Surveillance and monitoring of human African trypanosomiasis in the elimination era.

Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor of Philosophy by

Gala Garrod

March 2022

Declaration of work done

I hereby declare that except where specific reference is made to the work of others, the contents of this thesis are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This thesis is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the contribution statements at the beginning of this thesis. This thesis does not exceed the maximum permitted word length: it contains fewer than 100,000 words including appendices and footnotes, but excluding the bibliography.

Gala Garrod

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Abstract

Surveillance and monitoring of human African trypanosomiasis in the elimination era

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Human African trypanosomiasis (HAT) is a parasitic disease caused by trypanosomes transmitted by tsetse flies (*Glossina spp*). Two forms of the disease exist: gambiense HAT (gHAT) and rhodesiense HAT (rHAT) caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense* respectively. A WHO-led programme, centered around case detection, treatment and vector control has driven down the annual number of HAT cases reported globally from a peak of >35,000 cases in the 1990s to 663 in 2020. The WHO aims to eliminate transmission of gHAT by 2030 and eliminate rHAT as a public health problem. As case numbers decline, current surveillance strategies, based largely on case detection, become more difficult and expensive. This thesis examines the utility of a xenomonitoring system to monitor trypanosomes in tsetse being integrated into HAT surveillance.

Towards this aim, two qPCR-based assays were developed to screen for pathogenic trypanosomes in tsetse. One assay allows both human pathogenic subspecies of *T. brucei s.l.* to be detected simultaneously. The assay was as sensitive as currently available PCR methods whilst reducing the number of assays required from three to one.

A second assay was developed to identify tsetse carrying *T. brucei s.l.* as well as *T. vivax* and *T. congolense,* pathogens of veterinary importance. In laboratory tests, the assay showed high specificity (100%) and sensitivity (96.9%) but sensitivity was much reduced (71.1%) when tested on wild-caught tsetse. Further optimization of the assay will be required for future use as a xenomonitoring method.

Extraction of trypanosome DNA from tsetