaging of bioluminescent trypanosomes in live mice** ....................................... 145

5.3.2 **Mouse perfusion and IVIS imaging of whole organs** ......................................... 150

5.4 **Discussion** ........................................................................................................... 151

6 **Summary & future directions** ................................................................................ 158

BIBLIOGRAPHY .............................................................................................................. 167

APPENDIX 1: SUPPLEMENTARY DATA FOR CHAPTER 3 .............................................. 204

APPENDIX 2: SUPPLEMENTARY DATA FOR CHAPTER 4 ............................................... 222

APPENDIX 3: SUPPLEMENTARY DATA FOR CHAPTER 5 ............................................... 228

APPENDIX 4: MEDIA AND BUFFERS .............................................................................. 232
1 GENERAL INTRODUCTION
1.1 Background

The Trypanosomatida (Phylum: Euglenozoa) is an ancient lineage of protozoan parasites infecting species across taxonomic kingdoms. These organisms have been widely studied owing not only to their peculiar biology but also the devastating diseases caused by the genera *Leishmania* and *Trypanosoma*. One example is human African trypanosomiasis (HAT), also known as sleeping sickness, which is caused by two subspecies of *T. brucei*, namely *T. b. gambiense* and *T. b. rhodesiense*. While still a significant source of morbidity and mortality in affected communities, newly-reported HAT infections have been steadily declining (Franco et al., 2020). This is not the case for the animal variants of the disease, however, which continues to place a significant burden on livestock-keeping communities, impacting upon health and livelihood (Swallow, 2000; Grace et al., 2009; Odeniran and Ademola, 2018).

1.1.1 Animal African Trypanosomiasis

Animal African trypanosomiasis (AAT), or nagana, is a livestock disease caused primarily by *T. congolense* (subgenus: Nannomonas) and *T. vivax* (subgenus: Duttonella), and also less frequently by *T. b. brucei* (subgenus: Trypanozoon). These species, known as Salivarian trypanosomes, are transmitted to a host in the infected saliva of *Glossina* spp. (tsetse flies) during a bloodmeal (Figure 1.1). Mechanical transmission, however, can also take
place particularly in the case of *T. vivax* (de Araújo Melo et al., 2017; Desquesnes and Dia, 2004).

AAT infects a wide range of important species, with the parasites being co-distributed across much of sub-Saharan Africa (Figure 1.2) (Auty et al., 2015). This overlap in geographical range is likely due to coinfection within the tsetse vector, which has been found to harbour more than one parasite species, or strain, at a given time (Adams et al., 2006; Weber et al., 2019). Such coinfections have also been reported in host mammals. For example, 14% of trypanosome-positive cattle were reported to be simultaneously infected with *T. congolense* and *T. vivax* in Nigeria (Takeet et al., 2013). This matched prevalence estimates in Tanzania, where combinations of *T. congolense*, *T. vivax* and *T. brucei* were detected in 14% of infected cattle (Haji et al., 2015). It is worth mentioning that such cases are likely underestimated, as diagnosing AAT in the field is fraught with difficulty (Anderson et al., 2011; Cox et al., 2010). Another factor which may have implications for accurate estimations of prevalence is the effect of coinfections for both the host and the parasite (discussed in section 1.4).
Figure 1.1: AAT is an economically important disease that is caused by African trypanosomes. The wasting disease affects numerous important livestock species (left; image: FAO/ G. Cecchi), and can be transmitted by the saliva of tsetse flies during a bloodmeal (right; image: IAEA).

1.1.2 Burden and control

A characteristic symptom of AAT is anaemia, but infected animals may also present with splenomegaly, pyrexia, ataxia and weight loss (Taylor and Authie, 2004). Acute cases can lead to death within weeks, while chronic cases can lead to months, or years, of progressive wasting and infertility in untreated animals. In fact, AAT is considered to be the most detrimental livestock disease in sub-Saharan Africa, leading to billions of dollars in losses every year through direct death, as well as the loss of animal productivity and draught power (Shaw et al., 2014; Swallow, 2000). These losses then indirectly impact upon the livelihoods of livestock-keeping communities, who depend on animals as ‘mobile banks’ for sources of social and medical expenditure (Mungube et al., 2012; Randolph et al., 2007). Currently, control strategies rely on a
combination of vector interventions and drugs, with diminazene aceturate and isometamidium chloride being the treatments of choice in most cases. Treatment failure has been increasingly noted, however, and can be ascribed to the development of drug-resistance in parasites, as well as improper dosing practices or counterfeit drugs (Delespaux and de Koning, 2007; Delespaux et al., 2008; Mamoudou et al., 2008; Richards et al., 2021).

As such, much attention has recently been given to the hunt for novel trypanocides or vaccines, such as the promising antigen candidate that was recently identified for *T. vivax* (Autheman et al., 2021). Any such developments require a thorough understanding of basic trypanosome biology, with many gaps still remaining in our knowledge, particularly for *T. congolense* and *T. vivax*. 
Figure 1.2: The distribution of AAT-causing species in Africa. The confirmed and inferred distribution of *T. congolense* subspecies are shown (left), with *T. vivax* being co-distributed in the same geographical areas (right). Adapted from (Auty et al. 2015).

### 1.1.3 Morphology and cell biology

While African trypanosomes share the same basic morphology, there are notable differences between species such as parasite size. *T. brucei* and *T. vivax* range between 18-31µm in length, with the flagellum being free at the anterior of the cell (Figure 1.3) (Bargul et al., 2016; Giordani et al., 2016). *T. congolense* is smaller in size (9-22µm) and the does not present with a free flagellum (Figure 1.3) (Giordani et al., 2016). The flagellum is a highly conserved structure that is necessary for cell motility (Bastin et al., 1998). It runs along the cell body and is composed of a paraflagellar rod (PFR) and a canonical 9+2 axoneme microtubule arrangement (Bastin et al., 1996a; Langousis and Hill, 2014).
Figure 1.3: Morphological differences between African trypanosome species. *T. brucei* and *T. vivax* are both larger than *T. congolense*, and also exhibit a free flagellum at the anterior-end. Adapted from Giordani et al. (2016).

The flagellum emerges through the flagellar pocket, a posterior invagination of the plasma membrane, which is an important structure and site of endo/exocytosis (Field and Carrington, 2009). Additionally, the basal body of the flagellum is attached to the tripartite attachment complex (TAC) which has an important role for mitochondrial genome segregation during cell division (Ogbadoyi et al., 2003; Schneider and Ochsenreiter, 2018). The process of cell division is peculiar among eukaryotes, with the segregation of single copy organelles being tightly regulated processes (Matthews, 2005; Hammarton et al., 2007; Wheeler et al., 2019).

One such organelle is the kinetoplast, a characteristic network of DNA (kDNA) comprised of mini- and maxi-circles (Figure 1.4) (Ryan et al., 1988; Shapiro and Englund, 1995). Kinoplast segregation occurs during the S- or
G2-phase, and is facilitated by the TAC, prior to mitosis (Robinson and Gull, 1991; Schneider and Ochsenreiter, 2018; Siegel et al., 2008). Chemical staining of the kinetoplast (K) and nucleus (N), using dyes such as 4',6-diamidino-2-phenylindole (DAPI), permits visualisation of this process thereby allowing researchers to accurately track the cell cycle position of trypanosomes (e.g., 1K1N, 2K1N or 2K2N) (Figure 1.4).

It is not only the genome architecture of trypanosomes that are peculiar, but also their modes of gene expression, as a number of key differences are present when compared to other eukaryotic organisms (Daniels et al., 2010). For example, trypanosome genes are arranged in multi-gene clusters which are polycistronically transcribed (Martínez-Calvillo et al., 2010; Muhich and Boothroyd, 1988). Interestingly, transcription rates of these clusters apparently remain constant throughout the trypanosome lifecycle, and must therefore be regulated post-transcriptionally (Clayton, 2002). Another notable feature is the presence of an RNA Polymerase I-mediated gene expression – usually involved in transcription of ribosomal RNA – that transcribes protein coding genes in trypanosomes (Günzl et al., 2003). As well as the conventional nucleolar location, this polymerase is also located in the expression site (ES) body, an extranucleolar structure, which tightly regulates expression of genes including those that encode variant surface glycoproteins (VSGs) (Navarro and Gull, 2001).
Figure 1.4: Cell biology of trypanosomes. (A) Cross-section illustrating the internal structures and organelles of *T. brucei*, with the kinetoplast highlighted. Adapted from (Vickerman et al. 1993); (B) Electron micrograph of the kinetoplast of *C. fasciculata*, another trypanosomatid, showing the characteristic circular kDNA (Klingbeil and Englund 2004). (C) Diagram depicting the distribution and segregation of the single kinetoplast (K) and nucleus (N) of *T. brucei*, prior to cytokinesis, which can be visualised by DAPI-staining.

The external cell surface of *T. brucei* is covered by a dense monolayer of VSGs, constituting 95% of the total surface protein content, which are anchored into the plasma membrane via GPI lipid anchors (Trevor et al., 2019; Ziegelbauer and Overath, 1992). *T. brucei* VSGs are composed of a homodimer of two 50-60 kD subunits, with a long, exposed N-terminal domain which is the primary target of the host immune response (Auffret and Turner, 1981; Schwede and Carrington, 2010). Antibodies attach to the dense VSG coat, which shields important invariant surface antigens, and are swept towards the anterior flagellar pocket, by a process known as hydrodynamic flow (Ziegelbauer and Overath, 1992; Engstler et al., 2007; Schwede and Carrington, 2010). Here, antibody-VSG complexes are endocytosed,
prolonging parasite survival during the developing immune response against each parasite antigen type (Engstler et al., 2007; Field and Carrington, 2009). For *T. brucei*, it has been shown that a single infection comprises of a heterogenous mix of trypanosome sub-populations that each express a given VSG-type (Mugnier et al., 2015). Initially, an infection may consist of a few dominant VSG-types, with the number of expressed types increasing as the infection progresses over time. This allows sub-populations expressing an alternative VSG type to establish dominance within the host, subsequently causing the characteristic waves of parasitaemia seen during trypanosome infections (Horn, 2014). This periodic shift from one VSG type to another is called antigenic variation.

1.2 Antigenic variation

The gene encoding the expressed VSG, together with ES-associated genes (ESAG) are contained within a polycistronic unit called the bloodstream expression site (BES), of which there are around 15-20 in the genome (Faria et al., 2021). Only one BES is expressed at a time, and consequently a single VSG isoform will be observed on the surface of that trypanosome cell (Navarro and Gull, 2001). The expression of a different VSG, facilitating host immune evasion, involves transcriptional changes amongst the BESs early in the infection which is accomplished by silencing of the active BES, and the subsequent activation of another (Van der Ploeg et al., 1984). This method of antigenic variation is eventually exhausted, given that there are roughly 15
BESs, necessitating an alternative method of accomplishing antigenic variation. Calling on a repertoire of ~2000 VSG encoding genes, recombination can occur with the active BES through VSG gene flanking repeat sequences (Marcello and Barry, 2007; Van der Ploeg et al., 1984). The available VSG repertoire can be further expanded by the formation of ‘VSG mosaics’ which occurs through recombination of two VSGs to form a novel VSG coding gene that is antigenically distinct (Hall et al., 2013; Lythgoe et al., 2007). These events take place stochastically, with transcriptional switching being favoured during the early stages of infection (Mugnier et al., 2015). This is then followed by gene conversion of silent VSGs into the active BES, and finally by the mosaic formation in the late stages of the infection.

It is important, however, to mention that antigenic diversity is not generated by recombination in *T. vivax*, and that the repertoire of VSGs may be more limited in that species (Jackson et al., 2012; Silva Pereira et al., 2020). This can be illustrated by so-called antigenic exhaustion, where certain VSG-types re-emerge later during the course of infection (Barry, 1986; Nantulya et al., 1986; Jackson et al., 2012). Interestingly, this reduced VSG repertoire of *T. vivax* has been suggested as the cause of self-curing that has been observed in infected animals, although such a mechanistic link has not been shown. Interspecies differences are not restricted to the morphology and VSGs of African trypanosomes, but are also evident in the lifecycles of those species.
1.3 The trypanosome lifecycle

African trypanosomes are extracellular, usually dixenous parasites, comprising distinct mammalian and insect-phases to complete their lifecycle (Figure 1.5). These different environments, and the need to regulate growth in vivo, involves signalling between parasites, as well as significant remodelling of cells and changes in metabolism. Much of this knowledge has been inferred from *T. brucei* which, as will become apparent, has certain limitations.

1.3.1 Host stages

*T. brucei* metacyclic cells are introduced to a new mammalian via the host skin and subsequently the bloodstream, during feeding by an infected tsetse vector (Caljon et al., 2016). Introduced trypanosomes undergo a morphological transition, to proliferating slender cells, which is accompanied by metabolic changes involving mitochondrial repression; changes in nutrient preferences; and subsequent glycosomal conversion of glucose to pyruvate (Smith et al., 2017; Vickerman, 1965). Once a certain density of slender cells is reached, trypanosomes transition to cell-cycle arrested stumpy forms (Figure 1.5) through a mechanism resembling quorum-sensing (QS) (Matthews, 2021) (section 1.3.2). We therefore refer to *T. brucei* as a pleomorphic parasite given its morphological heterogeneity in the mammal host. Stumpy forms have historically been considered to be pre-adapted for an insect environment, and therefore required for vector acquisition and the completion of the trypanosome lifecycle (section 1.3.3) (Dean et al., 2009).
Figure 1.5: Dixenous lifecycle stages of *T. brucei*. Newly acquired stumpy cells transition to procyclic cells in the tsetse midgut, before invading the salivary glands as epimastigotes where they are restructured to form metacyclic cells. During a tsetse bloodmeal, metacyclic cells are introduced to the mammalian bloodstream, where they transition to proliferative slender forms. Once these cells reach a certain density, they become cell-cycle arrested stumpy cells, which are pre-adapted for the acquisition by a feeding fly (Silvester et al., 2017a).

Controversially, new evidence suggests that slender cells are not only capable of tsetse transmission, but that these cells are as efficient as stumpy cells at progressing through the tsetse host (Matthews and Larcombe, 2021; Schuster et al., 2021).

The *T. brucei* cell obtains the required resources, such as glucose and iron, directly from the mammalian bloodstream. Iron, which is important for a
number of processes (Taylor and Kelly, 2010), is acquired in the form of host transferrin and imported into the cell via transferrin receptors (TfRs). Curiously, TfR diversity has been proposed as an adaptation to multihost survival, which is supported by studies where switching from bovine to canine serum resulted in the expression of different ESAGs (Bitter et al., 1998; van Luenen et al., 2005). Recently, however, research has shown that a single TfR binding site can bind transferrin from multiple hosts, and that TfR diversity may, in fact, be in response to selective pressure exerted by the host immune system (Trevor et al., 2019).

While the bloodstream is the primary niche of *T. brucei*, these parasites have also been detected in a number of extravascular host tissues. For example, the adipose tissue has been shown to be a major host niche for trypanosomes, where they exhibit altered metabolic activity, which presents a potential source of re-establishment into the host bloodstream (Trindade et al., 2016). *T. brucei* have also been detected in the skin and gonads of mice (Caljon et al., 2016; Capewell et al., 2016; Carvalho et al., 2018). Historically, *T. congolense* and *T. vivax* have been noted as strictly vascular parasites (Banks, 1978), but limited evidence has shown that this may not necessarily be the case (Masocha et al., 2007; Silva Pereira et al., 2019).
1.3.2 Slender to stumpy formation

Parasites can regulate their growth in vivo to avoid prematurely killing the host, and to promote transmission to a new host. This is indeed the case for *T. brucei* exhibiting monomorphism following serial passage, which exhibit extreme virulence and rapidly kill infected hosts (Ashcroft, 1960; Sendashonga and Black, 1986). Instead, the parasite has evolved an elegant mechanism for regulating its growth, which hinges on QS-mediated stumpy-formation.

Early stages of the infection are dominated by slender, proliferative cells, which exhibit antigenic variation. Once a certain density threshold is reached within the population, slender cells differentiate to stumpy forms which are morphologically shorter and broader, with reduced flagellar length. These cells are quiescent, and arrested in the G0/ G1-phase of the cell-cycle, thereby limiting parasitaemia within the host (Ziegelbauer et al., 1990; Matthews and Gull, 1994; Matthews, 2021). The host immune system will readily clear these cells, as they do not undergo antigenic variation, contributing to the cyclical dynamics of trypanosome growth in vivo. This response is driven by the environmental sensing of a parasite-derived signal, or ‘stumpy-induction factor’ (SIF), which accumulates as the population increases (Matthews, 2021; Reuner et al., 1997). Researchers have long searched for the elusive factor, with some evidence implicating the cyclic AMP (cAMP) signalling pathway. Indeed, cAMP levels fluctuate throughout the course of an infection, rising with increased parasitaemia and lowering once stumpy-formation is induced (Mancini and Patton, 1981; Vassella et al., 1997). Further evidence for the role
of cAMP in stumpy-formation was provided in experiments involving cell-permeable cAMP, which could induce differentiation in a monomorphic cell line (Breidbach et al., 2002; Reuner et al., 1997). While seemingly compelling, other studies have pointed out that it may not be cAMP conferring the differentiation signal, but instead analogues of the hydrolysis products of cAMP (Laxman et al., 2006).

Recently, a protein family called TbGPR89 was identified on the surface of slender form T. brucei, which was shown to regulate stumpy-formation through the transport of oligopeptides (Figure 1.6) (Rojas et al., 2019). First, the authors showed that ectopic expression of the protein drove early stumpy differentiation in developmentally competent parasites, but not those that have lost the ability to undergo QS. This was followed by structural analysis indicating a role for TbGPR89 in oligopeptide transport, which was confirmed in Escherichia coli and T. brucei, and evidence that GPR89 is located on the cell surface. Finally, trypanosome oligopeptidases were shown to accelerate stumpy formation in vitro when overexpressed, while also providing or generating a paracrine signal which could be detected during in vivo coinfections. These data provide the most compelling evidence yet for a mechanistic model of SIF-signalling in T. brucei.

Definitive evidence for pleomorphism is lacking in T. vivax and T. congoense but there is limited evidence of apparent ‘long-form’ trypanosomes in late-stage infections (Gardiner and Wilson, 1987; Hoare, 1972; Nantulya et al., 1978). It is worth highlighting that these parasites do exhibit density-dependent cell cycle arrest, in the absence of stumpy formation, and that the
genes regulating QS in *T. brucei* are conserved in *T. congolense* (Shapiro et al., 1984; Silvester et al., 2017b).
Figure 1.6: SIF-signalling, and subsequent stumpy-formation in *T. brucei*, is mediated by the surface protein GPR89 family. Slender parasites release oligopeptides into the host environment, which accumulate as parasite numbers increase. These oligopeptides are transported into the parasite cell, via GPR89, which induces stumpy formation through the QS-regulatory pathway (Rojas et al., 2019).
1.3.3 The QS pathway and gene expression changes in *T. brucei*

A complex signalling cascade, and subsequent stumpy-formation, is initiated once the SIF-signal is received at the cell surface. The components of this QS pathway were identified by utilising an RNA-interference (RNAi) library in monomorphic *T. brucei* cells (Figure 1.7) (Mony et al., 2014). Using this approach, library cells were grown in RNAi-uninduced or induced conditions, which would selectively knock-down genes representative of the entire genome. If the expression of a component integral to the QS pathway was depleted, cells would continue to proliferate. The inserts in such cells were sequenced, allowing for identification of those genes involved, and were used to identify the individual components of the pathway. A number of these genes were validated experimentally and found to be stumpy-inducers such as *RBP7* which encodes a predicted RNA-binding protein, and *HYP2* which currently has no identified product but is likely involved in gene regulation (Erben et al., 2014; Mony et al., 2014).

Orthologues of these genes are present in *T. congolense*, despite their absence of stumpy-formation, indicating a potential role in growth-regulation. In fact, a *T. congolense* orthologue of *HYP2* was found to restore the ability of null mutant *T. brucei* cells to respond to the QS signal, and form stumpy cells (Silvester et al., 2017b). Additionally, such evidence is indicative of the potential for interspecies signalling in trypanosomes, which is discussed in more detail elsewhere (section 1.5.4). A number of important genes are
differentially regulated in slender versus stumpy cells, including *ESAG9* family transcripts and *PAD1* (protein associated with differentiation) (Barnwell et al., 2010; Dean et al., 2009). PAD1 is only expressed on the surface of stumpy cells, and has been exploited as a useful marker for stumpy formation. Other upregulated genes include those involved in mitochondrial respiration which are required for an environment scarce in glucose - the tsetse midgut (Brown et al., 1973; Mantilla et al., 2017; Michelotti and Hajduk, 1987).
Figure 1.7: The SIF-sensing pathway of *T. brucei*. A monomorphic line of trypanosomes was treated with pCPT-cAMP, which can induce stumpy-like formation. Inducible knock-down by specific RNAi inserts of target genes led to cells being blind to the signal, and as such, they continued to proliferate in the presence of cell permeable cAMP. Sequencing of these inserts allowed the researchers to reconstruct the signalling pathway. Some of these components have been validated experimentally (in bold) (adapted from Mony et al., 2014).
1.3.4 Tsetse stages of *T. brucei*

Trypanosomes that are newly acquired following a tsetse bloodmeal are introduced to the harsh environment of the fly’s midgut. Stumpies in the blood meal, however, are pre-adapted to overcome these conditions which include tsetse immune responses, and altered resources, to establish in the new host (Haines et al., 2010; Nolan et al., 2000; Rico et al., 2013). Surviving stumpy cells respond to their new tsetse environment by responding to cues, such as *cis*-Aconitase (CCA), and subsequently transition into procyclic forms (Figure 1.5) (Dean et al., 2009; Engstler and Boshart, 2004; Szöör et al., 2010). Generated procyclic trypomastigotes do not express a VSG coat, but are instead covered by GPI-anchored proteins known as procyclins (GPEET and EP) (Roditi et al., 1989; Turner et al., 1988a; Vassella et al., 2001). As mentioned, the midgut is devoid of glucose necessitating procyclic forms to undergo extensive metabolic remodelling to utilise the available resources such as threonine and proline (Besteiro et al., 2005; Doleželová et al., 2020; Smith et al., 2017). Procyclic cells migrate to the proventriculus, where asymmetrical division results in two distinct epimastigote forms (Sharma et al., 2008). The short-form of the epimastigotes migrate to the tsetse salivary glands, attach and proliferate, before again differentiating into metacyclic trypanosmatigotes which are adapted to the mammalian bloodstream. Metacyclic cells express a VSG coat, albeit from a reduced repertoire, to aid in immune evasion once reintroduced into a mammal host (Barry et al., 1983; Turner et al., 1988b).
There are key differences in the insect stages of other trypanosomes, when compared to *T. brucei*, such as the route taken through the tsetse fly after acquisition (Figure 1.8). *T. congolense* cells also replicate in the midgut but invade the proboscis instead of the salivary glands. *T. vivax*, conversely, does not replicate within the tsetse midgut, and is instead restricted to the mouthparts. Interestingly, mechanical transmission by other insect-species have been shown, contributing to the spread of both *T. brucei* evansi and *T. vivax* (Desquesnes et al., 2013; Osório et al., 2008).

### 1.3.5 Tsetse vectorial capacity

Successful completion of the lifecycle, as detailed above, hinges on the vectorial capacity of a tsetse fly – the ability to acquire and transmit trypanosomes. We know, for example, that different species are more efficiently acquired by tsetse, with *T. vivax* being the most transmissible, followed by *T. congolense* and *T. brucei* (Gitonga et al., 2017; Van den Bossche et al., 2004). Additionally, variation exists within species, with the Savannah subgroup of *T. congolense* being more transmissible than the Forest type (Reifenberg et al., 1997).

The exact mechanisms governing vectorial capacity are not known and remain contentious. Firstly, the proportion of stumpy forms in the host bloodstream, and not the total number of parasites, has been found to determine *T. b. brucei* and *T. b. gambiense* transmissibility (Wijers and Willett, 1960).
Figure 1.8: Species-specific progression of trypanosomes through the tsetse-vector. Following a bloodmeal, newly acquired *T. brucei* and *T. congolense* parasites will replicate within the tsetse midgut (2), before invading the proventriculus (3) and eventually migrating to the salivary glands (4) and proboscis, respectively. *T. vivax* is restricted to the mouthparts of the tsetse fly, with this parasite also being capable of mechanical transmission. Created in BioRender.

This model, however, has recently been challenged by research showing that a single trypanosome, acquired during a bloodmeal, is sufficient for lifecycle progression within the tsetse vector (Schuster et al., 2021). For *T. congolense*, which lacks morphological stumpy-formation, enhanced transmissibility was not associated with the high parasitaemia but, instead, with the timing of acquisition (Akoda et al., 2008). These data are supported by another study, where it was noted that *T. congolense* were more transmissible during the acute stages of infection, as opposed to the chronic stages of the infection (Masumu et al., 2010). Next, differences were also observed among *T.
congolense field stains isolated from cattle in the same geographical area, where high parasite virulence was found to be associated with increased transmissibility (Masumu et al., 2006a). Finally, vectorial capacity can be ascribed to differences between the different tsetse species themselves, such as habitat selection and feeding behaviour.

1.4 Strain virulence

Trypanosome virulence is usually defined by either their pathogenesis in the host or in terms of parasite growth dynamics, both of which can vary significantly between species, subspecies, or even strains. The most well-known example of this variation is evident in the human-infective subspecies responsible for sleeping sickness, where acute, severe disease is prominent in \textit{T. b. rhodiense} infections, while \textit{T. b. gambiense} results in chronic disease. Such variation is also evident for different strains of \textit{T. congolense} (subgroup: Savannah), which were isolated from livestock and wildlife in Zambia (Masumu et al., 2006b; Van den Bossche et al., 2011). In the study, 62 strains were established in mice, and the virulence of each was scored based on the prepatent period, duration to the peak of parasitaemia, and host survival. These data resulted in strains being categorized as follows: low virulence (n = 23); medium virulence (n = 25); and high virulence (n = 13). It bears mentioning that mice are not the natural host of \textit{T. congolense}, and that such virulence differences may not be fully representative of the situation in the field.
The basis for differential virulence between trypanosome strains is not currently known but may involve the host immune response or phenotype (e.g., disease tolerant animals), the presence of coinfecting organisms (section 1.5), parasite genetics or a combination of these factors. An example of how genetics can impact the virulence of a parasite and pathology in the host can be illustrated using studies involving Toxoplasma gondii. Genomic analyses identified allelic differences and a loss of upstream regulatory elements, which altered the expression of a serine-threonine kinase (ROP18) and subsequently led to different outcomes in a mouse model (Khan et al., 2009). In T. brucei, regions of the genome were identified that determined variation in virulence (Morrison et al., 2009a). This was achieved by first crossing field-derived strains (T. brucei TREU927 and T. brucei STIB247) within tsetse, and then identifying major quantitative trait loci (QTL) that corresponded to the virulence phenotype – in this case the extent of observed hepatomegaly and splenomegaly that result from infections in mice. While this QTL – which is located on chromosome 3 and covers more than 300 genes – highlights the importance of genetic factors in determining virulence, the search for individual virulence factors remains a priority.

1.4.1 Virulence factors in African trypanosomes

A number of virulence factors, or candidate virulence factors, have been identified in African trypanosomes, with some having been investigated as therapeutic targets (Autheman et al., 2021; La Greca and Magez, 2011). The
most obvious of these are the membrane-anchored VSGs, which not only contribute to antigenic variation but have also been shown to be immunogenic. Residues of the GPI-anchors that tether VSGs to the parasite membrane are cleaved and released into the mammalian bloodstream, where they interact with host macrophages and contribute to pathology (Leppert et al., 2007; Magez et al., 2002). Also contained within the VSG-expression sites (ES) are genes encoding TfRs, which allow trypanosomes to scavenge host transferrin from the bloodstream (Kariuki et al., 2019). These proteins are also GPI-anchored, and have been proposed to participate in host immune evasion owing to the diversity represented in the family (Trevor et al., 2019). To date, they have not been investigated for a role in modulating virulence.

Another surface-bound protein in *T. brucei* that interacts with host macrophages to modulate host immune responses, is trypanosome-suppressive immunomodulating factor (TSIF) (Gómez-Rodríguez et al., 2009). Interestingly, TSIF induces tumour necrosis factor (TNF) and NO production by macrophages, while simultaneously suppressing T-cell proliferation and type 2 immune responses. To date, TSIF between different strains have not been investigated for differential effects on virulence and have also not been investigated in other African trypanosomes. Sialidases (SA) and trans-sialidases (TS) have a well-documented role in pathogenesis, leading to desialylation of host erythrocytes, and have also been proposed as vaccine targets (Coustou et al., 2012; Nok and Balogun, 2003). Notably, knock-down of SA/TA expression levels in *T. congolense* resulted in an impairment of a number of virulence parameters in mice, including a reduction in parasitaemia,
while no apparent growth defects were noted in vitro (Coustou et al., 2012). Variation in the genes encoding SA/TA have already indicated different catalytic properties, and a potential role in differential virulence between strains warrants investigation (Koliwer-Brandl et al., 2011). Trypanosomes can also interact with the host environment through the expression of secreted factors, which have been further implicated as determinants of virulence. In one study, the secretome and gene expression differences were assessed between two genetically similar \textit{T. b. gambiense} strains that exhibited differential virulence (Holzmuller et al., 2008). It was found that the more virulent strain was capable of increased arginase induction of host macrophages, which subsequently improved parasite growth in vivo. Similar work was also performed on two strains of the animal-infective \textit{T. congolense}, and identified increased expression of nine proteins in the more virulent IL3000 strain, notably the immune-modulator calreticulin (Grébaut et al., 2009). Interestingly, the same study also found that the two strains expressed different isoforms of cysteine proteases (CP), among others. CPs, such as congopain, are associated with immunosuppression and anaemia within the host and their role as potential virulence modulators are further underscored by the divergent nature of the genes encoding these proteins among differentially virulent \textit{T. congolense} subgroups (Lalmanach et al., 2002; Rodrigues et al., 2014).

One area that has not been explored is the potential link between QS-responses and virulence in field isolates of trypanosomes. Such a link is plausible given that the literature indicates trypanosomes with a growth advantage are more virulent, while those exhibiting uncontrolled growth rapidly
kill their host (Turner, 1990). Furthermore, studies in bacteria have already linked QS to virulence through altered gene expression levels, while the up-regulation of non-QS genes in trypanosomes have been shown in virulent strains (Antunes et al., 2010; Grébaut et al., 2009). Taken together, this provides strong motivation for investigating changes in QS genes in trypanosomes, especially when considering the implications of such changes in the field.

1.4.2 The consequences and drivers of altered virulence

Virulence is important to consider and any alterations of such is likely to have consequences in the field (Ebert, 1998). For example, improved uptake of more virulent T. congolense strains by the tsetse vector has already been shown (Masumu et al., 2006a), with altered virulence having potential implications for transmission. Other investigations have started to elucidate the factors that give rise to altered virulence in trypanosomes, notably sexual recombination between genetically different lines (MacLeod et al., 2001; Morrison et al., 2009b). Host species and the duration of infection has also been shown to have an impact, as evidenced by T. congolense parasites being more virulent following chronic infection in goats (Joshua, 1990). Most strikingly, however, is the myriad of implications for both host and parasite, including altered virulence, which result from coinfections.
1.5 Coinfection

Hosts are rarely infected with a single pathogen. In fact, infections with multiple organisms, or coinfection, is the norm in natural systems. Here, a particular host will harbour a community of microorganisms (infracommunity) – commensal and pathogenic – comprising different taxa, various species, and strains of a given species. Coinfection has far-ranging effects on both host and parasite (section 1.5.2), which is ultimately determined by the composition of the infracommunity.

1.5.1 Factors which shape the coinfecting community

A number of biotic and abiotic factors work in concert to shape the community which may coinfect a host (Figure 1.9). The first of these is the environment, given that parasites have certain resource requirements and optimal environmental conditions in which they thrive, which will determine their individual distributions. This is evidenced by, for example, the free-living stages of sheep helminths which require optimal soil conditions to survive (O’Connor et al., 2006). Climate is important where parasites require a vector species for transmission, such as in the case of tsetse flies which are highly sensitive to variations in temperature and humidity (Longbottom et al., 2020; Pagabeleguem et al., 2016). We see the highest incidence of coinfection, then, where all of the conditions are favourable, allowing hosts, parasites and their vectors to overlap (Murray et al., 2015; Osakunor et al., 2018; Raso et al., 2004).
Figure 1.9: Some of the biotic and abiotic factors which can influence the composition of a coinfected community. These include (i) interaction between microbes; (ii) availability of vector species; (iii) the condition of the host; (iv) host genetics, and (v) environmental factors. Created in BioRender.

However, it is not only the availability of parasites and vectors that determines coinfection risk, but factors attributed to the hosts themselves. Genetics is one such host factor that can determine the composition of a microbial community, with notable examples being heritable selection for reduced parasite abundance in livestock, and trypanotolerant cattle (Diez-Tascón et al., 2005; Hanotte et al., 2003). Examples in the literature also indicate how poor diets
can lead to increased parasite burdens, while the opposite is true during feed-supplementation (Boulay et al., 1998; Squire et al., 2019). Interestingly, a recent study indicated that the association between nutrition and coinfection is more complex, and that the direction of the effect is not only dependant on nutritional content, but also the composition of the microbial community already present in the host (Budischak et al., 2015).

Timing of infection is also important, and the first pathogen to establish within a host often enjoys a competitive advantage which, in turn, may determine the composition of the coinfecting community. This can be seen in mice, where a pre-existing *T. brucei* infection limits secondary establishment of *Plasmodium bergei* in the liver (Sanches-Vaz et al., 2019). But, an existing infection may also facilitate infection by a second pathogen. This has been observed in humans, where the fungal pathogen *Candida albicans* modifies the host environment to facilitate infection by *Streptococcus* spp., and in mosquitoes where one strain of *P. chabaudi* enhances vector acquisition of additional strains (Pidwill et al., 2018; Pollitt et al., 2015). These examples point towards the diverse mechanistic interactions that can occur between coinfecting pathogens and, apart from merely shaping the community, also has implication for both host and parasite.
1.5.2 Microbial interaction and the implications for host and parasite

Interactions between coinfecting microbes can be broadly defined as being direct or indirect in nature. To directly interact, organisms require close interaction, as illustrated by co-cultures of the insect stages of *T. brucei* and *L. donovani*. While the mechanism remains unknown, cell-to-cell contact between these trypanosomatids resulted in impaired growth and death of *Leishmania* (Coppens et al., 1992). Direct interaction between coinfecting microorganisms may also involve the transfer of toxins or secreted products from one to another. A striking example of such direct interaction is the targeted release of hydrogen peroxide by the bacterium *Streptococcus pneumoniae*, which is lethal to a competitor, *Staphylococcus areus* (Selva et al., 2009).

Microorganisms can also interact indirectly with one another through, for example, modulation of the host immune system. Such immune-mediated interaction was shown between *Schistosoma mansoni* and *Helicobacter pylori* during experimental murine infections, where early colonisation by the parasite redirected T-cells which allowed for increased stomach colonisation by the bacterium (Bhattacharjee et al., 2019). Resource competition is another means by which coinfecting microorganisms compete. In fact, a network analysis of 300 published coinfection studies in humans indicated that parasites are more likely to compete via shared resources than through immune-mediated means (Griffiths et al., 2014). One example is the
competition between helminths and malaria parasites for resources in a human host, where deworming resulted in reduced competition for red blood cells and, as a result, significantly increased *P. vivax* densities (Budischak et al., 2018). Another example of such interspecies competition for valuable resources can be seen in the case of an opportunistic bacterial pathogen of humans, *Pseudomonas aeruginosa*. By secreting a siderophore with a high affinity for iron called pyoverdine, *P. aeruginosa* actively limited the access of coinfecting organisms such *Candida albicans* and *Aspergillus fumigatus* to host resources, thereby gaining a fitness advantage (Purschke et al., 2012; Sass et al., 2019).

Competition may have evolutionary consequences for pathogens, as explained by the competitive exclusion theory which holds that two organisms cannot occupy the same niche indefinitely when their resource requirements overlap (Hardin, 1960). Indeed, competition avoidance is seen as the driving force behind the varying tissue specificity seen in different filarial parasites of humans in sub-Saharan Africa (Molyneux et al., 2014). Coinfection may also select for altered virulence in microorganisms that is predicted to lead to enhanced transmission potential and a competitive advantage (Bremermann and Pickering, 1983). The aforementioned examples highlight the impact of microbial interaction on the infecting organisms but it should be noted that such interactions may also have implications for the host. In these scenarios, hosts may experience increased pathology, or an amelioration of disease severity, which is dependent on the timing of infection, immune response and composition of the coinfecting community. As an example, a meta-analysis of
co-infections in mice found that subsequent helminth infection could either enhance or inhibit malaria disease, depending on host immune responses (Knowles, 2011).

1.6 Trypanosome-trypanosome coinfections

Various combinations of AAT-causing parasites were found to infect hosts in Western Kenya, while as many as 6.9% of total trypanosome infections comprised more than one species in the Luangwa Valley of Zambia (Anderson et al., 2011; Thungi et al., 2010). It is not only different species of trypanosome that coinfect but also strains of the same species. In fact, microsatellite analyses of *T. brucei* samples from different regions of the African continent showed that mixed strain infections occurred frequently (Balmer and Caccone, 2008). Similarly, mixed-trypanosome infections also occur in the tsetse vector, with a recent study from Cameroon indicating that >10% of infected flies carried more than one species (Kamdem et al., 2020). Data regarding the inter- and intra-specific interactions between coinfecting trypanosomes and their interaction with the host are scant, even though there is a significant prevalence of mixed infections in the field. Studies have begun to shed light on the subject, however, and indicate how coinfection can influence tissue tropism, virulence, and transmission.

Tissue tropism has been observed in trypanosomes, and some evidence exist for competition avoidance by means of niche compartmentalisation. Where multiple strains of *T. cruzi* infected mice, for example, more
disseminated tissue invasion was observed (Perez et al., 2018). Similarly, a lack of competition was observed between strains of Crithidia bombi, a bumblebee-infective trypanosomatid, when localised to different tissues (Schmid-Hempel, 2001). Limited work has explored the tissue distribution of African trypanosomes within coinfected animals, but one study in goats indicated the delayed appearance of T. brucei in the blood when hosts were already infected with T. congolense (Dwinger et al., 1989).

The impact of trypanosome coinfection on parasite virulence is well documented. This can be highlighted by experimental infections comprising different lines of T. brucei, where the negative effects of a virulent strain were ameliorated in the host by the presence of another, avirulent strain (Balmer et al., 2009). Similar cross-strain modulation of virulence, or consequences of subsequent infection with a different strain, have also been documented in T. congolense (Masumu et al., 2009; Morrison et al., 1982). What is less clear, however, is whether trypanosome coinfection may select for altered parasite virulence. This may indeed be the case, as evidence from other organisms such as P. chabaudi suggests that coinfection may lead to the selection of parasites exhibiting increased virulence (Bell et al., 2006; de Roode et al., 2005). It may seem counterintuitive that selection would favour enhanced virulence, since premature host death may limit parasite success but may be explained by the resultant effects on transmissibility. High virulence has been linked to increased vector acquisition of T. congolense strains (Masumu et al., 2006a), for example, with enhanced transmission potential also observed in virulent P. chabaudi strains.
Signalling between trypanosomes, and subsequent growth responses and implications for transmission potential were recently discovered to occur between two trypanosome species (Silvester et al., 2017b). First, the authors showed that *T. congolense* can regulate its growth in a density-dependent manner and that the genome contained orthologues of QS-genes previously identified in a *T. brucei* RNAi screen. Confirming their functional equivalence, at least one conserved *T. congolense* gene was able to restore the ability of a *T. brucei* null mutant for the orthologous gene to differentiate into stumpy forms. Following this, they sought to investigate the potential for interspecies communication, and showed that *T. brucei* exhibited accelerated differentiation into non-proliferative stumpy forms in vitro and in vivo, in the presence of *T. congolense*. Importantly, this interaction was demonstrated to be mediated via shared QS signalling since the phenomenon was lost when a component of the QS signalling pathway was silenced in the coinfecting *T. brucei* line. Hence, *T. brucei* can respond to shared QS signals in coinfections with *T. congolense*, with potential consequences for their virulence and transmission potential. Whether *T. congolense* conversely responds to *T. brucei* is not clear.
1.7 Aims

The aim of this thesis was to investigate strain virulence in *T. congolense* and the impact of inter-species interaction between coinfecting African trypanosomes. The three main questions and their objectives were as follows:

1. What are the mechanisms behind differential strain virulence in *T. congolense* field strains? This entailed:
   a. Genomic analysis of QS-genes between field strains of *T. congolense* of differing virulence.
   b. Identification of transcriptome differences between strains of low and high virulence.
   c. Comparison of the abundance of transferrin-receptor transcripts, relative to alpha-tubulin (RAT), between trypanosome strains.

2. How do coinfecting trypanosomes interact and what are the lifecycle implications? This entailed:
   a. Determination of the coinfection prevalence of different African trypanosome species in field samples.
   b. Investigation of the coinfection dynamics of *T. brucei* and *T. congolense* in a murine model.
   c. Examination of the impact of chronic coinfection on *T. brucei* virulence.
3. How does coinfection alter the distribution of coinfecting trypanosomes, within a live host?
   a. Generation of *T. brucei* lines expressing a luciferase-reporter gene.
   b. In vivo imaging of trypanosomes in a murine model.
2 MATERIALS AND METHODS
2.1 Trypanosome strains

Seven *T. congolense* (Savannah) field strains were isolated from the Luangwa Valley of Zambia, and provided to us in stabilate form by Vincent Delespaux (Vrije Universiteit Brussel, Brussels). These strains are of sylvatic and domestic origin, and have been categorised based on their virulence in mice (Masumu et al., 2006b; Van den Bossche et al., 2011) (Table 2.1). These strains were used for genomic- and transcriptomic-analyses as discussed in Chapter 3 of this thesis. Field strain MF1 CL1 was also utilised in a coinfection experiment of Chapter 4.

Laboratory adapted *T. congolense* (strain IL3000) were used during in vivo coinfection experiments. The strain was derived from the ILC-49 strain originally isolated from a cow in the Trans Mara, Kenya (Wellde et al., 1974). The line used for in vivo experiments in this thesis was obtained from Annette MacLeod (University of Glasgow).

*Trypanosoma brucei brucei* EATRO 1125 (AnTat 1.1 90.13) expressing a Ty1 epitope-tagged PFR (hereafter referred to as *T. brucei* EATRO 1125 PFR-Ty) allowed for discrimination between species during coinfection experiments in mice (Bastin et al., 1996b; Silvester et al., 2017b). A *T. brucei* EATRO 1125 line expressing a red-shifted luciferase reporter gene was generated to use during in vivo imaging experiments (*T. brucei* AMLuc 4.2) (sections 2.12 and 2.13).
Table 2.1: Origin of *T. congolense* (Savannah) field strains. The seven strains utilised in this study were originally isolated from wildlife and livestock in Eastern Zambia, and fall into either low or high virulence categories (Masumu et al. 2006b; Van den Bossche et al. 2011).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSORO M19 C1</td>
<td>Domestic</td>
<td>Low</td>
</tr>
<tr>
<td>KAPEYA 357 C2</td>
<td>Domestic</td>
<td>Low</td>
</tr>
<tr>
<td>MF1 CL1</td>
<td>Sylvatic</td>
<td>Low</td>
</tr>
<tr>
<td>MSORO M7 C3</td>
<td>Domestic</td>
<td>Low</td>
</tr>
<tr>
<td>BT0206</td>
<td>Sylvatic</td>
<td>High</td>
</tr>
<tr>
<td>MF2 CL5</td>
<td>Sylvatic</td>
<td>High</td>
</tr>
<tr>
<td>MF3 CL1</td>
<td>Sylvatic</td>
<td>High</td>
</tr>
</tbody>
</table>

2.2 Trypanosome cultures

Pleiomorphic bloodstream form *T. brucei* EATRO 1125 (AnTat1.1 90.13) expressing a luciferase reporter (hereafter referred to as *T. brucei* AMLuc 4.2) were maintained in HMI-9 medium supplemented with 20% FCS (Gibco) and 100U/ml penicillin/streptomycin, at 37°C and 5% CO₂ (Hirumi and Hirumi, 1989). Drugs were also used to select for transfectants, and to maintain inducibility of the original *Trypanosoma brucei brucei* EATRO 1125 (AnTat 1.1 90.13) line (Table 2.2). Parasite cultures were maintained below a density of 1x10⁶ cells/ml. Stabilates were generated by centrifugation of 2x10⁶ cells at 1500 x g, before resuspension in 500µl HMI-9 containing 14% glycerol. Cryovials were placed in cell freezing containers (BioCision) at -80°C for 24
hours to maintain a constant rate of freezing. Thereafter, cryovials were moved to cryoboxes at -80°C and liquid nitrogen for long-term storage. Thawing of stabalites involved incubating cryovials at 37°C for 1 minute before adding the contents to 2.5ml of HMI-9. This was followed by centrifugation at 1500 x g for 5 minutes to pellet the cells and remove the glycerol. These cells were then resuspended in 10ml of fresh HMI-9. The same procedure was followed for thawing bloodstocks but instead flasks were incubated upright to allow red blood cells to settle at the bottom. The top layer of HMI-9 could be transferred to new flasks the following day, and topped up with fresh media, to remove red blood cells from the culture.

*T. congolense* IL3000 bloodstream forms were maintained in TcBSF3 medium supplemented with 25% goat serum, 5% serum plus (Sigma-Aldrich) and 100U/ml penicillin/streptomycin, at 34°C and 5% CO₂ (Coustou et al., 2010). Adherent cells were flushed from the wall of culture flasks every 2-3 days, and resuspended in fresh media. Parasite cultures were maintained below 1x10⁷ cells/ml. Stabilates were handled as for *T. brucei*, except that they were frozen down at densities of 3-6x10⁶ cells/ml and in freezing-mix comprising of TcBSF3/30% glycerol. The recovery of frozen stocks was performed according to the same procedure as for *T. brucei*.

Epimastigotes of *T. vivax* were cultured in TV3 media. Instead of being passaged, adherent cells were washed with 1x PBS, and topped up with fresh media every 2-3 days. Once adherent cells reached confluence, the media was removed before gently tapping on the flask to dislodge the cells. These were re-suspended in TV3 media and passaged. Only confluent cells were
used to make stabilates by centrifuging dislodged cells at 800 x g for 5 minutes. These were re-suspended TV3/10% glycerol. Thawing of stabilates involved the same procedure as described for *T. brucei*.

### 2.3 Trypanosome infections in mice

All experiments were performed in female MF1 mice (unless otherwise stated) aged six weeks or older, which were house in the animal unit at Ashworth Laboratories, University of Edinburgh. All procedures were performed by myself under the PIL number I34B9CA9B, and according to UK Home Office regulations (Project Licence P262AE604). Julie Young assisted with the initial inoculation of *T. congolense* field strains, into MF1 mice, from frozen stocks.

Blood stocks for all parasites were generated by intraperitoneal (IP) injection of $1 \times 10^2$ - $1 \times 10^4$ trypanosomes in 200µl of warm HMI-9 medium into mice. The parasitaemia was monitored from three days post-infection (p.i) by means of the rapid matching method of trypanosomes in blood collected by tail snips (Hebert and Lumsden, 1976).
Table 2.2: Maintenance of \textit{T. brucei} cell lines. Selective drugs (InvivoGen) and the required concentrations to maintain trypanosomes cells in HMI-9 media.

<table>
<thead>
<tr>
<th>\textit{T. brucei} cell line</th>
<th>Selective drug</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EATRO 1125/ PFR-Ty</td>
<td>G418</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Hygromycin</td>
<td>2.5</td>
</tr>
<tr>
<td>AMLuc 4.2</td>
<td>G418</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Hygromycin</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Puromycin</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Parasites were grown to a density of 3.1-6.3×10^7 cells/ml, after which the experiments were terminated by placing mice in a holding chamber and introducing isofluorane anaesthetic. Once the animals were deemed unconscious and unresponsive (reflexes assessed by squeezing a paw with tweezers), they were removed from the chamber and placed in the supine position with a nose-cone delivering anaesthesia. Whole blood was extracted by means of cardiac puncture (CP) using a 0.6 x 25mm Microlance needle (BD) and a 2ml syringe containing 200µl of 2% citrate to prevent blood from clotting. The extracted blood was diluted 1:1 with freeze mix (14% glycerol in HMI-9 medium) and stored at -80°C.

Recovery of whole-blood for experiments and sequencing was performed as described above and parasites were isolated while passing blood through a DEAE-C anion exchange column (DE52; GE Healthcare) at
pH 7.8 with PSG buffer. This allows red blood cells to be retained in the column, while trypanosomes are collected in the flow-through. The resin can be cleaned by adequate flushing with dH2O.

**PSG Buffer:**

3mM NaH2PO4.2H2O

44mM NaCl

83mM D-glucose

Adjust the final pH to 7.8

### 2.4 Extraction of genetic material

For the extraction of genomic DNA, parasites were first purified from whole-blood or taken from culture and pelleted at 1500 x g for 5 minutes. The cells were washed using sterile 1x PBS and pelleted again, before extraction using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer’s recommendations. The quality and quantity of DNA was assessed using a Nanodrop Spectrophotometer (Thermo Fisher Scientific Inc.) and stored at -20°C for general storage or -80°C for long-term storage or prior to sequencing.

RNA extractions were performed by purifying trypanosomes from whole-blood, followed by centrifugation at 1500 x g for 5 minutes. The pelleted cells were lysed by resuspension in 600µl RLT buffer (Qiagen) supplemented with
10µl β-mercaptoethanol/ml and stored at -80°C. Thawed lysate was processed using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s recommendations, before being resuspended in 40µl of dH2O. An additional DNase treatment step was performed, after the extraction process, using the Turbo DNase-free kit (Invitrogen). The quality of RNA was assessed using a Nanodrop™ Spectrophotometer (Thermo Fisher Scientific Inc.), while quantification was performed using a Qubit 2.0 fluorometer (Invitrogen) and the Qubit RNA BR Assay Kit (Invitrogen). Samples were stored at -80°C and repeated cycles of freeze-thawing was avoided.

Ethanol precipitation was performed in cases where further purification or increased concentration of genomic material was required. Briefly, genetic material was precipitated overnight at -80°C in 3M sodium acetate (0.1 x volume) and ice-cold 70% ethanol (2.2 x volume). The samples were then centrifuged at 15000 x g for 20 minutes, after which the pellet was dried, and resuspended in an appropriate volume of sterile dH2O.

2.5 Whole-genome sequencing

For whole-genome sequencing (WSG) experiments, 1x10^3 cells (in 200µl of HMI-9 media) of three *T. congolense* field strains (BT0206, M19 C1, 357 C2) were each IP-injected into three mice. Four additional field strains (Table 2.1) had already been sequenced in the Matthews’ Laboratory (University of Edinburgh). Whole-blood was harvested once parasitaemia reached at least
1.9x10^8 cells/ml, and was pooled for each strain (when required) to provide enough material for sequencing. At least 1µg DNA per strain was sent to BGI Tech Solutions (Hong Kong) for WGS using the HiSeq 4000 (Illumina, Inc.) 150PE service. At least 30x genome coverage was generated for each sample. The processing of raw data was kindly performed by Dr Alasdair Ivens (University of Edinburgh). Subsequent genome annotations and visualisations were performed in Artemis (release 16.0.0) (Carver et al., 2008), with between-strain alignment of amino acid sequences executed in Clustal Omega (Sievers et al., 2011).

2.6 RNA-seq analysis

Material was generated for RNA sequencing (RNAseq) by selecting two *T. congolense* field strains of high virulence (BT0206; MF2 CL5) and two of low virulence (MF1 CL1; M7 C3), and injecting 1x10^3 cells (in 200µl of HMI-9 media) into three mice per strain. Whole blood was harvested once parasitaemia reached 2.5x10^8 cells/ml, and subjected to RNA extraction. The purity and quantity of the extracted RNA was assessed and subsequently visualised on a 1.2% RNA gel. Briefly, 1µg RNA was added to 9µl formamide, 3µl of formaldehyde, 2µl of 10x MOPS and of 2µl of RNA loading buffer (see below). Samples were incubated at 65°C before being separated by gel electrophoresis using a 1.2% RNA gel (see below), for 90 minutes and 150V, in 1x MOPS buffer.
At least 1µg of RNA per strain was sent to BGI Tech Solutions (Hong Kong) for ‘Transcriptome Sequencing’, using the BGISEQ PE100 service, which generated 4Gb of clean data for each per sample. Dr Alasdair Ivens analysed raw RNA sequencing data. Quality assessment of these data was performed using FastQC (Andrews, 2010). Reads were aligned to the *T. congolense* IL3000 reference genome ([ftp://ftp.sanger.ac.uk/pub/project/pathogens/gff3/2018-08/](http://ftp.sanger.ac.uk/pub/project/pathogens/gff3/2018-08/)) using Bowtie 2 to get count numbers of reads mapping to each gene, and were normalised to the reads per kilobase of transcript per million mapped reads (RPKM) (Langmead and Salzberg, 2012). Six group-wise comparisons were performed between strains, with an additional comparison between the ‘high virulence’ and ‘low virulence’ groups.

**RNA Loading buffer**

30% Formamide

16.6% Formaldehyde

1x MOPS

0.01% Bromophenol blue

10% Glycerol
RNA gel

1.2% Molecular grade agarose (Bioline, Meridian Life Sciences®).

1x MOPS

3% Formaldehyde

2.7 RAT-analyses

Data generated for RNA-seq was utilised for ‘Relative Abundance to alpha-Tubulin (RAT) analysis, according to the protocol described by (Kelly et al., 2017). mRNA ‘Transcripts per million transcripts’ (TPM) is calculated by dividing read counts by gene length (in kilobases), to yield ‘reads per kilobase (RPK)’, and then dividing this number by a ‘per million’ scaling-factor. To compare abundance between strains, TPM for TfRs were normalised against alpha-tubulin (gene).

2.8 Field data

DNA had previously been extracted from whole-blood, which was obtained during a two-year longitudinal cohort study of cattle in Eastern Kenya (de Clare Bronsvoort et al., 2013). Aliquots of these samples were kindly provided by Drs Mark de Clare Bronsvoort (University of Edinburgh) and Phil Toye (ILRI, Nairobi). A subset of these samples, namely those which were previously identified as ‘trypanosome positive’ or ‘showing clinical disease’
were further investigated for the presence of AAT-causing parasites to determine the species composition. Two protocols were used to achieve this, targeting the internal transcribed spacer region 1 (ITS-1) (Cox et al., 2005), or the intergenic regions of the tubulin gene array of trypanosome genome (kindly provided by Professor Mark Carrington, University of Cambridge). PCR reactions were performed in 0.2ml PCR tubes (Corning) or 96-well plates (Bio-Rad Laboratories, Inc.), using SimpliAmp thermocyclers (Thermo Fisher Scientific Inc.). All primers (Table 2.3) used in this study were produced by Sigma-Aldrich and all reactions were performed using OneTaq (NEB, Inc.) according to the manufacturer’s recommendations.

DNA fragments were separated and visualised by means of DNA gel electrophoresis, using 1.5-2% gels. These were prepared by adding 1.5-2g of agarose powder (Bioline, Meridian Life Science) to 100ml of 1x TAE buffer (see below) and heating until completely dissolved. Ethidium bromide (0.5 µg/ml) was added and the gel was poured into a cast within an electrophoresis tank. The tank was filled with 1x TAE buffer once the gel had set, after which DNA was added alongside a 100bp DNA ladder (NEB, Inc.) to allow for size estimation. Electrophoresis was performed using 100V until the bands were resolved, and visualised using a G:box UV transluminator (Syngene).
Table 2.3: Species identification of field-collected trypanosomes. Two nested-PCR tests were utilised, targeting either the ITS-1 or tubulin regions of the genome. Primers are written in the 5’ to 3’ direction.

<table>
<thead>
<tr>
<th>Target region</th>
<th>Primers</th>
<th>Conditions</th>
<th>Notes &amp; Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS-1</td>
<td>(FWD1): GATTACGTCCCTGCCATTTG (REV1): TTGTTCGCTATCGGTCTTCC</td>
<td>Reaction 1: 95ºC for 7 min; (94ºC for 1 min; 55ºC for 1 min; 72ºC for 2 min) x 30 cycles; hold at 4ºC</td>
<td>Nested PCR reaction 1. Product used as template for reaction 2 (1:100 dilution).</td>
</tr>
<tr>
<td></td>
<td>(FWD2): GGAAGCAAAAAGTCGTAACAAGG (REV2): TGTGGTTTTTTTGTCTCCGCTG</td>
<td>Reaction 2: 95ºC for 7 min; (94ºC for 1 min; 55ºC for 1 min; 72ºC for 2 min) x 30 cycles; hold at 4ºC</td>
<td>1413-1513bp (T. congolense); 1204-1224bp (T. brucei sl); 611bp (T. vivax); and others (Ahmed et al., 2013).</td>
</tr>
<tr>
<td>Tubulin</td>
<td>(FWD1): GGTGAGTTCTCCGAGGCCCGTG (REV1): CCGTGTCGTCGCTGATCAC (REV2): CCGTGTCATCGCTGATCAC (REV3): CCGTGTCGTCGATCAC</td>
<td>Reaction 1: 94ºC for 1 min; (94ºC for 30s; 60ºC for 30s; 68ºC for 30s) x 30 cycles; 4ºC hold</td>
<td>Equimolar concentration of three reverse primers added to master mix; Nested PCR reaction 1. Product used as template for reaction 2 (1:100 dilution).</td>
</tr>
<tr>
<td></td>
<td>(FWD2): CTGATCACYTCCCAGAAGTTT (REV4): GAGGAGGAYGTKGGAGGATCATA</td>
<td>Reaction 2: 94ºC for 1 min; (94ºC for 30s; 60ºC for 30s; 68ºC for 30s) x 30 cycles; 4ºC hold</td>
<td>586bp (T. vivax); 456bp (T. congolense); 424bp (T. brucei)</td>
</tr>
</tbody>
</table>
1x TAE Buffer

40mM Tris-Acetate

1mM EDTA

Adjust pH to 8.0

2.9 Quantitative PCR

Primers amplifying the ITS-1 region of the trypanosome genome were used as described by Silbermayr et al., (2013). Probes were modified to contain different fluorophores to allow for simultaneous detection of trypanosome species in a mixed infection (Table 2.4). All primers and probes used in this study were produced by Sigma-Aldrich and all reactions were performed using Luna Universal qPCR mix (NEB, Inc.) according to the manufacturer’s recommendations. Reactions were performed in 96-well plates in volumes of 25µl, and quantitative PCR (qPCR) reactions were performed on a LightCycler 96 instrument (Roche Molecular Systems, Inc.). qPCR conditions were as follows: (1) Initial denaturation at 95°C for 60 sec; followed by 40 cycles each of (2) 95°C for 15 sec; and (3) 60°C for 30 sec.
Table 2.4: Primer and probe sequences to detect and quantify different trypanosome species. The primers and probes were first described in Silbermayr et al., (2013) and target the ITS-1 region of the trypanosome genome. A probe for the detection of *T. brucei* was adapted to contain a different fluorophore, to facilitate simultaneous detection of species within the same sample.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer/ Probes</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryps_KS-for</td>
<td>CGT GTC GCG ATG GAT GAC TT</td>
<td></td>
</tr>
<tr>
<td>Tryps_KS-rev</td>
<td>CTC CCA TGC GCC GTT TG</td>
<td>Incorrect primer sequence provided in original paper</td>
</tr>
<tr>
<td>Tryps_KS-T.cong-p</td>
<td>FAM - TTG CAG AAT CAT CAC ATT GCC CAA TCT TTG - BHQ1</td>
<td></td>
</tr>
<tr>
<td>Tryps_KS-T.brucei-p</td>
<td>Cy5 - TGC GAT TGG TAT CAA TTG CAG AAT CAT TTC - BHQ3&lt;br&gt;</td>
<td>Probe labelled with Cy5 failed to detect <em>T. brucei</em></td>
</tr>
<tr>
<td></td>
<td>FAM - TGC GAT TGG TAT CAA TTG CAG AAT CAT TTC - BHQ1</td>
<td></td>
</tr>
</tbody>
</table>
2.10 Immunofluorescence microscopy

All of the cells assessed by immunofluorescence analysis were in vivo derived and fixed in paraformaldehyde (Sigma-Aldrich). To achieve this, 2x10^6 cells were pelleted for 5 minutes at 2450 x g, followed by two washing steps in 1x PBS. The cells were resuspended in 125µl of ice-cold 1x PBS, before 8% paraformaldehyde in PBS was added and then incubated on ice for 10 minutes. The cells were again centrifugated at 1500 x g for 5 minutes, after which the supernatant was removed and the pellet resuspended in 130µl of 0.1M glycine (in 1x PBS). This was followed for an incubation period of 4°C for at least 1h, after which the cells were pelleted at 1500 x g for 5 minutes and resuspended in 1x PBS (10µl per 1x10^5 cells) for storage at 4°C.

Fixed cells were adhered to Polysine slides (VWR) for 1 hour. Each well was treated with 20µl of 0.1% Triton-X/1x PBS for 2 minutes, before being aspirated and washed with 1x PBS. Wells were then blocked with 2% BSA/1x PBS for 45 minutes, before the application of the appropriate primary antibodies for 45 minutes at 37°C. Primary antibodies were diluted in 2% BSA/1x PBS and used as follows: αBB2 (1:5) for species discrimination by means of TY-epitope tagging the T. brucei PFR; αPAD1 (1:1000) for PAD1 detection (Bastin et al., 1996b; Dean et al., 2009). Each well was washed five times by application of 1x PBS, followed by aspiration, before incubation with secondary antibody for 45 minutes at 37°C in a humidity chamber. Secondary antibodies were diluted in 2% BSA/1x PBS as follows: α-rabbit Alexa Fluor 488 (1:500) (Invitrogen) and α-mouse Alexa Fluor 568 (1:500) (Abcam). Wells with
secondary antibody only acted as controls. Each well was treated with 4′,6-diamidino-2-phenylindole (DAPI) (10µg/ml), which stains DNA and allows for visualisation of the kinetoplast and nucleus to determine cell-cycle stage. This was again followed by 5 wash steps, with 1x PBS, before slides were mounted with a cover slip by application of Mowiol containing 2.5% DABCO (see below).

The cells were visualised with a Zeiss Imager Z2 microscope, using either a Retiga 2000R (QImaging) or Prime BSI camera (Teledyne Photometrics) camera systems. All image analyses were performed using Fiji (Schindelin et al., 2012).

Mowiol + DABCO

10% (w/v) mowiol 4-88 reagent (Calbiochem®)

25% (w/v) glycerol

100mM Tris, pH8.5

2.5% (v/v) DABCO
2.11 Trypanosome coinfection dynamics

2.11.1 Trypanosome interaction experiments

For trypanosome interaction experiments, mice were infected in three groups as follows: (1) coinfection group (n = 3); (2) *T. congolense* MF1 CL1 control (n = 3); and (3) *T. brucei* EATRO 1125 PFR-Ty control (n = 3). Groups 1 and 2 were injected with 2x10^6 cells *T. congolense* cells on day 0, followed by a *T. brucei* superinfection of 2x10^5 cells, on day 3, into group 2. The *T. brucei* control group was also infected with 2x10^5 cells on day 3. Parasitaemia was scored from day 3 post-infection (section 2.3) and 10µl of blood was collected and subsequently paraformaldehyde fixed. Species-discrimination and the percentage of PAD1-positive cells were determined as described above (section 2.10) between days 4-8 of the experiment.

2.11.2 Long-term coinfection experiments

To investigate long-term coinfection dynamics of trypanosomes, mice were infected in three groups as follows: (1) coinfection group (n = 6); (2) *T. congolense* IL3000 control (n = 6); and (3) *T. brucei* EATRO 1125 PFR-Ty control (n = 6). Group 1 was coinfected with 3.2x10^3 *T. brucei* and 2.5x10^3 *T. congolense* cells on day 0. Groups 2 and 3, which acted as single infection controls, were injected with 3.2x10^3 *T. brucei* and 2.5x10^3 *T. congolense* cells on day 0. The experiment progressed for 38 days, with the condition of the animals being assessed daily and parasite levels being monitored from day 3.
post-infection (section 2.3). At peak parasitaemia, 10µl of blood was harvested for subsequent paraformaldehyde fixation from each subject in the coinfection group. Species discrimination was performed as described (section 2.10) with cell-cycle status and PAD1-levels being determined where parasitaemia allowed for a sufficient number of scorable cells. Whole-blood was harvested from the coinfection group at the end of the experiment for further analyses.

2.11.3 Changes in *T. brucei* virulence after chronic coinfections

Whole-blood was collected from chronically coinfected mice (section 2.11.2) by CP on day 38 (d38) and frozen at -80ºC. Parasite stocks were then used to seed two separate cultures of fresh HMI-9 as follows: (1) *T. brucei* EATRO 1125 PFR-Ty injected into mice on day 0; (2) *T. brucei* EATRO 1125 PFR-Ty collected from blood samples from d38. Parasite cultures were allowed to grow to approximately $1 \times 10^5$ cells/ml, before being passaged and drug-treated with G418 (0.5µg/ml). This was to ensure that any residual *T. congoense* would be eliminated from d38 samples, while also subjecting d0 parasites to the same conditions to limit variation between treatments. Care was taken to ensure that both d0 and d38 parasites were in the same growth-phase, and the number of passages were strictly limited. Cells were harvested from flasks and counted on a haemocytometer, prior to injection into mice.
To assess whether *T. brucei* from chronic coinfections exhibit altered virulence, cells from stabilates were IP injected into female MF1 mice in two groups: (1) $1 \times 10^3$ d0 cells ($n = 3$ mice); (2) $1 \times 10^3$ d38 cells ($n = 3$). The parasitaemia of both groups were monitored from 3 days p.i and the infection profiles were assessed for differences in prepatent period, time to peak-parasitaemia, and the number of parasites in the first peak. Mice were also weighed daily and were humanely terminated following the first peak of parasitaemia.

2.12 Trypanosome transfection

2.12.1 Transformation of XL-1 *Escherichia coli* competent cells

A plasmid containing red-shifted luciferase and ampicillin resistance genes (pTbAMLuc) was kindly provided by Dr Martin Taylor (LSHTM) prior to transformation into XL-1 blue *Escherichia coli* (Agilent Technologies, Inc.), and used to generate *T. brucei* AMLuc 4.2 used for in vivo imaging experiments. Competent cells were removed from storage at $-80^\circ$C and thawed on ice, while dried plasmid was eluted from filter paper in dH$_2$O. Thereafter, 1µl of eluted plasmid was added to 90µl of *E. coli* cells and incubated on ice for 30 minutes, before being heat shocked at $42^\circ$C for 30 seconds. These cells were then added to 800µl of LB broth and placed in a shaking incubator at $37^\circ$C. After 1 hour, the cells were pelleted by means of centrifugation at 2400 x g for 90
seconds, and 800µl of supernatant was removed. The cells were resuspended in the remaining liquid, plated onto agar plates containing ampicillin (100µg/ml) and subsequently incubated overnight at 37°C. Single colonies were picked from the agar plates and added to 10ml of LB broth containing 100µg/ml ampicillin, and again placed in a shaking incubator at 37°C, overnight.

The following day, 10ml cultures were pelleted at 3000 x g for 5 minutes and resuspended in 100µl of solution I (see below). 150µl of solution II was then added to each tube and mixed several times by inversion. After 5 minutes, 200µl of ice-cold solution III was added, again mixed by inversion, and incubated on ice for 5 minutes. The samples were then transferred to 1.5ml Eppendorf tubes, and were centrifuged at 15,000 x g for 15 minutes at 4°C. The supernatant was then transferred to a new Eppendorf tube, before being centrifuged again at 15000 x g for 10 minutes at 4°C. The supernatant was again transferred to fresh tubes and 900µl of ice-cold absolute ethanol was added, before being incubated at -80°C for 30 minutes. The tubes were again centrifuged at 15000 x g for 30 minutes at 4°C, after which the ethanol was removed and replaced with 200µl of 70% ethanol. This was followed by a final round of centrifugation at 15000 x g for 5 minutes at 4°C, with the ethanol being subsequently carefully removed, and the DNA pellets allowed to air-dry. The DNA was resuspended in 30µl dH₂O containing 330µg/ml RNase, and quantified using a Nanodrop™ Spectrophotometer (Thermo Fisher Scientific Inc.).
Solution I

50mM Glucose

25mM Tris, pH 8.0

10mM EDTA, pH 8.0

Autoclave

Solution II

0.2M NaOH

1% SDS

Solution III

3M Potassium acetate

5M Glacial acetic acid

### 2.12.2 Transfection and drug-treatment

Prior to transfection, 20µg of plasmid DNA was linearised with SacI and KpnI restriction enzymes (1 unit/µg DNA) (NEB) in 1x CutSmartBuffer (NEB), and incubated overnight at 37°C. Verification of a successful restriction digestion was achieved by means of visualisation of two fragments (4000bp;
2500bp) after gel electrophoresis. Concurrently, a culture of *T. brucei* EATRO 1125 was maintained below a density of 1x10⁶ cells/ml (see section 2.2).

Once ready for transfection, a total of 3x10⁷ cells were centrifuged at 1400 x g for 5 minutes and resuspended in 1ml of ‘Roditi transfection buffer’ (RTB) (see below). The cells were again pelleted at 2370 x g for 5 minutes and resuspended in 100µl of Amaza transfection buffer (Amaza Basic Parasite Nucleofector™ Kit 2; Lonza). The mixture was transferred to transfection cuvettes and 10µl of linearised plasmid was added, before electroporation in an Amaza Nucleofector™ II using the Z-001 programme. Cells were transfected with both fragments (see above) to avoid a loss of pDNA associated with gel purification, as incorporating the undesirable fragment (2500bp) into the trypanosome genome was considered unlikely. A separate cuvette acted as a control, with dH₂O instead of DNA being added (mock). The transfected cells were then transferred to 30ml of pre-warmed HMI-9 media, with a serial dilution of the original flask (1:10; 1:100) also being prepared in 30ml volumes. Drug-treatment of these cells occurred after a 6-hour recovery period, or the next day.

Cultures were mixed with an equal volume of fresh HMI-9 containing 2x volume of selection drugs (Table 2.2) and transferred to 24-well plates (Corning Costar), with one plate being prepared for each dilution (undiluted; 1:10; 1:100). Each plate contained a well of untransfected cells as a drug-treatment control, as well as a well of mock transfectants. Those wells where parasites increased, after all of the cells died in the controls (3-8 days after
transfection), were transferred to fresh HMI-9 with selective drugs. The cultures which survived two successive passages were selected and frozen (section 2.2) for future analyses.

RTB

90mM Sodium phosphate

50mM HEPES, pH7.3

5mM KCl

0.15mM CaCl$_2$

2.13 In vivo localisation of trypanosomes

2.13.1 Selection of clones

Four *T. brucei* AMLuc clones (4.1–4.4) were selected following drug-treatment of transfectants. An adapted protocol described by (McLatchie et al., 2013) was used to assess luciferase expression and select the most suitable clones for in vivo imaging experiments. These clones were seeded into separate flasks with HMI-9 media and grown to a density of $5 \times 10^6$ cells and then pelleted at 1000 x g for 5 minutes. The media was removed and cells were resuspended in 100µl of reconstituted Bright-Glo substrate (Promega). $1 \times 10^6$ cells of each clone were added to a well in the first column of a 96-well
flat-bottomed plate (Corning) and serially diluted along each row (1x10^5 to 1x10^2 cells). The cells were left to lyse at room temperature for two minutes before determining the fluorescence, measured in relative light units (RLU), of each clone and dilution using a Varioskan Flash plate reader (Thermo Fisher Scientific Inc.).

2.13.2 *T. brucei* AMLuc growth

To determine if there were any growth defects associated with transfection, the growth of *T. brucei* AMLuc 4.2 was compared in vivo to the parental *T. brucei* EATRO 1125 (AnTat1.1 90:13) line. Female CD1 mice were each infected with trypanosomes (see section 2.3), in two groups, as follows: (1) 1x10^3 *T. brucei* AMLuc 4.2 cells (n = 3); (2) 1x10^3 *T. brucei* EATRO 1125 (AnTat1.1 90:13) line. The parasitaemia of mice in each group was determined from 3 days post-infection and followed up to day 10 of the experiment.

2.13.3 In vitro IVIS visualisation of *T. brucei* AMLuc

The *in vitro* bioluminescence and detection limit of *T. brucei* AMLuc (clones 4.1 and 4.2) was determined, using the IVIS Lumina III imaging system (PerkinElmer). Prior to imaging, a 200x stock solution of D-luciferin (PerkinElmer) (30mg/ml) was prepared in sterile water and stored at -20°C. Aliquots of this stock solution were quick-thawed and used to prepare a 1:100 working solution in warm HMI-9 media.
Next, $1 \times 10^5$ cells of *T. brucei* AMLuc were added to wells in the first column of a 96-well plate (Corning) and serially diluted across columns in 100µl of HMI-9, generating a range of $10^5$ to $10^2$ cells. Each well was topped up with 100µl of D-luciferin working solution (final concentration 150µg/ml) and incubated at 37°C for 10 minutes prior to imaging.

### 2.13.4 IVIS calibration for in vivo imaging of trypanosomes

The in vivo bioluminescence and imaging profile (optimal time to image) of *T. brucei* AMLuc 4.2 was determined using the IVIS® Lumina III imaging system (PerkinElmer). Prior to imaging, a fresh stock solution of D-luciferin (PerkinElmer) (15mg/ml) was prepared in Ma$^{2+}$/Ca$^{2+}$-free Dulbecco’s Phosphate Buffered Saline (DPBS) (Sigma-Aldrich®) and filter-sterilised. The working solution can be stored at 4°C for three weeks.

Three days before imaging, female MF1 mice were infected with *T. brucei* AMLuc 4.2 as follows: (1) no parasites ($n = 1$); (2) $1 \times 10^3$ *T. brucei* AMLuc 4.2 cells ($n = 1$); (3) $1 \times 10^4$ *T. brucei* AMLuc 4.2 cells ($n = 1$). Parasitaemia was also monitored as described (section 2.3), from 3 days p.i, with in vivo imaging taking place on days 3 and 6. Mice were weighed and IP injected with 150mg/kg of D-luciferin before each round of imaging. Sustained anaesthesia was administered to animals and imaging was performed every 5 minutes, from 10 to 30 minutes post-injection, to determine an imaging curve for future
experiments. Viscotears (Bausch & Lomb) was applied, during imaging, to ensure that the eyes of mice remained moist. Animals were allowed to recover on a heat-pad following imaging, and monitored for any adverse effects.

2.14 Ex vivo imaging of mouse organs

Mice were placed in an induction chamber and anaesthetised with an initial concentration of 4% isofluorane, which was later adjusted to 1.5-3% for maintenance of anaesthesia. Animals were then transferred to a perfusion pad with a nose cone for sustained administration of isofluorane. Mice were placed on their back with their limbs pinned to ensure that the animals remained in place during all subsequent procedures.

A small incision was made in the abdominal wall midline, using sharp scissors, followed by two lateral-wards incisions towards the mouse axillae to expose the ribcage. The diaphragm was cut below the xyphoid process and opened before the ribcage was removed to expose the chest cavity. The vasculature was flushed by inserting a needle and syringe containing perfusare (heparinised PBS) into the left ventricle and cutting the right atrium. Successful perfusion resulted in blanching of the organs. Organs of interest were carefully removed using clean forceps, scissors, and scalpels.

Once harvested, organs were placed in a petri-dish and soaked in 1xPBS to avoid dessication. Thereafter, organs were soaked in 100-300µl of
D-luciferin (PerkinElmer®) (15mg/ml) for 10 minutes, before imaging using the IVIS® Lumina III platform (PerkinElmer®).

Perfusate

1 liter of 1x PBS

54mg Heparin (185.8 units/mg stock)

### 2.15 Statistical analysis and production of figures

The statistical analyses in this thesis were performed in GraphPad Prism (version 9.2.0). Results with a p value ≤ 0.05 were considered to be statistically significant.

Graphs were generated in GraphPad Prism (version 9.2.0), and diagrams were produced using BioRender.com. Gel and microscope images were manipulated (i.e. adjusting brightness and contrast; cropping; adding scale bars) using Fiji Software (Schindelin et al., 2012).
3 INVESTIGATING THE MOLECULAR DETERMINANTS OF DIFFERENTIAL STRAIN VIRULENCE IN T. CONGOLENSE
3.1 Introduction

Virulence differences between trypanosomes are well-documented but poorly understood. Previous investigations have focused on trypanosome epidemiology – recording the prevalence of strains with different virulence characteristics in different geographic areas – or differences in transmission efficiency, while neglecting the underlying mechanisms behind such differences (Masumu et al., 2006b; Chitanga et al., 2013; Nakamura et al., 2021). One area which has yet to be explored is the potential link between QS responses and virulence in trypanosomes. Such a link is plausible given that trypanosomes exhibiting uncontrolled growth have been shown to rapidly kill their host (Turner, 1990). Furthermore, studies in bacteria have also linked QS to virulence through altered gene expression levels, while secretome differences have been shown between strains of *T. congolense* strains that exhibit different virulence profiles (Antunes et al., 2010; Grébaut et al., 2009).

Taken together, this provides motivation for a thorough investigation of the link between strain virulence and changes in the genome, or transcriptome, of trypanosome strains. Previously, *T. congolense* (subtype: Savannah) strains which exhibit different virulence phenotypes, measured in terms of mouse survival, were isolated from the Luangwa Valley, in Zambia (Masumu et al., 2006b; Van den Bossche et al., 2011). The ability to establish these lines in mice, the identification of *T. congolense* QS gene orthologues, and the availability of next-generation sequencing (NGS) technologies, allows us to explore this link.
3.2 Whole-genome sequencing of *T. congolense* field strains

Seven field strains of *T. congolense* (subtype: Savannah) (Chapter 2; Table 2.1) were successfully established in mice and bloodstocks were frozen in the laboratories of the Institute of Immunology and Infection Research (IIIR), University of Edinburgh, before genomic material for sequencing could be generated. Five additional field strains, from stabilate, could not be established in mice (Appendix 1.1). This may be due to difficulties that are routinely associated with adapting field strains for infections in mice, or the quality of stabilates which had been stored at -80°C for a prolonged period.

The genomes of four strains had already been generated before undertaking this project (MF5 CL2; MF3 CL1; MF1 CL1; M7 C3). To generate genome data for the additional *T. congolense* strains, three groups of female MF1 mice (n = 2 per group) were each injected IP with $1 \times 10^3$ cells from a given strain (BT0206; 357 C2; or M19 C1). The parasitaemia was monitored from three days post-infection and grown to comparable levels between strains, before whole-blood was harvested for DNA extraction and subsequent whole-genome sequencing (WGS) at BGI Tech Solutions Hong Kong (BGI).
3.2.1 Strains exhibit differential virulence in mice

Clear differences in parasite virulence were noted between these three strains, as measured by the prepatent period and the time to reach the termination threshold (Figure 3.1). BT0206, a strain of sylvatic origin, was the most virulent, with parasites being first detected at day 3 for both mice (2.3-4.7x10^7 parasites/ml), and the end-point of infection being reached a day later (1.8-2.5x10^8 parasites/ml). This was followed by strains 357 C2 and M19 C1 – which were both isolated from domestic livestock – where parasites were first detected at day 4 (both 5x10^5 parasites/ml) with experimental end-points being reached on day 6 (both between 7.25x10^7 and 2.5x10^8 parasites/ml). These data confirm the results of a previous study, which categorised BT0206 as a strain of high virulence, and 357 C2 and M19 C1 as low virulence strains (Masumu et al., 2006b; Van den Bossche et al., 2011).

The four strains previously established in our laboratory also presented with different growth characteristics (Appendix 1.2) which corresponded to the virulence categories to which they were originally assigned, namely high virulence (MF2 CL5 and MF3 CL1) and low virulence (MF1 CL1 and M7 C3).
Figure 3.1: *T. congolense* infections in MF1 mice. Three groups, each consisting of two female MF1 mice, were infected with one of the following field strains: BT0206, 357 C2 or M19 C1. Parasites were allowed to grow to a threshold of 1-2\times10^8 cells before experiments were humanely terminated for whole-blood collection, parasite isolation, and subsequent DNA extraction. Infections for two strains (357 C2; M19 C1) had to be repeated to generate enough DNA for sequencing. Virulence profiles (measured in terms of prepatent period; time to reach threshold parasitaemia) confirm that BT0206 = high virulence; 357 C2 and M19 C1 = low virulence. Disease severity or pathology were not assessed between groups.
3.2.2 Sample preparation and sequencing

Mouse blood was passed through a DEAE resin column to separate out trypanosome cells for subsequent DNA extraction (Lanham and Godfrey, 1970). The amount of the DNA in each sample was analysed using a Qbit (2.0) spectrophotometer – as each sequencing reaction by BGI required at least 1µg of DNA – and the quality of those samples were assessed by means of a nanodrop spectrophotometer, measured in absorbance values, and thereafter by gel-electrophoresis. A sufficient quantity of genomic DNA was obtained for BT0206 following the first round of infections using a commercial kit (Table 3.1). The quality of the DNA was assessed in our laboratory and confirmed by BGI before performing WGS. Additional infections had to be performed to obtain enough genetic material for the sequencing of two further stains, namely 357 C2 and M19 C1. This may be ascribed to, in part, a lower number of parasites being purified through the DEAE resin column for the two low virulence strains. Previous observations from our laboratory (pers. comm. Dr Stephen Larcombe) further suggest that T. congolense adheres to the inside of resin columns, hampering purification from mouse blood. Thus, infections were repeated for 357 C2 and M19 C1, as described above, and parasite DNA was subsequently extracted by means of the phenol-chloroform method in an effort to improve yields of genomic material. DNA quantities for both strains were low, but a sufficient amount of DNA was obtained for 357 C2 following ethanol precipitation for WGS (Table 3.1). A third round of infections, phenol-chloroform extractions, and ethanol precipitation, yielded enough DNA for
WGS of strain M19 C1. The requisite quality checks were performed on material from both strains in our lab (as for BT0206), and confirmed by BGI upon receipt prior to sequencing.

Table 3.1: Preparation of DNA from *T. congolense* field strains for whole-genome sequencing. At least 1µg of DNA is required for WGS, necessitating repeat infections for strains 357 C2 and M19 C1, and the use of the phenol-chloroform extraction method for both. Quantification and quality checks were performed in our laboratory and confirmed by BGI prior to sequencing. The differences in these final concentrations can be ascribed to issues related to the purification of parasites from whole mouse blood.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extraction Method</th>
<th>Concentration (ng/µl)</th>
<th>Total amount for WGS (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT0206</td>
<td>Qiagen Dneasy Kit</td>
<td>51.4</td>
<td>3.08</td>
</tr>
<tr>
<td>357 C2 (repeat exp.)</td>
<td>Phenol-chloroform + EtOH precip.</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>M19 C1 (repeat exp.)</td>
<td>Phenol-chloroform + EtOH precip.</td>
<td>13.2</td>
<td>1.3</td>
</tr>
</tbody>
</table>
3.3 Genome analyses

Sequenced reads for the three stains (BT0206, 357 C2 and M19 C1) were downloaded from BGI, quality checked, and de novo assembled by Dr Alasdair Ivens. The genome sequences of seven total *T. congolense* field strains, as well as the IL3000 reference genome, were then visualised and annotated using Artemis (release 16.0.0) (Carver et al., 2008).

3.3.1 Differences exist between the encoded protein sequences of QS gene orthologues of *T. congolense* field strains

Several components of the QS pathway had been previously identified in *T. brucei*, along with orthologues in *T. congolense* (Mony et al., 2014; Silvester et al., 2017b). The predicted protein sequence of each orthologue in each *T. congolense* strain was aligned to the reference *T. congolense* (IL3000) genome in Clustal Omega (Sievers et al., 2011) and scrutinised for mutations, resultant amino acid changes, or deletions (Appendix 1.3).

Point mutations, deletions, and frameshifts were detected in individual strains – such as MF3 CL1 which contained a deletion between positions 332-348 in the amino acid sequence of TclL3000.0.18960 (adenylosuccinate lyase) – but would likely not contribute to strain virulence (Appendix 1.3). Rather, we anticipated that phenotypically meaningful differences would be shared across
strain categories (i.e., low or high virulence strains), with those being prioritised for further assessment (Figure 3.2a). For example, three proteins, comprising a hypothetical protein (TcIL3000.11.1420), protein kinase (TcIL3000.0.44450) and a putative kinetoplastid-specific phosphatase (TcIL3000.0.52520), contained amino acid substitutions that were shared by at least two strains of the same virulence category (Figure 3.2a). One particularly interesting substitution – because of its presence across three of the four low virulence strains used in our study (MF1 CL1; M7 C3; and M19 C1) – as noted in the amino acid sequence of TcIL3000.11.3610 which encodes adenylosuccinate synthetase (AdSS). Here, at position 415, those low virulence strains contain a serine residue (Ser415), compared to alanine in the reference IL3000 and high virulence field strains (Figure 3.2b).
77

Figure 3.2: Amino acid substitutions in *T. congolense* field strains compared to the laboratory strain IL3000. (A) Gene products of interest, where an amino acid substitution is present across more than one strain, within the same virulence category. (B) Alignment of the predicted protein sequences of the *T. congolense* AdSS (*TcAdSS*) indicated an alanine to serine substitution at position 415 (Ser415), across three low virulence strains (highlighted in teal). See Appendix 1.4 for variation at this position in the genomes of other species.

### 3.3.2 Adenylosuccinate synthetase protein structure

To investigate the impact of the Ser415, which is present in three low virulence strains, we modelled *T. congolense* AdSS (*TcAdSS*) using the I-TASSER server (Yang and Zhang, 2015). First, a 3D model was generated based on the predicted protein sequence of TcIL3000.11.3610 (c-score = 2.10; estimated TM-score = 0.46 ± 0.15; estimated RSMD = 12.8 ± 4.2Å) and aligned to *Plasmodium falciparum* AdSS (PDB 1p9b) for which a crystal structure had been resolved previously. The results of this analysis indicated that some regions did not align well with the *Plasmodium* protein, notably the region of interest which contained Ser415 (position 380-430) (Figure 3.3a).
interrogate this alignment further, we visualised TcAdSS superimposed onto 1p9b in PyMOL (v2.5), and noted that the trypanosome protein contained additional alpha-helices which were not present in its Plasmodium counterpart (Figure 3.3b). Upon further investigation, using a BLASTP search, it was found that the region of interest was missing from the protein sequences encoding AdSS of other organisms, and seemingly specific to trypanosomatids.

The lack of a resolved trypanosomatid AdSS crystal structure, prompted us to utilise the AlphaFold DB (Jumper et al., 2021; Varadi et al., 2022). This database of protein structure predictions allowed for the alignment of TcAdSS, to the T. cruzi counterpart. The region of interest, containing Ser415, once again did not align well to the predicted T. cruzi protein (Appendix 1.5). This did not allow any possible impact of the change to be modelled, therefore, and it was decided not to pursue this variant further.
Figure 3.3: I-TASSER modelling of *T. congolense* AdSS (*TcAdSS*), aligned to the *Plasmodium* protein 1p9b. (A) The estimated accuracy values (measured in Angstrom) indicated that the region of interest, containing Ser415, does not align to 1p9b. (B) Visualisation in PyMOL (v2.5) indicated that *TcAdSS* (teal) contains additional alpha-helices (numbered 1-3), when compared to 1p9b (grey), with residues 380-430 highlighted (salmon).
3.4 RNA-sequencing

3.4.1 Preparation of material

In addition to analysing genes specifically implicated in QS signalling at the genomic level, we broadened the analysis of the *T. congolense* isolates of differing virulence by analysing their complete transcriptomes. Specifically, we selected four *T. congolense* field strains (high virulence: BT0206 and MF3 CL1; low virulence: M7 C3 and MF1 CL1) for investigation. These parasites were injected into mice, in triplicate, and allowed to reach comparable levels of parasitaemia before whole-blood was collected.

Parasites were isolated through a DEAE resin column, and RNA was subsequently extracted. The quality of the RNA was assessed prior to performing RNA-seq analysis. First, 1µg of RNA per sample was loaded onto a denaturing gel to confirm that the samples were not degraded. Ethidium bromide staining resulted in 5 bands per sample, which is expected for *T. congolense* RNA (Appendix 1.6). Thereafter, the quality and quantity of the RNA was assessed, using a nanodrop spectrophotometer.

3.4.2 RNA-seq results

Parasite RNA was prepared and 1µg per replicate (n=3 per strain) was sent to BGI Tech Solutions, Hong Kong, for quality assessment and subsequent analysis. Library preparation was performed at BGI and
sequencing on the BGISEQ platform was performed (PE 100bp service) resulting in 4 Gb of clean data. The raw reads were downloaded and the quality of those were assessed by Dr Alasdair Ivens, using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). All of the reads were of high quality and did not contain primer content. The reference IL3000 genome was also downloaded, and gene models were extracted from the genome file using simple scripts and a bedfile comprising 11791 loci generated for subsequent count summarisation with bedtools (v2.23.0). Alignments were preformed using Bowtie 2 (Langmead and Salzberg, 2012), with outputs being sorted and indexed using Samtools (v1.12) (Li et al., 2009) and stored as compressed bam files.

Next, read counts were normalised to reads per kb/ map (rpkm) and group-wise comparisons were performed in R/ Bioconductor software package limma between T. congolense strains (Ritchie et al., 2015). The fold changes (FC) of transcripts between strains were recorded as Log2FC where a value of 1 or larger indicates a transcript that is at least 2-fold more abundant in one strain versus another. Similarly, a Log2FC of 1, or lower than 1, indicates a transcript which is at least 2-fold less abundant.
3.4.3 Transcripts are differentially expressed between
*T. congolense* field strains

The comparison performed was ‘high virulence’ relative to ‘low virulence’, where a positive Log2FC represents transcripts that are more abundant – whereas a negative value is accordingly less abundant – in strains of high virulence compared to those of low virulence (Figure 3.4a). Certain exclusion criteria were applied to identify the transcripts that were the most significantly differentially expressed between the two categories, and this process is summarised below (Figure 3.4b). First, a statistical threshold of \( p < 0.05 \) was applied, which resulted in 1142 transcripts that were differentially expressed (641 up-regulated, 501 down-regulated between high and low virulence isolates). Next, all transcripts with a Log2FC between -1 and 1 were excluded, resulting in 628 transcripts of interest (319 up-regulated, 209 down-regulated). Finally, transcripts that were described as having no protein product were excluded, along with those related to VSG, resulting in a short-list of 299 transcripts across a number of categories (Figure 3.5a). The transcripts with the most significantly different abundances were ‘hypothetical proteins’ (55%), followed by ‘VSG-associated congolense-specific ORF’ (8.1%) which were not excluded given that BLASTP results indicated non-VSG related protein identity in *T. brucei* (Appendix 1.7). These may be incorrectly annotated as VSGs in the *T. congolense* IL3000 genome, and warrants further investigation. Other prominent categories included ‘transferrin receptor-like’
Figure 3.4: RNA-seq analyses of *T. congolense* field strains and workflow for the identification of the most significantly differentially expressed transcripts. (A) Initially, 1142 transcripts were found to be differentially expressed in high virulence strains relative to low virulence strains. (B) A curated list of candidates was created, by first excluding those transcripts with a p-value > 0.05, followed by transcripts with a < 2-fold change in transcript levels. Finally, transcripts related to VSG were removed, as these may confound the search for differentially expressed genes. This process resulted in a final list of 299 transcripts (Image generated in BioRender).
(6.9%), ‘zinc-finger proteins’ (3.8%) and ‘retrotransposon hot spot proteins’ (2.6%). Notably, the short-list did not contain transcripts corresponding to the QS-gene orthologues previously identified in *T. congolense*.

The direction and extent of fold-changes associated with each class of transcript in the shortlist (n = 299) was scrutinised further (Figure 3.5b). Interestingly, a large subset of TfR-like proteins were upregulated in high virulence strains when compared to low virulence strains. In fact, TfR-like transcripts were over-represented in a subset of those transcripts with > 4-fold increased abundance in high virulence strains (Table 3.2). A second class of transcripts showed a clear direction and expression pattern in high virulence compared to low virulence strains, namely SecA DEAD-like domain containing proteins.
Figure 3.5: Identification of transcripts with significant fold changes following RNAseq of *T. congolense* field strains. (A) Characterisation of the 299 transcripts, with 'hypothetical protein' being returned as the largest group (55%), followed by 'VSG-associated, congolense specific ORF' (8.1%) and 'transferrin receptor-like' (6.9%). Transcripts labelled as 'VSG-associated, congolense-specific ORF' were not excluded, given that BLASTP results indicated non-VSG identity in *T. brucei*. (B) Expanding on the fold-changes associated with 8 classes of transcripts, 'Transferrin receptor-like' transcripts were generally upregulated in high virulence strains, and also represented the transcripts with the highest increased fold-change in high virulence strains (Log2FC = 2.86); 'SecA DEAD-like' transcripts were down-regulated in high virulence strains.
Table 3.2: Genes exhibiting the largest changes in expression in high virulence compared to low virulence strains. Twenty-six transcripts were identified with fold changes between -4 and 4 (Log2FC > 2), with transferrin receptor-like proteins being over-represented in this list.

<table>
<thead>
<tr>
<th>Feature ID</th>
<th>Description</th>
<th>Log2FC</th>
<th>pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcIL3000_0_43130</td>
<td>Transferrin receptor-like, ESAG6-like</td>
<td>2.86</td>
<td>9.63E-12</td>
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<tr>
<td>TcIL3000_0_50290</td>
<td>mitochondrial carrier protein</td>
<td>2.54</td>
<td>1.16E-09</td>
</tr>
<tr>
<td>TcIL3000_0_29030</td>
<td>geranylgeranyl transferase type II beta subunit, putative</td>
<td>2.48</td>
<td>7.99E-10</td>
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<td>Transferrin receptor-like, ESAG6-like</td>
<td>2.39</td>
<td>1.20E-08</td>
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<td>Transferrin receptor-like, ESAG6-like</td>
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<td>2.48E-11</td>
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<tr>
<td>TcIL3000_0_32750</td>
<td>expression site-associated gene (ESAG) protein, putative</td>
<td>2.36</td>
<td>1.06E-08</td>
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<td>2.36</td>
<td>1.64E-08</td>
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<td>TcIL3000.11.13190</td>
<td>cyclin 1, serine peptidase family S51, peptidase E, putative</td>
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<td>4.29E-08</td>
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<td>Trypanosomal VSG domain containing protein, putative</td>
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<td>1.56E-07</td>
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<tr>
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<tr>
<td>TcIL3000_0_15070</td>
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<td>TcIL3000_0_41820</td>
<td>SecA DEAD-like domain containing protein, putative</td>
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3.4.4 Transferrin receptor abundances on the trypanosome cell surface do not differ between field strains

While the primary role of TfR’s is to scavenge host iron from their environment, these proteins have also been implicated in host immune evasion (Kariuki et al., 2019; Trevor et al., 2019). Given that TfR-like proteins are upregulated in high virulence strains in our RNA-seq screen (section 3.4.3), we sought to explore a link between these proteins and differential strain virulence. This led to the hypothesis that TfR’s may be more abundant on the cell surface of virulent stains, compared to those of low virulence.

To test this hypothesis, transcript abundance was used to infer protein abundance by normalising the TPM of a given transcript, to alpha-tubulin (Kelly et al., 2017). For example, a given transcript with TPM-value that is 2 times greater than that of alpha-tubulin, has a relative abundance to alpha-tubulin (RAT) of 2. A transcript with TPM-value that is 5 times greater than that of alpha tubulin, has a relative abundance to alpha-tubulin (RAT) of 5, and so forth. The TPM- and RAT-values were calculated for 24 genes encoding TfR’s in T. congolense, after applying exclusion-criteria to our RNAseq data. These values were then normalised to T. congolense alpha-tubulin (TcIL3000_0_31230), and compared between strains (Figure 3.6). No significant differences were observed in TfR abundance, when comparing BT0206 (high virulence; median RAT = 0.008) to MF1 CL1 (low virulence;
median RAT = 0.0008) (p = 0.1). Similarly, no significant differences were observed when comparing BT0206 (high virulence; median RAT = 0.008) to M7 C3 (low virulence; median RAT = 0.0004) (unpaired T-test: p = 0.5; t = 0.7; df = 96).
Figure 3.6: RAT-scores for different *T. congolense* strains. The abundance of a subset of TfR transcripts – identified when applying exclusion criteria to RNAseq data - relative to alpha-tubulin (RAT) was calculated, and represented on the y-axis. No significant differences in abundance were detected between field strains, as confirmed by the results of two separate unpaired T-tests: BT0206 (high virulence; median RAT = 0.008) vs. MF1 CL1 (low virulence; median RAT = 0.0008) (unpaired T-test: p = 0.1; t = 1.6; df=46); BT0206 (high virulence; median RAT = 0.008) to M7 C3 (low virulence; median RAT = 0.0004) (unpaired t-test: p = 0.5; t = 0.7; df = 96).
3.5 Discussion

We sequenced the genomes of three *T. congolense* field strains, which adds to the four genomes previously sequenced in our lab. By comparing these genomes with respect to the sequences of genes known to be implicated in QS, we detected a number of point mutations in the adenylosuccinate lyase of MF3 CL1, which resulted in a frameshift and deletion at positions 332-348 (Appendix 1.3). This deletion is unlikely to contribute to differential strain virulence, however, as it was only observed in a single strain.

A number of further differences were detected in the genes shown to be implicated in QS, notably *TcAdSS* where an alanine to serine substitution was observed (Ser415) in three low virulence strains (MF1 CL1; M7 C3; M19 C1) (Figure 3.2). While it is true that variation exists at this position when comparing species (*T. brucei* = Thr415; *T. congolense* = Ala415 or Ser415; *T. vivax* and *T. cruzi* = Pro415), it nevertheless remains plausible that a Ser415 substitution may have virulence consequences. For example, such a change alters the physico-chemical properties at this residue (from non-polar to polar) and allows for phosphorylation by protein kinases, thus allowing for functional alteration of the protein (Cohen, 2002). Furthermore, it has previously been shown that deficiencies in a AdSS homologue found in *Leishmania donovani* impacted upon parasite growth and infectivity, with depletion of AdSS by RNAi in *T. brucei* also resulting in growth inhibition (Boitz et al., 2013). Protozoa lack purine biosynthetic pathways and, instead, depend on scavenging of these nucleosides from the host, which involves a number of enzymes such as
AdSS, which catalyzes inosine monophosphate (IMP) to adenosine monophosphate (AMP) (Raman et al., 2004).

Our attempts to model TcAdSS onto existing structures in order to scrutinise this mutation, were hampered due to additional trypanosomatid-specific sequences and alpha-helices, when compared to AdSS from other organisms. In particular, the region of interest which contains Ser415 did not align to 1p9b (from Plasmodium), or other AdSS orthologues, and we could not draw any conclusions based on structural changes resulting from the substitution. To date, no crystal structure has been resolved for TcAdSS, or any other AdSS from trypanosomatids, further complicating structural investigations. As such, we utilised the AlphaFold DB to align TcAdSS to the predicted AdSS from T. cruzi. The alignment remained poor, however, particularly in the region of interest which contained Ser415 and consequently no meaningful conclusions could be made.

To further explore the virulence characteristics of the respective lines, we performed RNAseq from T. congolense field strains that were isolated from infected mice to investigate transcriptome differences between these lines. Initial analyses indicated that 299 transcripts were significantly differentially expressed in high virulence strains when compared to low virulence strains. Notably, TfR-like transcripts were well-represented in those transcripts that were upregulated, suggesting their expression has a potential role in strain virulence or is a consequence of it. Trypanosomes use TfR to scavenge host transferrin, and upregulation of these genes in Leishmania spp. has been
associated with increased iron uptake from host macrophages (Das, 2009; Kariuki et al., 2019). It is plausible that strains which exhibit increased expression of TfR could exhibit increased growth, when compared to other strains, through their increased ability to scavenge iron. Interestingly, TfR-like transcripts had previously been shown to be upregulated in *T. congolense* IL3000 at peak parasitaemia, indicating that elevated cell density may contribute to the observed transcriptional changes (Silvester et al., 2018). While TfR-like protein transcripts were among the most significantly up-regulated RNAs in that study, my own work reveals that such increased abundance is more pronounced in the high virulence strains. Beyond expression levels, little information exists for TfR regulation in *T. congolense*, as opposed to *T. brucei*. In *T. brucei*, TfR genes are located within VSG expression sites (ES) which show reduced transcription during stumpy formation (Zimmermann et al., 2017). Consequently, TfR expression is correspondingly reduced at high parasitaemias. TfR expression has also been shown to be regulated by the 3’ UTR of ESAG6 (Batram et al., 2014; Benz et al., 2018) but sequences homologous to the 3’-UTR of *T. brucei* ESAG6 could not be found in *T. congolense*, although this could be contributed to by the incomplete assembly of the IL3000 reference genome. Hence the relationship between cell density, cell virulence and transferrin receptor expression remain to be clarified.

Another group of transcripts, namely ‘SecA-DEAD-like domain containing proteins’, were found to be down-regulated in *T. congolense* field strains of high virulence. Interestingly, a previous study of the laboratory
IL3000 strain showed that these transcripts were enriched at peak-parasitaemia, when compared to the ascending phase of parasite growth (Silvester). In combination, these data suggest a role of ‘SecA-DEAD-like domain containing proteins’ in the density-dependant regulation of parasite growth, but this remains unconfirmed. Unfortunately, no additional data pertaining to these trypanosome transcripts exist in the literature, but studies in other taxa have been illuminating. SecA is a well-defined protein in plants and bacteria, with reported helicase and translocase activities (Koyama; Lill; Anantharam). Its essential role has been proven in Eschericia coli, for example, where ATP-binding results conformational changes of the protein, that subsequently allows for the translocation of protein across channels in the cytoplasmic membrane (Koyama; Lill). While the T. brucei genome does not encode a SECA gene, an inhibitor of SecA activity in E. coli was found to inhibit protein translocation in T. brucei, hinting at uncharacterised proteins which have a similar function (Patham et al., 2009; Sugie et al., 2002).

We sought to investigate whether increased expression of TfR in high virulence strains amounted to higher predicted TfR abundance on the cell surface using a ‘relative to alpha-tubulin’ analysis, as previously described (Kelly et al., 2017). Alpha tubulin transcript abundance provides insight into the relative growth of trypanosomes and so would attempt to normalise for virulence differences between the strains. The results, however, indicated that there were no significant differences in TfR abundance between strains. While this result seemingly contradicts that of our differential expression data, it is worth mentioning that these results may be ascribed to the different
normalisation methods of each analysis. Briefly, our differential expression analysis is based on normalisation within a group of samples utilising the complete dataset, whereas RAT-analysis depends on normalisation based on the length based on a single gene, namely alpha-tubulin. In other words, TfRs may not be upregulated in high virulence strains when compared to alpha-tubulin within those strains (RAT), but the extent of that upregulation may differ between strains in relation to global transcript abundance. While both RPKM and TPM have been widely used in normalisation of differential expression data, researchers have been cautioned against directly comparing datasets using these different methods (Zhao et al., 2020).
4 THE DYNAMICS AND INTERACTIONS BETWEEN COINFECTING TRYpanosomes
4.1 Introduction

It is the norm, rather than the exception, for hosts in natural settings to be simultaneously infected with pathogens from different taxa, species, or strains of the same species. Furthermore, it is well-established that such coinfection leads to both direct- and indirect-interaction between microbes, and limited evidence has shown that this is indeed true for trypanosomes. Trypanosomes have been seen, for example, to avoid within-host competition, by means of niche compartmentation – the selective occupation of different tissues or locations (Perez et al., 2018; Schmid-Hempel, 2001). Parasite virulence may also be impacted in coinfection scenarios, as illustrated by less-virulent strains of *T. brucei* that ameliorate the disease severity caused by a more aggressive strain (Balmer et al., 2009). Evidence has also started to unravel the molecular basis of these interactions, such as inter-trypanosome communication which is mediated through QS responses (Silvester et al., 2017b). Such interactions are likely to have long-term implications for parasite biology, growth, and evolution, yet remain understudied.

In this chapter, I endeavoured to better understand trypanosome coinfections in a natural setting, and to explore the effects of such infections on parasite growth, virulence, and transmission potential. First, I assessed the occurrence of trypanosomes from a longitudinal cohort study in Western Kenya, with a particular interest in the prevalence of different combinations of coinfections between African trypanosomes (de Clare Bronsvoort et al., 2013). I also describe here the work towards studying changes in parasite density of
different species, within a particular host over time, by adapting a qPCR protocol described earlier (Silbermayr et al., 2013). Next, we sought to confirm the relevance of an earlier finding – that *T. congolense* IL3000 (lab strain) interacts with *T. brucei* through QS signalling (Silvester et al., 2017b) – by investigating the effect of a short-term, in vivo coinfection between *T. congolense* MF1 CL1 (field strain; sylvatic origin) (Masumu et al., 2006b; Van den Bossche et al., 2011) and *T. brucei*. This was followed by pilot-studies and dose-finding experiments, to find suitable *T. congolense* field stains and *T. brucei* lines for long-term coinfections. These data were used to inform a long-term coinfection, between these two species of African trypanosomes, to investigate the effect on host survival, parasite growth dynamics, as well as trypanosome transmission potential. Finally, *T. brucei* EATRO 1125 PFR-Ty, recovered from the preceding long-term coinfection, was assessed to explore the evolutionary pressure of coinfections on parasite virulence.

### 4.2 Trypanosome coinfections in the field

Surveys have confirmed that different trypanosomes – species and strains - frequently coinfect hosts and tsetse flies in the field (Adams et al., 2006; Takeet et al., 2013; Weber et al., 2019). Historically, cross-sectional studies have produced insights into trypanosome species abundance at a given time, but these are not capable of illuminating coinfections which are below the threshold for detection at that time. A longitudinal study, however, presents a unique opportunity to investigate the dynamics between mixed
infections over time in the field. To understand the prevalence of such coinfections, in the Busia region of Western Kenya, we utilised samples from a previous longitudinal cohort study which followed 548 zebu calves between 2007 and 2009 (de Clare Bronsvoort et al., 2013). The study showed that calves in this region were infected with a plethora of pathogens which included trypanosome species.

We sought to confirm the species identity of those previously identified parasites, using two different PCR protocols that target the ITS-1 and tubulin regions of the trypanosome genome. We also had a particular interest in identifying calves which were coinfected with African trypanosome species, and to follow the changing dynamics of such coinfections over time within individual animals using an adapted qPCR protocol.

4.2.1 Multiple trypanosome species co-circulate in Western Kenya

Previously, the IDEAL project followed 548 zebu calves in Western-Kenya for a period of 2 years, with the purpose of identifying pathogens that circulate in this region (de Clare Bronsvoort et al., 2013). Veterinarians visited calves every 5 weeks to collect blood samples, with genomic material being extracted and subsequently analysed by researchers at the University of Pretoria, South Africa. Tests indicated that over 50 pathogens infected the study cohort, including viruses, bacteria, and parasites (de Clare Bronsvoort
et al., 2013). A subset of these samples that were previously identified as ‘trypanosome positive’ through a combination of PCR tests and microscopy techniques, were further investigated by us, using a nested-PCR protocol targeting the ITS1-region of the parasite genome (Ahmed et al., 2013; Cox et al., 2005). The assay uses differences in band size to identify different trypanosome species. A total of 608 samples (from 127 animals) were assayed, and revealed that a number of trypanosome species – including African trypanosomes – infected cattle in the region (Figure 4.1). The most prevalent species infection that was detected was *T. theileri* (26%) which was followed by *T. vivax* (4.9%); *T. simae* (1.3%); *Trypanozoon* (*T. b. brucei* s.l) (0.8%); and *T. congolense* (0.2%) (Figure 4.2a). Interestingly, many of the samples also contained clear bands at either 300, 400, or 700bp, which do not identify any specific trypanosome species (18%). Coinfections were also detected in this subset of samples, with *T. theileri* being readily found in mixed trypanosome infections (total = 46 out of 157) (Figure 4.2b). The most frequent coinfection combinations were between *T. theileri* and different unidentified species (6.7%), followed by *T. theileri* and *T. simae* (0.5%). No coinfections between African trypanosome species (*T. brucei; T. congolense; and T. vivax*) were observed, but *T. vivax* and *T. brucei* were detected with either *T. theileri*, *T. simae* or unidentified trypanosomes (prevalences below 1% in each combination). The small number of coinfections between African trypanosomes prompted us to explore a second data set – surviving animals which received additional clinical visits by veterinarians as a result of disease symptoms.
Figure 4.1: Gel image from an ITS-1 screen for trypanosome coinfections. The gel image shown is plate 17 (1 of 6), comprising 85 individual samples derived from cattle blood, taken during a longitudinal cohort study in Western Kenya (de Clare Bronsvoort et al., 2013). Orange arrows denote the 500 and 1000bp of the DNA Ladder used; Red arrows denotes *T. congolense* (1413-1513bp) and *T. brucei* (1207-1224bp) positive controls; Blue arrows denote positive *T. vivax* samples (611bp). These data illustrate the low prevalence of African trypanosomes in the sampled region, as well as the lack of detectable coinfections between these species.
Figure 4.2: Trypanosome species prevalence following detection by ITS-1 PCR assay.
(A) A total of 608 samples (127 animals) were screened and species identification was performed based on gel-electrophoresis and band-size, indicating that *T. theileri* was the most prevalent trypanosome species in these samples. African trypanosome prevalence was low, accounting for 4.9%; 0.8% and 0.2% for *T. vivax*, *T. brucei* and *T. congolense*, respectively. Interestingly, bands (700; 400; and 300bp) were detected which did not correspond to any known trypanosome species (accounting for 18%) and these are labelled as 'unidentified'. (B) No coinfections were detected between African trypanosomes in our screen, with *T. congolense* found to coinfect with *T. theileri* in a single infection. The most prevalent coinfections detected in this study, were between *T. theileri* and 'unidentified' trypanosomes.
An additional 382 samples (60 animals) were assessed using an alternative assay which targets the tubulin region of the genome to detect African trypanosomes (kindly provided by Prof. Mark Carrington, University of Cambridge) (Figure 4.3a). The most prevalent species in this subset of samples were *T. vivax* (14%) followed by *T. brucei* (1.3%) and *T. congolense* (0.5%) (Figure 4.3b). There were again no observable coinfections between African trypanosome species.
Figure 4.3: Screening for African trypanosomes, using a tubulin-targeting PCR assay.
(A) Samples (n = 384) from clinically ill animals were screened using a nested PCR protocol which targets the tubulin region of the trypanosome genome. An example gel contains: L which denotes DNA ladder; lanes 1-3 contained positive controls (T. vivax = 586bp; T. congolense = 456bp; T. brucei = 424bp); lanes 4-11 contained field samples; and lane 12 served as negative control. Lanes 5 and 11 (green arrows) tested positive for T. vivax and T. brucei, respectively. (B) A low prevalence of T. brucei and T. congolense infections (1.3% and 0.5% respectively) was observed among these samples. A high number of T. vivax infections were detected, however, accounting for 14% of infections. Like our ITS-1 screen, no coinfections could be detected between African trypanosome species. Unlike the ITS-1 screen, no unidentified bands were detected using the tubulin assay.
Towards estimating changes in parasite dynamics infecting individual animals, over time

We sought to identify samples which returned confirmed coinfections between African trypanosomes. Those calves would be followed longitudinally by screening all samples attributed to a particular animal and assessed for changes in the growth dynamics of the coinfecting trypanosome species. While we did not observe coinfections between African trypanosomes in either sample set (trypanosome positive animals; clinically ill animals), we nonetheless endeavoured to adapt a qPCR assay which could be used to quantify parasite DNA at a given time, for future use.

To this end, we utilised the primer and probe sequences, targeting the ITS-1 region of the trypanosome genome (Silbermayr et al., 2013). The protocol successfully detects simultaneous infections with different trypanosomes but uses the same fluorophore and quencher pair (6-FAM; BHQ-1) for each of the three probes used to detect those parasites. Consequently, positive samples need to be screened again by means of a single-plex PCR to identify trypanosome species. We adapted the protocol from Silbermayr et al. (2013) to use different fluorophores for each individual species-specific probe to aid in simultaneous detection and identification of two different African trypanosomes. Care was taken to ensure that the two probes did not contain dyes at the 5’-end which would result in spectral overlap ($T. brucei = $ Cy5; $T. congolense = 6-FAM$). These new probes were tested on samples which contained DNA from either $T. congolense$ field strain BT0206
or T. brucei EATRO 1125, with initial runs failing to detect either species even
after optimisation of run parameters (Figure 4.4a). Further investigation
demonstrated that the reverse primer (Tryps KS-rev) sequence in the original
paper was incorrect, and new primers were subsequently ordered which
resulted in positive detection of T. congolense. T. brucei DNA was not
detected, even after multiple rounds of trouble-shooting which involved
generating new DNA stocks, testing fresh reagents, and confirming the
specificity of the T. brucei probe by sequencing. Interestingly, using a new
FAM-labelled T. brucei probe (as described in the original paper) resulted in
positive detection (Figure 4.4b).
Figure 4.4: Adapting a qPCR protocol for the simultaneous detection of African trypanosomes. (A) DNA from neither *T. congolense* nor *T. brucei* could be detected initially with troubleshooting involving testing different run parameters, changing DNA template concentrations, and confirming the specificity of the *T. brucei* probe to qPCR product. *T. congolense* DNA was detected after noticing that an incorrect primer sequence was reported in Silbermayr et al. (2013). *T. brucei* could not be detected by using a Cy5-labelled probe. (B) Reverting to a FAM-labelled probe, as recommended by the authors of Silbermayr et al. (2013), resulted in the positive detection of *T. brucei* DNA (light blue; red; dark blue; brown) between 16 and 20 cycles. *T. congolense* detection is shown in orange, and negative control in teal.
4.3 Selection of strains for coinfections studies

*Trypanosoma congolense* field strains exhibit strikingly different virulence phenotypes (Masumu et al., 2006b; Van den Bossche et al., 2011). As such, we sought to assess the suitability of these different lines for use in both short-term and chronic coinfections, in mice (Table 4.1). The aim of our short-term coinfection experiments were to extend previous work performed in our laboratory (Silvester et al., 2017b), where *T. brucei* responded to QS signals from *T. congolense* IL3000, to a field setting. As such we sought to repeat these experiments using a field derived strain of *T. congolense*. High-virulence field strains, such as BT0206 or MF2 CL5, were not considered given their rapid growth, and high parasite numbers, previously seen in mice between 3- and 4-days post-infection (Chapter 3; Figure 3.1). Instead, we required a low virulence strain that would peak at tolerable levels of parasitaemia (1-5x10^8 parasites/ml) and thus selected a strain of sylvatic origin, MF1 CL1. Next, pilot studies informed the optimal time to perform superinfections with *T. brucei* EATRO 1125 PFR-Ty (Appendix 2.1), to ensure that this species would be in the exponential growth phase, while *T. congolense* was at peak parasitaemia, allowing us to investigate whether interspecies QS occurred between the two species. In all subsequent short- and long-term coinfections we used a *T. brucei* EATRO 1125 line containing a PFRA protein tagged with a Ty1-epitope (*T. brucei* EATRO 1125 PFR-Ty), thereby facilitating accurate species identification (Bastin et al., 1996b; Silvester et al., 2017b).
For chronic infections, we needed to assess the ability of various strains to grow in mice over a prolonged period, which first required single strain infections, as well as dose-finding experiments. To this end, we initially selected two low-virulence *T. congoense* field strains to be used in conjunction with *T. brucei* for long-term coinfections, namely M19 C1 and M7 C3. Three mice were first injected intraperitoneally (IP), with M19 C1 parasites, and parasitaemia was monitored daily until day 15, when the animals were sacrificed due to showing symptoms of disease, along with increasingly high numbers of parasites (Figure 4.5a). The suitability of M7 C3 was next assessed, by again injecting two mice with parasites of that strain. The animals did sustain parasites for 13 days before starting to clear the populations but unfortunately resulted in the death of one animal on day 11 (Figure 4.5b). This together with the poor condition of remaining mouse in the group, prompted us to end the experiment. Given that M19 C1 and M7 C3 were not suitable for chronic experiments, given animal welfare concerns and the stipulations of our animal licence, we abandoned the use of field strains (including MF1 CL1) for long-term infections. Instead, I opted to use the laboratory *T. congoense* strain IL3000 for use in a subsequent pilot long-term infection, as this line had previously been used in such experiments. Here, we inoculated four groups of mice (n = 3 each) with trypanosomes, and monitored parasitaemia for 30 days (Figure 4.5c). The first group, inoculated with $2 \times 10^4$ cells of *T. brucei* EATRO 1125 PFR-Ty, were able to sustain a long-term infection for 30 days. Using twice that dose, for the same species, resulted in animals having to be culled before 30 days. Mice in a coinfection, inoculated with $4 \times 10^4$ cells (2$\times 10^4$ *T.
*brucei* EATRO 1125 PFR-Ty + 2x10⁴ *T. congolense* IL3000) were able to sustain parasites for the full 30 days of the pilot trial. Interestingly, variation was noted in the infection profiles of mice that were inoculated with 2x10⁴ *T. congolense* strain IL3000 only. One had to be culled at day 10, while another sustained infection for 30 days. The third mouse in the group did not have a detectable parasite in blood smears. As such, we opted to use a line of IL3000 parasites obtained from Dr Liam Morrison (Roslin Institute, University of Edinburgh), which reproducibly grew in mice without resulting in deaths (pers. comm: Dr Stephen Larcombe), for all future a long-term infection experiments.

Table 4.1: Trypanosome lines used during coinfection experiments. Suitable species and strains were selected for short-term and chronic coinfections, based on their growth dynamics in vivo. Dose-finding was also assessed for some strains. The use of *T. congolense* field strains in long-term experiments were abandoned (after assessing M19 C1 and M7 C3), owing to animal welfare concerns.

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<tr>
<td>MSORO M7 C3</td>
<td>1e-3 to 1e-4 cells</td>
<td>Not suitable for chronic experiments in mice. Death of individual mouse recorded on d10; Termination of experiment on days 11-14 due to welfare concerns</td>
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Figure 4.5: Selection of lines for chronic infections. Two *T. congolense* strains of low virulence (M19 C1 and M7 C3) were assessed for their suitability in long-term infections. (A) M19 C1 were sustained in infections for 15 days, followed by successive peaks comprising of increasingly high parasitaemia. This observation, and the condition of the animals, prompted us to end the experiment at day 15. (B) One animal died during infections with strain M7 C3, and the condition of the remaining animal prompted us to end the experiment at day 14. (C) A pilot chronic infection was performed by inoculating mice in four groups as follows: (1) Coinfection = $2 \times 10^4$ *T. brucei* EATRO 1125 PFR-Ty cells and $2 \times 10^5$ *T. congolense* IL3000 cells; (2) $2 \times 10^4$ cells of *T. brucei* EATRO 1125 PFR-Ty only; (3) $2 \times 10^5$ cells *T. congolense* IL3000 only; and (4) $4 \times 10^4$ cells of *T. brucei* EATRO 1125 PFR-Ty only. Mice in the coinfection group as well as the *T. brucei* only group, were able to sustain parasites for 30 days. Significant variation was observed in the *T. congolense* only group, with animals being culled in this and the *T. brucei* groups (dose: $4 \times 10^4$ cells). Individual mice were designated Z, B, BB, R or RR to reflect their tail labelling band colour (Z= none, R= red, RR=two red bands, B=blue, BB=two blue bands).
4.4 *T. congolense* field strains induce stumpy-formation in *T. brucei* in a short-term coinfection

It had previously been shown that *T. brucei* responds to quorum sensing signals from *T. congolense* IL3000, in vitro and in vivo (Silvester et al., 2017b). As such, we sought to investigate the relevance of this phenomenon in the field, by performing a coinfection experiment, in mice, between *T. brucei* EATRO 1125 PFR-Ty and the *T. congolense* field strain MF1 CL1. Mice were infected in triplicate, comprising three groups. First, a coinfection group was set up by injecting three mice with 2.3x10⁶ *T. congolense* MF1 CL1 cells on day 0, followed by a superinfection with 2x10⁵ *T. brucei* EATRO 1125 PFR-Ty cells on day 3; Also, the experiment comprised of two single infection control groups: (i) *T. brucei* EATRO 1125 PFR-Ty control (2x10⁵ cells); and (ii) and *T. congolense* MF1 CL1 control (2.3x10⁶ cells).

Parasitaemia was monitored from day 3 post-infection, and species-discrimination was performed by means of immunofluorescence (IFA) based on tagging of the *T. brucei* PFR (Figure 4.6a). These data indicated reduced parasitaemia for *T. brucei* in the coinfection (day 7; 1x10⁶ cells/ml), when compared the *T. brucei* only control (day 7; 1x10⁸ cells/ml), while *T. congolense* did not exhibit such a reduction in parasite numbers (Figure 4.6b). Whole-blood was retrieved from mice by cardiac puncture on day 7, after which separated trypanosomes were paraformaldehyde-fixed and investigated by means of IFA, to determine PAD1 expression in each group. These data revealed that there was a significantly higher proportion of PAD1-positive cells.
on day 7 for *T. brucei* cells in the coinfection (mean = 65%), than for those in the single infection (mean = 25%) (unpaired test = p < 0.002; t = 7.2; df = 4) (Figure 4.6c). This was consistent with the previous study using *T. congolense* IL3000 (Silvester et al., 2017b).
Figure 4.6: The presence of a *T. congolense* field strain affects *T. brucei* growth and stumpy formation during an in vivo coinfection. (A) *T. brucei* EATRO 1125 expressing a Ty-epitope on the PFR (PFR-Ty), was used to discriminate between parasite species in coinfections in mice. *T. congolense* MF1 CL1 adjacent to *T. brucei*, without tagged PFR (Scale bar = 10µm). (B) PFR-tagging (shown in red) of *T. brucei* also allowed for estimations of the individual parasitaemia of each species within the coinfection. *T. brucei* exhibited reduced growth during coinfection with *T. congolense* MF1 CL1 than in infections with *T. brucei* alone. The growth of *T. congolense* was similar in the coinfection and control groups. (C). PAD1 expression (shown in green), on the cell surface, was determined for *T. brucei* in the different groups by means of IFA. This revealed that a significantly higher proportion of cells were PAD1-positive in the coinfection group (mean = 65%), than in the *T. brucei*-only control group (mean = 25%) (unpaired t-test: p < 0.002; t = 7.3; df = 4) despite the lower net parasitaemia of *T. brucei* in the coinfection compared to the monoinfection.
4.5 Long-term in vivo trypanosome coinfection

Pathogens that occupy the same niche will compete directly, through for example signalling, or indirectly by means of the host immune system (Bruce et al., 2000; Hoffman et al., 2006; Vieira-Santos et al., 2021). Such interaction had already been shown to occur between T. brucei, and both the laboratory IL3000 strain of T. congolense (Silvester et al., 2017b) and a field-derived strain (MF1 CL1) in the present study. Earlier results from a pilot study (section 4.3) also indicated that it was not the total dose that determined the survivability of the group, but instead the composition of the parasites that are injected. This information was then used to inform the design of an experiment which tested the effects of a long-term coinfection on both host and parasite.

The experiment comprised of three groups of mice being infected with different combinations of two trypanosome species (Figure 4.7). Six mice in each group were infected as follows: (1) T. brucei EATRO 1125 PFR-Ty (3.2x10^3 cells) + T. congolense IL3000 (2.5x10^3 cells); (2) T. brucei EATRO 1125 PFR-Ty only (3.2x10^3 cells) and (3) T. congolense IL3000 only (2.5x10^3 cells). Overall parasitaemia was monitored daily from three days post-infection (Appendix 2.2) and additional blood samples were collected at seven time-points (days 5; 16; 21; 25; 29; 33; and 38) across the 38-day experimental period for the coinfection group, to be used in IFA analyses (Figure 4.7). To discriminate between species in each of the mice in the coinfection group, trypanosome cells were scrutinised on the basis of Ty-epitope tagging of the T. brucei PFR, as assessed by IFA.
Figure 4.7: Diagram depicting the experimental design and procedures used for a long-term coinfection study. Three groups of mice (n = 6 each) were injected with trypanosomes on the same day, as follows: (1) *T. brucei* EATRO 1125 PFR-Ty (3.2x10^3 cells) + *T. congolense* IL3000 (2.5x10^5 cells); (2) *T. brucei* EATRO 1125 PFR-Ty only (3.2x10^3 cells); and (3) *T. congolense* IL3000 only (2.5x10^5 cells). Parasitaemia was monitored daily by means of blood smears in all groups, with additional blood-sampling taking place in the coinfection group at seven time points (d5; d16; d21; d25; d29; d33; d38). These additional blood samples were utilised for IFA to discriminate between species, by counting 2000 cells in each sample on the basis of Ty-epitope tagging the *T. brucei* PFR. These samples were also used for further IFA-analysis to determine PAD1-proportions in *T. brucei*, and DAPI-staining to determine the cell-cycle status of both species in the coinfection group (200 cells counted respectively).

**Group 1 (n=6):**
*T. brucei* EATRO 1125 + *T. congolense* IL3000

**Group 2 (n=6):**
*T. brucei* EATRO 1125 only

**Group 3 (n=6):**
*T. congolense* IL3000 only

Created in BioRender.
These samples were also used to determine the proportion of PAD1-positive *T. brucei* cells, as well as DAPI-staining to determine the cell-cycle status of both species in the coinfection group.

### 4.5.1 Coinfections reduce disease severity

The mean daily parasitaemia was monitored for each group of mice and this revealed that the coinfection group showed altered growth dynamics – in terms of prepatent period and number of peaks – when compared to the control groups. Peak parasitaemia was observed at day 5-6 post-infection for both the coinfection group and the *T. brucei* control group (Figure 4.8a; Appendix 2.2-2.4). The first wave of parasitaemia was cleared more quickly in the coinfection group (day 8) than in the *T. brucei* control group (day 12), however. The number of peaks of parasitaemia also differed between the coinfection and *T. brucei* control groups, with the latter exhibiting characteristic waves. The peak of parasitaemia for the *T. congolense* control group lagged compared the other two groups, occurring at day 9 post-infection, and notably did not clear completely during the course of the infection (Figure 4.8a). Both the coinfection group and the *T. congolense* control groups did not exhibit waves of parasitaemia, and instead resembled chronic infections after the initial, first wave.

Animals were humanely terminated during the course of the 38-day experiment, according to Home Office Regulations, once specified conditions of illness and parasitaemia were reached. Interestingly, coinfection
ameliorated parasite virulence when measured in terms of host disease and humane termination (Figure 4.8b). In the coinfection group, one animal was culled on day 24, and another on day 33, with 67% progressing up to day 38. This was followed by the T. congolense control group, where one animal was culled on 20, and another on day 29, again with 67% of the animals progressing up to day 38. The worst outcome was observed in the T. brucei control group, where four animals had to be culled on day 11 (33% survival), with all animals in this group being culled by day 29.

4.5.2 Altered growth dynamics and increased transmission potential during coinfections

To further investigate the basis of the altered parasite growth dynamics in coinfections versus single infections, additional blood samples were collected for IFA in the coinfection group. These seven time-points coincided with the peak of overall parasitaemia, and for each of those time-points (and each individual mouse) we counted 2000 cells to calculate the proportion of T. brucei versus T. congolense cells. Plotting these proportions indicated a dynamic interplay between the two species, with neither peaking at the same time (Figure 4.9; Appendix 2.5). The first part of the coinfection, in each individual mouse, was dominated by T. brucei (day 16; 70-90% of parasites).
Figure 4.8: Growth and disease outcomes were different between the three infection groups. (A) The prepatent period and number of peaks of parasitaemia were different between the three groups of mice, with both the confection and *T. brucei* control groups exhibiting an initial wave at days 5-6. The first peak of parasitaemia occurred at day 8 for the *T. congolense* control group. The *T. brucei* control also exhibited characteristic waves of parasitaemia, when compared to the coinfection and *T. congolense* control groups, which exhibited chronic growth after the initial peak of parasitaemia. (B) Host removal from the experiment was different in the different experimental groups, with the best outcome being observed in the coinfection group where significantly more animals (67%) remained in the experiment up to day 38 (Wilcoxon test: Chi-square = 15.82; P value = 0.0004; df=2). This was followed by the *T. congolense* control group, where animals were culled sooner than the coinfection group, but with 67% retained to day 38. The worst outcome was observed in the *T. brucei* control group, where no animal was retained past day 29.
Figure 4.9: *In vivo* parasite proportions over the course of a long-term coinfection. Six mice were coinfected with *T. brucei* EATRO 1125 PFR-Ty and *T. congolense* IL3000, and followed for 38 days, with additional sampling of blood taking place at days 5, 16, 21, 25, 29, 33 and 38 for IFA analyses. Two-thousand cells were counted at each of the seven time-points, allowing for the calculation of the proportion of each species on the basis of Ty-epitope tagging of the *T. brucei* PFR. The infections were initially dominated by *T. brucei* (day 5 = peak), but was gradually replaced by *T. congolense* (peak = day 21-33). Following clearance of *T. congolense*, a resurgence in *T. brucei* parasitaemia was observed in three of the mice remaining by day 38 (n=4). Individual mice were designated 1Z, 1R or 1RR and 2Z, 2R or 2RR to reflect their tail labelling band colour (Z= none, R= red, RR=two red bands). See Appendix 2.5 for mean parasitaemia of individual species in the coinfection group.
These number gradually reduced, and *T. congolense* became the dominant parasite in the host blood by day 21, in five out of six mice, peaking between days 25-33. Interestingly, once the population of *T. congolense* started to decline in numbers, *T. brucei* parasites re-emerged between day 29 and 33 (n = 5), gradually increased, and again became prevalent in the infection by day 38 (in three of the four remaining mice).

Next, we sought to investigate the cell-cycle status of parasites during a chronic coinfection, which was accomplished by DAPI-staining cells of each parasite species (Figure 4.10). The number of dividing cells (2K1N; 2K2N) were calculated for each species at the seven time-points where additional bleeds were taken (dependant on there being a sufficient number of cells), and compared to the inferred parasitaemia (species-proportion of the total parasitaemia in the coinfection) at a given time-point. The mean proportion of dividing (% 2K1N; 2K2N) *T. brucei* cells ranged between 1.6% (day 33) and 7.7% (day 5), and lowered as parasitaemia decreased (Figure 4.11a). Interestingly, this number remained low (day 38; mean = 3.7%) even as *T. brucei* parasitaemia was seen to increase. The proportion of dividing *T. congolense* cells, conversely, remained consistently high after day 16 (20%) and increased at day 38 (23%) (Figure 4.11b). Notably, this increase coincided with an increase of dividing *T. brucei* cells, between day 33 and 38.

Finally, we sought to investigate the transmission potential of *T. brucei* in the context of a coinfection. This was accomplished by determining the proportion of PAD1-positive cells, at the seven time-points where additional
bleeds were taken, and compared to the inferred parasitaemia at those time-points (Figure 4.12). Initially, the mean proportion of PAD1-positive cells was 30% (day 5), but gradually increased to between 61% (mean; day 16) and 78% (mean; day 21), coinciding with a decrease in parasitaemia. Additionally, the highest proportion of PAD1+ *T. brucei* cells were observed as *T. congolense* populations were becoming dominant in each coinfected animal. As mentioned earlier, a decrease in *T. congolense* parasitaemia (after day 33) coincided with a resurgence in *T. brucei* numbers. Interestingly, these increasing cells presented as a population with a high proportion of stumpy cells (day 38; mean = 71%).
Figure 4.10: Determining the cell-cycle status and transmission potential of trypanosomes during a long-term coinfection. (A) Representative microscope images of DAPI-stained nuclei (N) and kinetoplasts (K), and their inferred cell-cycle status (1K1N; 2K1N; 2K2N). 1K1N represents G1 and G0 phase cells, 2K1N are G2 phase cells and cells in early mitosis, whereas 2K2N cells are postmitotic. Scale bar = 10µm. (B) Comparison of PAD1-positive (green) and non PAD1-positive T. brucei cells. Note the Ty-epitope tagged PFR (in red) allowing for species identification. A cell was classified as 'stumpy' based on morphology, cell-cycle status (1K1N; K-N distance), and uniform staining with PAD1-antibody. Scale bar = 10µm; BF = Brightfield; BB2 = Antibody that detects Ty-epitope.
Figure 4.11: Cell-cycle stages of parasites in a chronic coinfection. Six female MF1 mice were coinfected with *T. brucei* EATRO 1125 PFR-Ty and *T. congolense* IL3000, and followed for 38 days. The proportion of each parasite at seven time-points were calculated by IFA based on Ty-epitope tagging of the *T. brucei* PFR, which also allowed for an estimate of species parasitaemia (as a proportion of overall parasitaemia). Formaldehyde-fixed cells were DAPI-stained, to allow for visualisation of the kinetoplast (K) and nucleus (N). Two-hundred cells were scored according to cell-cycle status (1K1N; 2K1N; 2K2N) at the seven time-points, for both *T. brucei* (A) and *T. congolense* (B). The mean proportion of dividing (2K1N; 2K2N) *T. brucei* ranged between 1.6 and 7.7%, while the proportion of dividing *T. congolense* was consistently high, and ranged between 20 and 23%. An increase in the % dividing *T. congolense*, on day 38, coincided with an increase in *T. brucei* parasitaemia. (Note the difference between the scales of the right-hand side y-axes, between panel A and B).
Figure 4.12: Proportion of PAD1-positive cells in a coinfection. Six female MF1 mice were coinfectd with *T. brucei* EATRO 1125 PFR-Ty and *T. congolense* IL3000, and followed for 38 days. The proportion of each parasite at seven time-points were calculated by IFA based on Ty-epitope tagging of the *T. brucei* PFR (detected with the BB2 antibody), which also allowed for an estimate of species parasitaemia (as a proportion of overall parasitaemia). Paraformaldehyde-fixed cells were labelled with PAD1-antibody, and 200 *T. brucei* parasites were scored at each time-point to calculate the proportion of PAD1-positive stumpy cells. The proportion of PAD1+ were higher in the presence of *T. congolense* cells (mean d5 = 30%; mean day21 = 78%). A high proportion of PAD1+ cells were noted at days 33 and 38, even as parasitaemia was increasing (mean 70.6 and 71.2%).
4.6 The effect of coinfection on parasite virulence

Theory predicts that coinfection promote the selection of parasites that are more virulent, or exhibit enhanced transmission potential (Bremermann and Pickering, 1983). Following on from the long-term experiment that we performed, we sought to confirm the outcomes of coinfection on the *T. brucei* EATRO 1125 PFR-Ty population, in MF1 mice, which required separating this species from *T. congolense* IL3000 in recovered mouse blood at the end of the chronic infection experiment (Figure 4.13). Briefly, whole blood was extracted from three mice which survived up to day 38, and frozen as stabilates (designated LT line). One such stabilate (isolated from mouse 2RR on d38), together with cells from the stabilate used to initiate the original long-term coinfections (designated the OG line), were used to seed flasks containing fresh culture media. Cells in both flasks were allowed to grow, and subsequently drug-treated, to kill off any residual *T. congolense* IL3000 cells in LT samples. This was possible because the *T. brucei* EATRO 1125 line that was used in these experiments had previously been engineered to be resistant to G418 and hygromycin (Engstler and Boshart, 2004). Care was taken to ensure a limited number of passages were performed for each sample, and both the OG and LT samples were drug treated for consistency, before an equal number of cells (1x10³ cells) from the OG line and the LT line were injected into two groups of mice (n = 3 per group).
Figure 4.13: Isolation of *T. brucei* EATRO 1125 PFR-Ty from whole-blood derived from a chronic coinfection. Whole-blood was extracted from three female mice by cardiac puncture, on day 38 of a long-term coinfection experiment, and frozen at minus 80°C (LT line). Parasites from one such stabilate (mouse 2RR) was used to seed a flask of fresh HMI-9 media. The original *T. brucei* EATRO 1125 PFR-Ty cells (OG line), used to infect mice in the preceding coinfection study, was used to seed a separate flask that acted as a control. Both lines were drug-treated with G418 to remove potential *T. congolense* IL3000 cells from the coinfection sample, and care was taken to limit the number of passages (n = 3). Cells from both flasks (1x10^3) were then injected IP into two groups of female MF1 mice (n = 3 per group), with parasitaemia and body condition being monitored for 11 days. Created in BioRender.
4.6.1 Coinfection selects for increased parasite virulence

Parasitaemia and body condition of mice were followed from three days post-infection, and compared between the two groups. The data indicated that the prepatent period, time to first peak, and mean parasitaemia at the peak, were initially similar between the two groups (Figure 4.1a). After day 7, however, mice infected with the OG line started clearing parasites, with *T. brucei* being completely absent in blood smears, whereas the LT group sustained high parasitaemia during the same period. In fact, the parasitaemia remained high in the LT group up to day 11. Differences in the host body condition of the two groups were also noted, where both groups had similar mean body weights up to day 7 (OG line = 30.4 gram; LT line = 30.2 gram), before mice in the LT group started losing weight with a mean body weight of 28 gram being recorded in this group by day 11 (Figure 4.14b). These data, together with the consistently high parasitaemia observed in the LT group, prompted us to humanely end the experiment at day 11.

Investigation by microscopy indicated that parasites in the OG group presented as morphologically stumpy cells on day 7, and that they were completely cleared from the host blood by day 10, while those in the LT group resembled a combination of intermediate and stumpy cells by day 10 (Figure 4.13c). This phenomenon was further explored, by determining the cell-cycle of trypanosomes, in both groups, on days 6 and 7 (Figure 4.15). Additional blood samples were taken on these days, and cells were paraformaldehyde-
fixed, before DAPI-staining was performed. Two-hundred cells were counted and scored (1K1N; 2K1N; 2K2N) for each day and individual mouse (n = 3 per group). The results indicated that more cells were dividing (% 2K1N; 2K2K) in the LT line (mean = 33%) than in the OG line (mean = 17%) on day 6, although this was non-significant (unpaired t-test: p = 0.06; t = 2.7; df = 4) (Figure 4.14a). On day 7, however, the LT line (mean = 12%) was observed to comprise of a significantly higher number of dividing cells, when compared to the OG line (mean = 1.6%) (Unpaired t-test: p = 0.04; t = 2.9; df = 4) (Figure 4.14.b). Taken together, these data indicate that cells in the LT line were not becoming growth arrested, and continuing to divide at days 6 and 7. This suggests they may have selected for reduced quorum sensing sensitivity after long term coinfection with *T. congolense*. Alternatively, this phenomenon may be explained by adaptation to a mouse host after a prolonged period, leading to improved growth. Future work will allow us to separate the effects of coinfection and long-term in vivo growth on parasite virulence (see Chapter 6).
Figure 4.14: *T. brucei* recovered from a long-term coinfection is more virulent. (A) Parasitaemia in the two groups were monitored from three days post-infection, and it was noted that the time to peak parasitaemia and parasite numbers were comparable between the two groups (days 3 and 8). From day 8, parasites in the OG line group were cleared, while those in the LT group persisted. (B) Body condition was comparable between both groups, up to day 7. Mice were seen to lose weight from day 8, in the LT group. (C) Parasites in the OG line resembled stumpy cells, by day 7, and were fully cleared from the blood by day 10. Conversely, no stumpy cells were observed at day 7 for parasites in the LT group, and these parasites had not been cleared from the blood by day 10, when they presented an ‘intermediate’ morphology. Scale bar = 30µm.
Figure 4.15: Comparing the cell-cycle status between two trypanosome-lines. Parasites collected from mouse blood, on day 6 and 7, were formaldehyde fixed and DAPI-stained. Two-hundred cells were scored according to cell-cycle status on both days for each mouse. Data shown are the mean percentage of dividing cells (% 2K1N; 2K2N) at a given time for both the OG (starting line) and LT lines (recovered; long-term) of *T. brucei* EATRO 1125 PFR-Ty, from a previous long-term coinfection. The mean proportion of dividing cells on day 6 was not significantly different (unpaired t-test: p = 0.06; t = 2.7; df = 4) between the OG line (17%) and the LT line (34%). The mean % proportion of dividing on day 7 was significantly different (unpaired t-test: p = 0.04; t = 2.9; df = 4) between the OG line (1.6%) and the LT line (12%).
4.7 Discussion

Analyses of field-derived samples indicated that multiple trypanosome species are co-circulating in Western-Kenya. This agrees with previous, cross-sectional surveys, in tsetse and host animals that have been performed in that geographic region (Njiru et al., 2004; Thumbi et al., 2010). The most prevalent species that was detected in our ITS-1 screen was *T. theileri*, a ubiquitous – and reportedly non-pathogenic – trypanosome species which infects a range of mammals in sub-Saharan Africa (Hoare, 1972; Soulsby, 1968).

Interestingly, a high number of recurring PCR-products (corresponding to 300; 400; and 700bp) were also detected which do not correspond to known band sizes in the ITS-1 assay. These may represent strains of species, such as *T. vivax* (band size = 611bp) with sequence variation that may perturb PCR amplification, or indeed previously unidentified species. In fact, the presence of unknown trypanosomes, or newly discovered species, have also been reported elsewhere (Adams et al., 2006; Auty et al., 2012; Gibson, 2007; Hamilton et al., 2009). We cannot confidently identify these bands in the absence of sequencing and phylogenetic analyses of the PCR products, which will be the focus of follow-up analyses. Initial screening using an assay targeting the ITS-1 region of the genome indicated a low prevalence of African trypanosomes, with no coinfections being found between these species. The overall prevalence of African trypanosomes, in this data set, was also low with *T. brucei* and *T. congolense* being particularly underrepresented. Our data agrees with a recent cross-sectional survey, using alternative primers, which
indicated that less 0.9% of samples were infected with *T. brucei*, and that no *T. congolense* infections could be found (Kivali et al., 2020). The authors stressed that they used primers that are specific to the Savannah type of *T. congolense*, and that other types may indeed be circulating. It should also be noted that a previously performed comparison, between different assays, indicated that the ITS-1 protocol under-performed when compared to species-specific primers, and that the prevalence of African trypanosomes may indeed by under-estimated (Ahmed et al., 2013).

The low incidence of African trypanosomes in our ITS-1 screen and the reported performance of that assay prompted us to screen a second, smaller data set, using primers specific for African trypanosomes that target the tubulin region of the parasite genome. The data set was for animals which showed clinical symptoms of disease and which were visited by veterinarians (de Clare Bronsvoort et al., 2013). However, we obtained similar results using these tubulin primers again indicating a low prevalence of African trypanosomes, an absence of *T. congolense* infections, and a lack of detectable coinfections between African trypanosome species. These are interesting findings, given that previous reports have indicated that coinfections do occur between these species in the Teso and Suba districts of Busia County, Kenya (Thumbi et al., 2010). Furthermore, surveys of infected tsetse flies in the same region also indicated a *T. congolense* prevalence of 1.4% (overall infection prevalence = 4.2%) (Adungo et al., 2020). At the same time, a recent study reported a decrease in the prevalence of African trypanosomes, which may explain our findings (Kivali et al., 2020).
Coinfecting organisms interact, either indirectly or directly, within the host, which have been shown to have implications for parasite traits such as virulence, transmission and niche localisation (Ademola and Odeniran, 2016; Nacher et al., 2001; Randall et al., 2013). The altered traits may, consequently, have implications for the epidemiology of those organisms, as well as the treatment and disease outcome in the hosts. Studies exploring these phenomena in trypanosomes are limited, however, and the mechanisms behind such interaction are poorly understood. The results of our short-term coinfection study, indicated that the parasitaemia of *T. congolense* MF1 CL1 was unaffected and similar in both the coinfection group and single infection control. The data indicated, however, that *T. brucei* EATRO 1125 PFR-Ty parasitaemia was reduced in the coinfection group and that a significantly higher proportion of those were stumpy cells when compared to the *T. brucei* only control. Coinfections, in other organisms, have been shown to either increase or reduce transmission potential (Susi et al., 2015; Tang et al., 2019). Indeed, our data suggests that coinfection between *T. congolense* and *T. brucei* may enhance the transmission potential of the latter, though this would have to be confirmed with tsetse-feeding experiments in future.

Chronic infections with trypanosomes occur frequently, through tissue adaptation and antigenic variation (MacGregor and Matthews, 2012; Matthews et al., 2015). As such, we sought to extend our observations of between species interaction to a long-term coinfection system in mice. We initially sought to perform these experiments with *T. congolense* field strains but the outcome of pilot studies indicated that mice were not amenable to long-term
infections with these strains. As such, we instead opted to use the laboratory IL3000 strain in combination with *T. brucei*. An initial observation, after 38 days, was that coinfection impacted upon disease outcomes in mice, where such mixed infections ameliorated the virulence of *T. brucei*. This finding is supported by previous studies, in African trypanosomes as well as other trypanosome species, where coinfections can lessen the effects observed during single infections (Balmer et al., 2009; Peterson et al., 2016). Parasite growth was also altered during our long-term coinfection study, in addition to changed host outcomes. Firstly, these data indicated that the growth dynamics were altered between the different infection groups where the first peak of parasitaemia in the coinfection group was cleared 3-4 days before that of the *T. brucei* only group. Next, we observed that overall parasitaemia remained high – and did not clear – in the coinfection group, but that the proportion of each species represented in the overall parasitaemia fluctuated over time. In other words, we observed a cycling between species, similar to peaks of parasitaemia, where *T. brucei* initially dominates, then being superseded by *T. congolense*, before becoming dominant again. Observations of the cell-cycle status after day 30 in the coinfection group indicated that the proportion of dividing *T. brucei* increased slightly, while *T. brucei* parasitaemia also increased. The magnitude of the increased *T. brucei* parasitaemia seems surprising given the low proportion of dividing cells (<5%) between day 33 and 38. The proportion of dividing *T. congolense*, on the other hand, remained high throughout the course of the infection, and was seen to increase as the *T. brucei* population increased. The observation that *T. brucei* was increasing in
population and that *T. congolense* dividing cells were increasing, seems to be counter-productive to both species as these conspecifics will likely compete for resources. However, evolutionary theory posits that parasites in a mixed infection may become ‘more reckless’ in their acquisition of resources than would be observed in single infection to gain a competitive advantage (van Baalen and Sabelis, 1995; Bremermann and Pickering, 1983; Frank, 1996).

At the same time, a high proportion of PAD1-positive *T. brucei* cells were observed at day 33-38 in the coinfection group of our long-term experiment. This agrees with earlier observations, where circulating *T. congolense* drove increased stumpy formation in *T. brucei*. However, if the levels of *T. congolense* are high and driving *T. brucei* to be growth arrested, then how can we account for the increased parasitaemia observed for the latter species? Together with the observations of cell-cycle status, this could imply that a population of *T. brucei* is replicating elsewhere, which then subsequently seeds the blood with transmission-ready cells. In fact, *T. brucei* has been shown to not only invade the skin and other organs, but has also been shown to replicate within the male reproductive organs of mice (Capewell et al., 2016; Losos and Ikede, 1972; Trindade et al., 2016).

Coinfection will select for enhanced virulence and the more virulent parasite will enjoy a competitive advantage, according to evolutionary theory (Bashey et al., 2013; Bell et al., 2006; Bremermann and Pickering, 1983). Conversely, intermediate virulence may be favoured, as a trade-off between virulence and transmission (Levin and Pimentel, 1981). To investigate whether the virulence characteristics of *T. brucei* (LT line) was altered following a long-
term coinfection, we infected mice with a line of parasites that were recovered from day 38 of the previous experiment. The experiment progressed for 11 days, and indicated that LT line *T. brucei* was indeed more virulent – mice infected with these parasites exhibited increased weight loss and did not clear parasites from the vasculature before the experiment had to be terminated. Cell-cycle analysis also indicated that these parasites were more proliferative on day 7 of the infection than parasites that had not previously been in a mixed infection. In other words, parasites in the LT line were continuing to grow, even in the presence of high parasite numbers which is traditionally accompanied by QS mediated cell cycle arrest. Trypanosomes need to balance their resource needs, while ensuring transmission potential, and it seems plausible that coinfecting trypanosomes might respond by gaining a transmission advantage, and increased virulence has been linked to increased transmission. As has already been shown, *T. congolense* can accelerate stumpy formation in *T. brucei*, which could select parasites which become less sensitive to QS signals in response. In unrelated organisms, such as fungi, coinfections have conclusively been shown to lead to increased transmission, through the enhanced production of spores (Susi et al., 2015). Studies utilising *Plasmodium chabaudi*, have also shown that more virulent strains can suppress those that are less virulent, and enjoy transmission success (de Roode et al., 2005; Bell et al., 2006). At present, we cannot tease apart the effects of long-term infection or coinfection on parasites.
5 WITHIN-HOST LOCALISATION AND COMPETITION AVOIDANCE BETWEEN COINFECTING TRYPANOSOMES
5.1 Introduction

Theory predicts that two organisms cannot indefinitely occupy the same niche if they were to compete for resources (Hardin, 1960). As such, parasites may selectively occupy different locations to avoid competition, which is thought to be the driver behind the tissue specificity observed in different human-infective filarial parasites (Molyneux et al., 2014). Evidence for such competition avoidance does exist among trypanosomatids as well. For example, in *Crithidia bombi*, a trypanosomatid of bumblebees, less competition was observed among those populations that were more disseminated across host tissues (Schmid-Hempel, 2001). Similarly, more disseminated infections were noted, including more parasites in the organs, when hosts were infected with multiple strains of *T. cruzi* (Perez et al., 2018). Tissue specificity has also been observed between African trypanosome species, where *T. brucei* is known to invade extravascular tissues such as the skin, lymph, adipose tissue, heart and the testes of mice (Capewell et al., 2016; Carvalho et al., 2018; Crilly and Mugnier, 2021; Trindade et al., 2016). Strikingly, *T. brucei* has also been shown to adapt to the specific niches that it may occupy, through metabolic and morphological changes (Dwinger et al., 1988; Mogk et al., 2014; Trindade et al., 2016). Both *T. vivax* and *T. congolense*, in contrast, are reportedly restricted to the vasculature, with limited evidence indicating that the latter species may invade other tissues (Abebe et al., 1993; Luckins and Gray, 1978).
In chapter 4 of this thesis, I observed that *T. brucei* EATRO 1125 PFR-Ty responded the presence of *T. congolense* (strains MF1 CL1 and IL3000) in separate coinfection experiments. This response is likely driven by QS, as illustrated previously during coinfections between *T. brucei* EATRO 1125 PFR-Ty and *T. congolense* IL3000 (Silvester et al., 2017). Furthermore, in the blood of chronically coinfected mice, we observed an increase in the parasitaemia of *T. brucei* cells which were growth-arrested, and transmission-adapted, while *T. congolense* numbers were already high in the vasculature. This opened up a host of interesting questions. Firstly, where were *T. brucei* cells growing and re-emerging from? Secondly, would competition avoidance between different trypanosome species, in a long-term coinfection, selectively drive increased invasion of host extravascular tissues by *T. brucei*? To answer these questions, I sought to generate a luciferin-reporting line of *T. brucei* cells, which could be used during in vivo imaging experiments. These parasites were validated in vitro, and were also used to establish an in vivo imaging model in our laboratory. I also discuss, here, plans for the future imaging of long-term coinfection experiments, which were impacted by the ongoing COVID-19 pandemic.

5.2 Generating a luciferin-reporting line of *T. brucei*

A luciferin-reporting line of *T. brucei* EATRO 1125 was sought to investigate the in vivo interactions of different trypanosomes during coinfections. To this end, Dr Martin Taylor (LSTHM) kindly provided us with a
plasmid (pTb-AMLuc; Figure 5.1a) for the stable integration of a red-shifted luciferase gene (REH9) into the rDNA locus of the *T. brucei* genome (Myburgh et al., 2013). The plasmid was digested with *SacI* and *KpnI*, resulting in two fragments – one larger, at ~ 4000bp (containing REH9), and another at ~ 3000bp (Figure 5.1b). Initial attempts to purify the REH9-containing fragment from agarose gels, post visualisation, delivered a low yield of linearised material, which resulted in no viable clones after transfection and drug-selection. The decision was made to avoid a loss of material during the purification step and instead transfect cells with both fragments, while simply confirming linearization using a small amount of material during gel electrophoresis.

### 5.2.1 Clone selection and in vitro validation

Trypanosomes (*T. brucei* EATRO 1125) were successfully transfected and drug-treated, yielding four viable clones (*T. brucei* AMLuc clones 4.1 - 4.4). We sought to assess the expression of red-shifted luciferase in the four clones by means of a luciferase assay to measure bioluminescence. Briefly, cells of each clone were seeded into flasks containing HMI-9 and grown to suitable density before centrifugation and resuspension in a luciferin containing substrate. Next, 1x10⁶ cells from each clone were seeded onto a 96-well plate, and serially diluted down to a concentration of 1x10³ cells, and allowed incubate at room temperature for two minutes prior to determining the RLU by means of a plate reader.
Figure 5.1: Transfection of *T. brucei* EATRO 1125 cells to generate a line of luciferin-reporting parasites. (A) Plasmid vector (pTb-AMLuc) showing the location of a red-shifted luciferase gene (*RE9H*) in red and puromycin N-acetyl transferase (PAC) in purple; blue denotes the GPEET2 procyclin, tubulin, actin and VSG intergenic region sequences to provide for RNA processing at the 5’-end of the PAC and 3’-end of *RE9H* (McLatchie et al., 2013). (B) Visualisation of linearised plasmid DNA following digestion with SacI and KpnI, resulting in two fragments (~4000bp fragment containing *RE9H*; and a smaller ~3000bp fragment). Initial attempts to purify the 4000bp fragment resulted in a loss of material and unsuccessful transfection, leading to the decision to transfec *T. brucei* EATRO 1125 cells with both fragments from the initial plasmid digest.
Figure 5.2: Determining the signal intensity of four luciferin-reporting *T. brucei* AMLuc clones. Bioluminescence was assessed by lysing cells, and performing luciferase assays by means of a plate reader. A serial dilution of cells (from $1 \times 10^6$ to $1 \times 10^3$) indicated that AMLuc 4.2 was the most bioluminescent clone (15 000 RLU), followed by AMLuc 4.1 (10 000 RLU). Untransfected *T. brucei* EATRO 1125 or HMI-9 media alone, served as controls.

The results indicated that the most bioluminescent line was clone 4.2, across different dilutions (Figure 5.2). Before moving forward with this clone for future experiments, we sought to assess the growth of this line and so initiated mice infections, in triplicate, to compare *T. brucei* AMLuc 4.2 with untransfected *T. brucei* EATRO 1125. Parasite growth was comparable between both lines in terms of prepatent period, peak of parasitaemia, and days until parasite clearance (Figure 5.3a) indicating that the expression of the luciferase reporter did not have an observable effect on the growth of parasites in vivo.

Next, we visualised luciferase activity of the *T. brucei* AMLuc lines (clones 4.1 and 4.2) in vitro, using the IVIS system. A total of $1 \times 10^5$ cells of
each clone was added to a 96-well plate, in duplicate, in 100µl of HMI-9 media, and serially diluted across the plate to deliver a range of $1 \times 10^2$-$1 \times 10^5$ cells. Each well was topped up with D-luciferin working solution, and incubated for 10 minutes, after which a time-series of images were taken (5-minute intervals). The results indicated that both lines could be successfully detected, using the IVIS system, and that in both cases (clones 4.1 and 4.2) the detection limit was determined to be $1 \times 10^2$ T. brucei cells (Figure 5.3b).
Figure 5.3: Investigating the properties of *T. brucei* EATRO 1125 AMLuc clones (A.) Mice infections were initiated to assess *T. brucei* AMLuc 4.2 for possible growth effects. This line was compared to untransfected *T. brucei* EATRO 1125, and growth was found to be similar between both groups, in terms of prepatent period, parasitaemia, and time to clearance. (B) Cells of the two most bioluminescent *T. brucei* AMLuc clones (4.1 and 4.2) were serially diluted across a 96-well plate (1x10^5 to 1x10^2 cells). Panels 1 to 4 indicate imaging across a period of 20 minutes (5-minute intervals), indicating no loss of signal. The results indicated that AMLuc 4.2 delivered the strongest signal, and that a dilution as low as 100 cells could be detected using the IVIS® system.
5.3 In vivo validation

The decision was made to move forward with *T. brucei* AMLuc 4.2, for future experiments and I sought to set up an in vivo mouse model using this line. Initial issues with the availability of the MF1 mouse strain, led to attempts at using other mouse strains, with suboptimal results such as significant variation between mice or a failure to establish infections at all (Appendix 3.1). As such, a new MF1 breeding colony was established by our laboratory, within our facilities at the University of Edinburgh, and work could once again progress.

5.3.1 Imaging of bioluminescent trypanosomes in live mice

The in vivo imaging of bioluminescent trypanosomes, in our laboratory, followed a protocol that was previously described by McLatchie et al. (2013). Two female MF1 mice were injected IP with either $1 \times 10^3$ or $1 \times 10^4$ *T. brucei* AMLuc 4.2 cells, with a third acting as parasite-free control (HMI-9 media only). Mice received additional injections (IP) with a substrate of the expressed luciferase protein D-luciferin, on days 3 and 6 post infection, followed by incubation at room temperature for 10 minutes prior to imaging (Figure 5.4).
Figure 5.4: Establishing an in vivo model for the imaging of *T. brucei* AMLuc 4.2, in mice. (1) Three groups of mice were injected (n = 1 per group), on day 0, as follows: (i) HMI-9 control; (ii) $1 \times 10^3$ *T. brucei* AMLuc 4.2 cells; (iii) $1 \times 10^4$ *T. brucei* AMLuc 4.2 cells. (2) Injection of D-luciferin ($15 \text{ mg.ml}^{-1}$) took place before imaging, on days 3 and 6 p.i. (3) Mice were anaesthetised, following D-luciferin injection, and (4-5) 10 minutes elapsed before imaging on the IVIS system. Image created in BioRender.
Parasites were successfully imaged using the IVIS platform, delivering a number of interesting observations (Figure 5.5). Firstly, differences in the intensity of bioluminescent signal could be detected between individual mice coded R or RR, which were infected with either $1 \times 10^3$ and $1 \times 10^4$ cells, respectively. This indicated that quantitative information could be derived and that the sensitivity of the detection would permit use of a lower infecting dose of parasites. Interestingly, a high parasite load was detected around specific body locations on day 6 for individual RR, which may correspond to the lymph nodes and spleen respectively (Figure 5.5; red arrows). Comparing data between the two doses, on days 3 and 6, also indicated that the most optimal time to image was between 10 and 15 minutes after injection of D-luciferin (Figure 5.6a). While the bioluminescent signal remained above $8 \times 10^7$ photons per second (p/s) at 10- and 15-minutes post-injection of D-luciferin, in mouse RR, signal intensity was slightly better for lower parasitaemias (mouse R) at 15 minutes post-injection. Signal intensity also corresponded to increased parasite load, as confirmed by microscopy on days 3 and 6 (Figure 5.6b and 5.6c). Interestingly, parasites could be detected by means of in vivo imaging, on day 3, before parasites were detectable on blood smears.
Figure 5.5: In vivo imaging of *T. brucei* AMLuc 4.2 on day 6 post-infection. Panels 1 to 5 indicate an imaging time-series, with intervals of 5 minutes, where panel 1 = 10 minutes p.i. with D-luciferin; 2 = 15 minutes p.i., and so forth. The mice were infected as follows: HMI-9 media control (Z); 1x10^3 trypanosomes (R); and 1x10^4 trypanosomes (RR). Colours indicate signal intensity and inferred parasite density, where purple represents areas of lowest bioluminescence, and red representing those with the highest signal intensity. The optimal imaging time was observed to be 15 minutes after injection with D-luciferin. Parasites were also seen to congregate around areas that correspond to the lymph nodes, and spleen (denoted by red arrows). Only ventral images were obtained for this initial experiment.
Figure 5.6: Optimisation of an in vivo imaging model using luciferin-reporting trypanosomes. (A) The optimal time for imaging of mice, as recorded on day 3, was determined to be at 15 minutes post-injection of D-luciferin although the signal was sustained well for up to 20 minutes. No signal was detected in the control group (Z; HMI-9 and substrate). (B and C) Bioluminescent signal increased proportionally, with a corresponding increase in parasitaemia, on days 3 and 6 of the experiment.
5.3.2 Mouse perfusion and IVIS imaging of whole organs

Four female MF1 mice were infected with different doses of *T. brucei* AMLuc 4.2 cells, to determine if excised organs could be imaged using the IVIS® system. Mouse Z served as a media only control (0 cells), with mouse B, R and RR being IP injected 100, $1 \times 10^3$ and $1 \times 10^4$ cells respectively. After six days, each mouse was injected with D-luciferin, 10 minutes before imaging, to reveal the in vivo distribution of live parasites (Appendix 3.2). Mice were anaesthetised and perfused the following day (7 days post-infection) to eliminate the presence of blood in the vasculature and organs. Individual organs of interest (brain; heart; lungs; kidneys; spleen; and abdominal fat) were excised from the mice, once the perfusions were completed, and placed in a petri dish with 1x PBS to avoid desiccation. Each organ was placed in different well of a 6-well plate, and a luciferin solution was administered onto each organ by means of a Pasteur pipette, 10 minutes before imaging. The results indicated that *T. brucei* AMLuc 4.2 could indeed be imaged and quantified in individual mouse organs (Figure 5.7). The strongest bioluminescent signal was detected in the abdominal fat ($5.2 \times 10^6$ - $2.3 \times 10^7$ p/s), liver ($4.8 \times 10^6$ - $2.2 \times 10^7$ p/s) and the spleen ($5.2 \times 10^6$ - $1.5 \times 10^7$ p/s) (Figure 5.8a). Parasites were detected at lower densities in the kidney and heart, with the lowest signal being detected in the brain (Figure 5.8a; Appendix 3.3). Interestingly, differences were observed in the parasite load of particular organs, on day 7, between the three mice. For example, the strongest signal
that was detected in the spleen and liver (1.5x10^7 p/s and 2.2x10^7 p/s), was observed in mouse B (IP dose = 100 cells). At the same time, the largest parasite load, on the same day, was detected in the abdominal fat (2.3x10^7 p/s) of mouse RR (IP dose = 1x10^4). Comparing these data to the vascular parasitaemia (B = 3.75x10^8 parasites/ml; R and RR = 5x10^8 parasites/ml) for each mouse on day 7 (Figure 5.8b), may be indicative of changes in tissue preference over time.

5.4 Discussion

Silvester et al. (2017b) found that the laboratory IL3000 strain of *T. congolense* could drive enhanced stumpy formation in *T. brucei*. This result was confirmed by us, using a field strain, and we further showed a dynamic cycling and interactions between these two species (chapter 4 of this thesis). To investigate how these interactions impact upon the within-host localisation of coinfecting species, we sought to establish a model for the in vivo imaging of trypanosomes that coinfect mice. These efforts were delayed, first, by the unprecedented nature of the COVID-19 pandemic, and the subsequent closure of laboratories and animal facilities at the University of Edinburgh. Next, issues with procuring the requisite MF1 strain of mice - and a failure to establish our trypanosome infection models in other strains of mice – further impacted upon the completion of these experiments.
Figure 5.7: Distribution of *T. brucei* AMLuc 4.2 in individual mouse organs. Three female mice were IP injected with 100 (B), 1X10³ (R) and 1x10⁴ (RR) *T. brucei* AMLuc 4.2 cells. Mouse Z served as a media only control. Animals were perfused and individual organs were excised for imaging using the IVIS system. The parasites could be successfully imaged in individual organs, with the strongest bioluminescent signal being detected in the abdominal fat, liver and the spleen.
Figure 5.8: Bioluminescence signal and parasite load in the tissues of individual mice. (A) The intensity of the bioluminescent signal between organs, on day 7, indicated that the abdominal fat \((5.2\times10^6 - 2.3\times10^7 \text{ photons/sec/cm}^3)\), liver \((4.8\times10^6 - 2.2\times10^7 \text{ photons/sec/cm}^3)\) and spleen \((5.2\times10^6 - 1.5\times10^7 \text{ photons/sec/cm}^3)\) harboured the highest parasite load. A lower parasite load was detected in the kidneys, heart and the brain. (B) Blood parasitaemia was monitored from 3 days p.i., indicating densities of \(3.8\times10^8\) cells/ml (mouse B) and \(5\times10^8\) cells/ml (mice R and RR) respectively, on day 7 (lower dose lagged by a day).
Nevertheless, significant progress was made towards establishing and optimising this model. We successfully generated *T. brucei* EATRO 1125 parasites which express a red-shifted luciferase gene, yielding four clones (designated *T. brucei* AMLuc clones 4.1 - 4.4). These lines produced varying expression levels of bioluminescent signal, which may be due to differences in gene copy integration into the trypanosome genome or variation in the integration locus. As such, we selected *T. brucei* AMLuc 4.2 for future experiments. The growth of that line was similar to the wild-type *T. brucei* EATRO 1125 in terms of growth profile, allowing it to be used in future coinfection experiments and for the results to be hopefully comparable to that obtained with non-reporter lines.

Next, the in vivo model was tested in our lab, by determining if *T. brucei* AMLuc 4.2 could be successfully imaged on the IVIS Lumina III system, and to determine the optimal time for imaging in future experiments. I found that parasites could be detected using the IVIS platform, even before parasites were noted in peripheral blood by means of microscopy – demonstrating high sensitivity. This result is similar to a finding by Capewell et al. (2016), where parasite bioluminescence was detected in mice on day 3 following tsetse feeding, while parasites were only detected in the blood on day 5. Some signal could be observed in the control mouse (Z; injected with HMI-9 and D-luciferin) which can be attributed to spill-over light emission from the experimental mice, and care will be taken to avoid this in future experiments by placing dividers between individual animals (Ritchie et al., 2020).
Analysis of bioluminescent signal, over time, indicated that the optimal time to image was 15 minutes after the injection of substrate. Signal intensity did correspond to pathogen load, between day 3 and 6, but a limited number of mice and imaging time-points meant that we could not expand on this correlation. We also observed a high parasite load on day 6 around areas which may correspond to the spleen and lymph nodes of mice. In fact, *T. brucei* is known to invade the extravascular tissues, including the lymphatic system, and has been previously detected in the lymph nodes through IVIS® imaging (Barry and Emergy, 1984; Capewell et al., 2016; Alfituri et al., 2019). Furthermore, these locations cannot be attributed to the site of injection, as these occurred on the opposite side of the mouse abdomen.

Bioluminescent imaging cannot resolve pathogen load in deep tissues as a result of the scattering of light (Luker and Luker, 2008). As such, mice were perfused and individual organs were excised, to determine if *T. brucei* AMLuc 4.2 could be imaged in the extravascular tissues using the IVIS® system. Parasites were successfully imaged and quantified, with the strongest signal being observed in the abdominal fat, liver and the spleen. Parasite invasion of these organs have been reported, previously (Capewell et al., 2016). Unlike other studies, I did not observe a high parasite load in the brain. It is worth mentioning that those studies used the GVR35 strain of *T. brucei* (Myburgh et al., 2013), which may invade the brain tissue more efficiently than AMLuc 4.2 that was used in this thesis. Differences in the parasite load of individual organs were observed between mice, on day 7. Comparing these data to the observed parasitaemia in the blood, may indicate a sequential progression of
tissue invasion over time, where the spleen and liver is colonised earlier than the abdominal fat. A number of studies have highlighted the changing distribution of trypanosomes throughout the tissues but none have quantified parasites in specific organs, over multiple time points.

The ultimate goal of this chapter is to image coinfections between \textit{T. brucei} and \textit{T. congolense} in vivo. Interactions between these two species of parasite may lead to competition avoidance, through shared resource requirements and host immune responses. These phenomena will be tested by conducting a long-term coinfection experiment, with mice being infected in two groups (\(n = 6\) per group), as follows: (1) coinfection group; (2) \textit{T. brucei} AMLuc 4.2 only. The experiment will progress for a total of 40 days, with three mice in each group being sacrificed (\(n = 6\)) at day 16 (following the first peak of \textit{T. brucei} parasitaemia) to image appropriate organs. This will test the hypothesis that coinfection precipitates competition avoidance, which will in turn drive increased tissue invasion by \textit{T. brucei} (Figure 5.9). The remaining three mice in each group (\(n = 6\)) will be imaged on until the conclusion of the experiment (day 40) to test the hypothesis that coinfection alters the distribution of parasites within the host.
Figure 5.9: Hypothesised model for competition avoidance during coinfections in mice. (Top) Coinfections with *T. brucei* AMLuc 4.2 and *T. congolense* IL3000 leads to increased competition, possibly through QS signals, between both species. Such competition may result in increased invasion of tissues, such as the spleen and lymph nodes, by *T. brucei*. (Bottom) Infections with *T. brucei* alone, and so in the absence of competition, may result in a lower parasite load within mouse organs.
6 SUMMARY & FUTURE DIRECTIONS
Trypanosome coinfections are common. This fact has been evidenced by countless epidemiological surveys of natural settings which have found hosts to be infected with different trypanosome species, and even strains of the same species. Such coinfections are important for a number of reasons. Firstly, coinfections may alter parasite growth or the outcome of disease, as is evidenced by less virulent trypanosomes ameliorating the effects of a more virulent strain when both infect the same host (Masumu et al., 2009; Morrison et al., 1982). Secondly, different trypanosome species can interact directly through, for example, shared QS signalling pathways with resultant effects on the growth dynamics and transmission potential of these parasites (Silvester et al., 2017b). Thirdly, transmission efficiency and virulence are coupled, with more virulent strains of *T. congolense* being found to more efficiently transmit to tsetse (Masumu et al., 2006a). Finally, the selective pressure exerted by such interactions could drive enhanced virulence in trypanosomes, which may have both competitive and transmission advantages. Evidently, many aspects of trypanosome-trypanosome infections remain understudied necessitating a better understanding of the mechanisms at play as well as real-world implications of such coinfections, which was the main aim of this thesis.

Differential virulence has been noted in field-derived strains of *T. congolense* (subtype: Savannah), where mouse survival time was used to separate strains into low, middle or high virulence categories (Masumu et al., 2006b; Van den Bossche et al., 2011). Scant data exists to explain the mechanisms that govern these phenotypic differences and we attempted, first, to explore genomic differences between these strains (section 3.2). A number
of differences were detected in the predicted protein sequence encoded by genes implicated in QS, and a particularly interesting finding was a shared amino acid substitution (A > S at position 415) that was present in AdSS of several low virulence strains (Figure 3.2). Attempts at modelling this protein to explore the impact of the mutation were hampered by the absence of a defined crystal structure for trypanosome AdSS (section 3.3.2). Aligning a modelled TcAdSS, against PDB 1p9b (P. falciparum) however, revealed that additional regions are present that are specific to trypanosomatids, and that the mutation of interest was located within that region. Future work should involve using appropriate molecular techniques, such as CRISPR, to introduce the Ser415 mutation to the IL3000 reference strain, and to investigate any resultant effects on the virulence of that line. In addition, the genome data generated for each of the strains analysed could be utilised for future investigations into the evolutionary history and relationships of T. congolense field strains, as well as further molecular and bioinformatic studies to explore regulatory elements, for example, and gene differences involving non-QS genes that are consistent between high and low virulence strains.

Further analyses of the virulence phenotypes observed between T. congolense field strains focused on the transcriptome differences between those parasites (section 3.4). These data indicated that there were differences in expression levels between the different strains and a set of criteria were then applied to find those transcripts with the most significant fold-changes (Figures 3.4). Two transcript classes were of particular interest, namely SecA DEAD-like domains, which were downregulated in high virulence strains when
compared to low virulence strains, and TfRs which were significantly upregulated in strains of high virulence (Figure 3.5; Table 3.2). A correlation between TfR and increased virulence was explored by quantifying the abundance of these receptors between different T. congolense strains and comparing expression levels to the relative abundance of alpha-tubulin (RAT) transcripts providing insight into the TfR transcript levels in relation to one measure of overall growth and activity. This analysis indicated, however, that no significant difference in TfR abundance was present between stains (Figure 3.6). Follow-up experiments could involve performing similar abundance analyses relative to another protein which remains stable throughout the trypanosome lifecycle, performing Western blotting on TfR between strains to visualise protein abundance, or utilising TPM as a normalisation method for differential expression analysis, prior to comparing those results with RAT data.

A further aim of this study was to explore the impact of coinfections between African trypanosomes and the implications of this for both host and parasite. First, data from a longitudinal cohort study in Western Kenya was explored (de Clare Bronsvoort et al., 2013) to determine the prevalence of different trypanosomes in this region and to identify any animals that harboured appropriate coinfections for follow-up analyses (section 4.2). Multiple species of trypanosome were found to co-circulate in this region, including African trypanosomes (Figures 4.2 and 4.3). While coinfections were noted, no animals were identified to be simultaneously infected with T. brucei, T. congolense and T. vivax. This does not mean that these coinfections do not
exist, as previous surveys in the same region had previously documented such
combinations between African trypanosomes (Thumbi et al., 2010).
Furthermore, sensitivity of the ITS-1 assay and the timing of sampling needs
to be taken into account. As such, further investigations should involve
rescreening of our ITS-1 and tubulin data using species-specific primers.
Failing that, future work could also involve repeating such longitudinal screens
in a geographic area where coinfections are indeed prevalent.

Shifting from the field to the lab, we sought to select strains for
appropriate in vivo studies (Table 4.1) and initiated both short and long-term
coinfections in mice. Species discrimination during each of these experiments
were accomplished by utilising a line of *T. brucei* EATRO 1125 containing a
PFRA protein tagged with a Ty1-epitope. An experiment to determine if *T.
brucei* could respond to QS signals from MF1 CL1 – a field-derived strain of *T.
congolense* – indicated that *T. congolense* suppressed *T. brucei* growth during
coinfections when compared to single infections, and that *T. brucei* stumpy
formation was significantly increased (Figure 4.6). These data confirm the
results of previous work between *T. brucei* and the laboratory strain IL3000 of
*T. congolense* (Silvester et al., 2017b), thereby showing the relevance of this
phenomenon in the field. Long-term coinfections, comprising *T. brucei* EATRO
1125 PFR-Ty and *T. congolense* IL3000 confirmed that parasite growth
dynamics and disease outcome in the host were altered when compared to
single infections (Figure 4.8a and b). Furthermore, it was shown that dynamic
cycling between the two species occur over the course of the experiment, with
the peak of parasitaemia in one species coinciding with the clearance of the
other from the host vasculature (Figure 4.9). Interrogating the cell-cycle status of these species during the course of the infection indicated that a high rate of dividing cells was seen for *T. congolense* throughout the infection, while that of *T. brucei* remained low. Interestingly, the number of dividing *T. brucei* cells remained low throughout the experiment, even as the overall numbers of this species increased between day 33 and 38 (Figure 4.11). The transmission potential of *T. brucei* was also assessed during the course of the long-term coinfection. Here, it was revealed that the proportion of *T. brucei* PAD1+ cells were indeed highest as *T. congolense* parasitaemia increased in the vasculature (Figure 4.12), in-line with the earlier findings where strains IL3000 (Silvester et al., 2017b) and MF1 CL1 drove increased stumpy formation. Curiously, the highest proportion of PAD1+ cells were observed at the end of the infection (day 38) where *T. brucei* cell numbers were found to be increasing, after *T. congolense* had dominated the infection. This counterintuitive observation may be interpreted by reinvasion of stumpy forms into the blood combined with replication of slender forms released from repression by *T. congolense*. Taken together, data from this long-term study suggests that not only does *T. brucei* replicate and differentiate outside the vasculature, but that such coinfections may alter the transmission potential of this species. It is important to mention that the timing of infection is important, with the established parasite species often enjoying a competitive advantage over the species that is introduced later (Karvonen et al., 2019). As such, future work should also include trypanosome coinfections in different timing
sequences and doses, as well of different species, to tease apart the important impact that coinfection may have in the field.

It has been proposed that coinfections may select for enhanced virulence in parasites. To test this theory, *T. brucei* parasites were retrieved from mice following long-term coinfections, and subjected to culturing with a selective drug to eliminate any *T. congolense* which may be residing in those samples (Figure 4.13). These were injected (LT line) into mice and compared to the original line (OG line) of *T. brucei* that was used to initiate the long-term coinfections. The results showed that the LT line of parasites that were retrieved from a long-term coinfection scenario exhibited increased virulence. Animals in the LT group experienced increased weight-loss, and microscopic investigation revealed that these parasites did not readily clear the vasculature, or exhibit stumpy morphology, when compared to the OG line (Figure 4.14). Comparisons of the cell-cycle status of both lines at peak parasitaemia (day 7) also indicated that a significantly higher proportion of LT line cells were dividing (2K1N; 2K2N) than OG cells (Figure 4.15). These results indicate that parasites in a long-term coinfection show a reduction in the capacity for growth arrest, possibly as a result of reduced sensitivity to QS signals. At present, we cannot tease apart the effects of infection duration and coinfection on parasites. Thus, a repeat experiment will take this into account by inoculating one group of mice with *T. brucei* EATRO 1125 PFR-Ty from a long-term monoinfection and another with trypanosomes from long-term coinfection. Next, the experiments should also be repeated to include groups
with long-term coinfected parasites from multiple sources, and time-points, to investigate if there is indeed evolutionary pressure driving increased virulence.

Coinfection may result in the altered tissue localisation within the host, as a means of competition avoidance. To explore the impact of such interaction on mixed infections between African trypanosomes, this thesis aimed to establish a model for the in vivo imaging of live parasites (Chapter 5). To this end, a line of bioluminescent *T. brucei* was engineered to express a luciferin reporter gene (Figure 5.1). Transfection experiments resulted in four clones, with further analyses identifying *T. brucei* AMLuc 4.2 as the most suitable line for future experiments (Figure 5.2). There were no detectable growth defects, when comparing *T. brucei* AMLuc 4.2 to untransfected parasites, and this line could also be successfully imaged using the IVIS system (Figure 5.3). These parasites were also successfully imaged in mice (Figure 5.5), with peak bioluminescence being detected at 15-minutes post-injection of the D-luciferin substrate (Figure 5.6). To adequately explore trypanosome invasion of the extravascular tissues, the model must be able to able to detect and quantify parasites in individual mouse organs. Therefore, mice were infected with *T. brucei* AMLuc 4.2 and later perfused, before organs of interest were excised and imaged. Parasites could be detected in a number of organs (Figure 5.7) such as the kidneys and heart, while the greatest parasite load was detected in the abdominal fat, liver and spleen across three experimental dosages (Figure 5.8). Interestingly, no parasites were detected in the brain, although it is likely that the experiment did not progress long enough to allow for parasites to invade this organ (Appendix 3.3). Future work should first assess the
stability of the reporter gene expression over time, in *T. brucei* AMLuc 4.2, and this will be investigated in future by means of long-term growth in culture. Next, in vivo imaging of a long-term coinfection experiment in mice, between *T. brucei* AMLuc 4.2 and *T. congolense* IL3000, will explore changes in parasite localisation. Particular attention will be given to quantifying *T. brucei* in the extravascular tissues to explore if coinfection leads to enhanced invasion of these spaces as a response to competition with *T. congolense* in the host blood. Finally, a luciferin-reporting line of *T. congolense* IL3000 cells will be generated in our lab, to replicate long-term coinfection studies performed with *T. brucei*. This will allow both *T. brucei* and *T. congolense* bioluminescent lines to be assayed in converse coinfection experiments to determine the location of each species, and to investigate if parasite distributions are altered when comparing single infections and coinfections.

In this thesis, I have presented novel insights into the mechanisms that govern virulence of different strains of trypanosomes derived from the field. Insights gained into the interactions between *T. brucei* and *T. congolense* also have implications for the broader management of disease – namely virulence and transmission. Important questions also remain to be answered, such as the impact of coinfection and tissue reservoirs of parasites on disease management interventions, including drug-treatment, with the data and models presented here providing a foundation for such investigations.


Banks, K.L. (1978). Binding of Trypanosoma congolense to the walls of small


Cox, A., Tilley, A., McOdimba, F., Fyfe, J., Eisler, M., Hide, G., and Welburn,


Evolution 52, 1869–1871.


Parasitology Today 3, 49–52.


Holzmuller, P., Biron, D.G., Courtois, P., Koffi, M., Bras-Gonçalves, R., Daulouède, S., Solano, P., Cuny, G., Vincendeau, P., and Jamonneau, V.


La Greca, F., and Magez, S. (2011). Vaccination against trypanosomiasis: can it be done or is the trypanosome truly the ultimate immune destroyer and
escape artist? Hum Vaccin 7, 1225–1233.

Lalmanach, G., Boulangé, A., Serveau, C., Lecaille, F., Scharfstein, J.,
Gauthier, F., and Authié, E. (2002). Congopain from Trypanosoma


Langousis, G., and Hill, K.L. (2014). Motility and more: the flagellum of

trypanosomes from man and other mammals using DEAE-cellulose. Exp
Parasitol 28, 521–534.

Hydrolysis products of cAMP analogs cause transformation of Trypanosoma
brucei from slender to stumpy-like forms. Proc. Natl. Acad. Sci. USA 103,
19194–19199.

variant surface glycoprotein of African trypanosomes is recognized by a
macrophage scavenger receptor and induces I kappa B alpha degradation


Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth,


MacGregor, P., and Matthews, K.R. (2012). Identification of the regulatory elements controlling the transmission stage-specific gene expression of


Marcello, L., and Barry, J.D. (2007). Analysis of the VSG gene silent archive in Trypanosoma brucei reveals that mosaic gene expression is prominent in
antigenic variation and is favored by archive substructure. Genome Res. 17, 1344–1352.


membrane structural linkage is responsible for mitochondrial genome positioning and segregation by flagellar basal bodies in trypanosomes. Mol. Biol. Cell 14, 1769–1779.


Peterson, J.K., Graham, A.L., Elliott, R.J., Dobson, A.P., and Triana Chávez,


Ritchie, R., Barrett, M.P., Mottram, J.C., and Myburgh, E. (2020). In Vivo


Trindade, S., Rijo-Ferreira, F., Carvalho, T., Pinto-Neves, D., Guegan, F.,
Aresta-Branco, F., Bento, F., Young, S.A., Pinto, A., Van Den Abbeele, J., et
al. (2016). Trypanosoma brucei Parasites Occupy and Functionally Adapt to
the Adipose Tissue in Mice. Cell Host Microbe 19, 837–848.


antigen during transformation of Trypanosoma brucei rhodesiense from

Turner, C.M., Barry, J.D., Maudlin, I., and Vickerman, K. (1988b). An
estimate of the size of the metacyclic variable antigen repertoire of

Van den Bossche, P., De Deken, R., Brandt, J., Geerts, S., Geysen, D., and
brucei/T. congolense infections by tsetse (Glossina morsitans morsitans).

Van den Bossche, P., Chitanga, S., Masumu, J., Marcotty, T., and
subgroup. A comparison between strains and transmission cycles. Parasite
Immunol 33, 456–460.


APPENDIX 1: Supplementary data for Chapter 3
Appendix 1.1: The origin and virulence of five *T. congolense* (subtype: Savannah) field strains. These strains were identified in and isolated in Zambia (Masumu et al., 2006b; Van den Bossche et al., 2011) and supplied to us in the form of stabilate. These lines could not be established in mice after successive rounds of inoculation and were consequently not used for further analyses.

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<td>Low</td>
</tr>
<tr>
<td>ALICK 589 C1</td>
<td>Domestic</td>
<td>Low</td>
</tr>
<tr>
<td>KASANDA 20C4</td>
<td>Domestic</td>
<td>Low</td>
</tr>
<tr>
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<td>Medium</td>
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<tr>
<td>BT0106</td>
<td>Sylvatic</td>
<td>High</td>
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</table>
Appendix 1.2: Growth characteristics of four *T. congolense* (subtype: Savannah) field strains. Mice (n = 3 per group) were cyclophosphamide-treated, prior to being infected with $1 \times 10^3$ cells of each strain. These data confirm the classification of these strains into different virulence categories, namely: High virulence = MF3 CL1 and MF2 CL5; Low virulence = MF1 CL1 and M7 C3.
Appendix 1.3: Genome comparisons between seven of *T. congolense* (subtype: Savannah) field strains. WGS allowed for the identification of amino acid substitutions, truncations or deletions, in 35 genes associated with QS, in different trypanosome strains. Genomes were visualised using Artemis (v16.0.0) (Carver et al., 2008) and alignments were performed in Clustal Omega (Sievers et al., 2011).

<table>
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<th><em>T. congolense</em> ID</th>
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| Adenosine kinase, putative    | Tb927.6.2360 | TcIL3000.6.1860 |
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- Protein sequences and amino acid changes are listed. The changes are specified by their location and the amino acid change (e.g., 11 L>R).
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117 N>S (MF2 CL5, MF3 CL1, M7 C3), 156 T>A (MF1 CL1, MF2 CL5, MF3 CL1, M7 C3), 345 D>E (MF1 CL1, MF2 CL5, MF3 CL1, M7 C3) are the amino acid changes in the DNA repair protein putative (Hyp 9).
Appendix 1.6: Aligned protein sequences for AdSS from different trypanosome species.

Serine residues are present at the same position for *T. vivax* (TvY486_1103820), *T. congoense* (TcIL3000.11.3610), *T. brucei* (Tb1125.11.3650), *T. b. gambiense* (Tbg972.114160), and *T. theileri* (TM35_000301140). A threonine substitution is present at the same position for *T. cruzi* (TcBrA4_0074250).
Appendix 1.5: Alignment of TcAdSS to the *T. cruzi* AdSS predicted by AlphaFold DB, indicating the region of interest (pos. 380-430) that contains an amino acid substitution between strains (Jumper et al., 2021; Varadi et al., 2021). Alignment remained poor in the absence of a resolved crystal structure, limiting any meaningful inferences based on these amino acid substitutions. IL3000 AdSS (Ala415) indicated in teal; TcAdSS (Ser415) indicated in salmon; *T. cruzi* predicted protein indicated in grey; Individual amino acids at position 415, indicated in yellow.
Appendix 1.6: Assessing the quality of extracted *T. congolense* RNA, prior to RNA sequencing. Separation of *T. congolense* RNA on a gel yields five characteristic bands (teal arrows). From left to right: (1) BT0206 Z; (2) BT0206 BB; (3) MF3 CL1 R; (4) MF3 CL1 RR; (5) M7 C3 Z; (6) M7 C3 Z (7) M7 C3 B.
Appendix 1.7: Transcripts with a Log2FC = 2, following RNA-seq, that were not removed after applying certain exclusion criteria. These were identified as VSG-associated, congolense-specific ORF, but BLASTP analyses indicated a different role in *T. brucei*.

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APPENDIX 2: Supplementary data for Chapter 4
Appendix 2.1: Timing of *T. brucei* superinfections to investigate inter-species signalling. An in vivo experiment was planned to investigate the interaction between *T. brucei* EATRO 1125 PFR-Ty, and the field-derived MF1 CL1 strain of *T. congolense*. This required determining the optimal time to perform infections and superinfections (in the coinfection group), to ensure the timing of peak-parasitaemia coincided between both species. A trial experiment was initiated, where nine female MF1 mice were infected via IP injection. Group 1 (coinfection group) and group 2 (*T. congolense* control) (n = 3 per group) were each infected with $2.3 \times 10^6$ MF1 CL1 cells on day 0. Infections with $2 \times 10^5$ *T. brucei* EATRO 1125 PFR-Ty cells were then performed in group 1 (coinfection group) and group 2 (*T. brucei* control) (n = 3 per group) on day 4. The experiment reached a humane endpoint on day 7, and parasites were purified from whole-blood of groups 1 and 3 to calculate the proportion of PAD1+ *T. brucei* cells. Unfortunately, not enough *T. brucei* cells could be recovered from group 1, indicating that *T. congolense* had already dominated the infection and suppressed *T. brucei*. As such, the decision was made to perform *T. brucei* superinfections on day 3 for all future coinfection experiments.
Appendix 2.2: Overall parasitaemia for individual mice, as measured during a long-term co-infection. Total parasite numbers (*T. brucei* EATRO 1125 PFR-Ty and *T. congolense* IL3000) were scored by means of tail-snips, and the method described by Herbert and Lumsden (1976). Two mice (1/R and 2/Z) were humanely terminated before the conclusion of the 38-day experiment.
Appendix 2.3: Parasitaemia for individual mice, as measured during a long-term infection with *T. brucei* only. Total parasite numbers (*T. brucei* EATRO 1125 PFR-Ty) were scored by means of tail-snips, and the method described by Herbert and Lumsden (1976). All mice in this group were humanely terminated before the conclusion of the 38-day experiment.
Appendix 2.4: Parasitaemia for individual mice, as measured during a long-term infection with *T. congolense* only. Total parasite numbers (*T. congolense* IL3000) were scored by means of tail-snips, and the method described by Herbert and Lumsden (1976). Two mice in this group (6/Z and 6/R) were humanely terminated before the conclusion of the 38-day experiment.
Appendix 2.5: Inferred parasitaemia of two trypanosome species, during a long-term coinfection. Total parasite numbers (Appendix 2.2) were scored by means of tail-snips, and the method described by Herbert and Lumsden (1976). Tagging of the *T. brucei* PFR allowed for species discrimination and subsequently allowed for the calculation of parasite numbers, of both species based on their relative proportions, over the course of the 38-day period.
APPENDIX 3: Supplementary data for Chapter 5
Appendix 3.1: Assessing the use of Balb/ C mice for in vivo trypanosome experiments.

Six female Balb/ C mice were each IP-injected with 1000 *T. brucei* EATRO 1125 PFR-Ty cells, to assess their suitability for future long-term infection studies. Parasitaemia was monitored from 3 days p.i, with significant variation being observed between mice. Additionally, mice presented with moderate symptoms, including piloerection, lethargy and hunched posture, leading to the decision to terminate the experiment on day 16 for animal welfare reasons.
Appendix 3.2: Distribution of *T. brucei* AMLuc 4.2 parasites in live MF1 mice. Four female mice were infected with either 100 (B); $1 \times 10^3$ (R), or $1 \times 10^4$ (RR) trypanosome cells. Mouse Z acted as a media only control. On day 6 p.i, the maximum bioluminescence measured for B = $3 \times 10^6$ p/s, notably around the spleen. The signal for R and RR peaked between $2-2.5 \times 10^6$ p/s, and was observed to be more widely distributed across the abdomen. No signal was observed in mouse Z (control) apart from spillover signal from mouse B. Care will be taken to use signal dividers in all future experiments.
Appendix 3.3: Reduced bioluminescence from specific organs of mice infected with *T. brucei* AMLuc 4.2. IVIS® imaging of excised mouse organs revealed low signal intensity from the kidneys, heart and brain of mice. Signal around the kidneys and heart of mouse R, may be attributed to residual fat around those tissues that could not be removed.
APPENDIX 4: Media and Buffers
4.1 HMI-9 Media

A bottle of HMI-9 powder (Life technologies) was supplemented with the following:

45mM NaHCO₃

256μM β-mercaptoethanol

The media was made up to 4 litres with autoclaved dH₂O, and the pH was adjusted to 7.5 before filter sterilisation.

Combine the following to prepare complete media:

10% heat-inactivated FBS (Gibco)

10% sterile dH₂O and 1% Penicillin/ Streptomycin (Gibco).

4.2 T. congolense TcBSF3 media

TcBSF3 media was prepared as described previously (Coustou et al., 2010). Basal media was prepared by mixing one bottle of MEM powder (Sigma-Aldrich®) with the following:

5.96g acid HEPES (Sigma-Aldrich®)

2.2g NaHCO₃ (Sigma-Aldrich®)
1g D-glucose (VWR™)

110mg sodium pyruvate (Sigma-Aldrich®)

10.68mg adenosine (Sigma-Aldrich®)

14mg hypoxanthine

4mg thymidine

14.1mg bathocuproinedisulfonic acid disodium salt (Sigma-Aldrich®)

Aguettant Versol water was added to a final volume of 1 litre.

The pH of the basal media was adjusted to 7.3, followed by filter sterilisation.

Combine the following to prepare complete media:

75% TcBSF3 basal media

20% Goat serum (Sigma-Aldrich®)

5% Serum plus (Sigma-Aldrich®)

0.0014% β-mercaptoethanol

0.8% 200mM glutamine

1% Penicillin/ streptomycin solution (Gibco)

The completed media was filter sterilised and was stored at 4°C for up to two weeks.
4.3 TV3 media

TV3 media for culturing of T. vivax epimastigotes was prepared as follows:

One bottle of Iscove's Modified Dulbecco's Medium (IMDM) containing 25mM HEPES, 25mM D-glucose, and 4mM glutamine (Gibco) was supplemented with:

10% heat-inactivated FBS (GIBCO)

10% heat-inactivated Goat serum (Sigma-Aldrich®)

0.03mM bathocuproinedisulfonic acid disodium salt (Sigma-Aldrich®)

0.14mM β-mercaptoethanol

0.4mM Cysteine HCl

0.2mM Hypoxanthine

10mM Proline

0.05mM Thymidine

4.4 PSG Buffer

3mM NaH₂PO₄·2H₂O

44mM NaCl

83mM D-glucose

Adjust the final pH to 7.8
4.5 10X MOPS

221mM MOPS

50mM Sodium acetate, pH7

10mM EDTA

Autoclaved and store in the dark at 4°C.

4.6 RNA gel loading buffer

30% formamide

6% formaldehyde

1x MOPS

0.01% bromophenol blue 10% glycerol

4.7 TAE buffer

0.4mM Tris-HCl

0.1mM sodium acetate

0.1mM EDTA
4.8 Miniprep solution I

50mM glucose

25mM Tris-HCl, pH8

10mM EDTA pH8

4.9 Miniprep solution II

0.2M NaOH

1% sodium dodecyl sulphate (SDS)

4.10 Miniprep solution III

3M potassium acetate

11.5% (v/v) Glacial acetic acid
4.11 Roditi Transfection Buffer

90mM Sodium phosphate

50mM HEPES, pH7.3

5mM KCl

0.15mM CaCl$_2$

Filter sterilise before use

4.12 Phosphate buffered saline (PBS)

137mM NaCl

3mM KCl

16mM Na$_2$HPO$_4$

3mM KH$_2$PO$_4$

Adjust the pH to 7.4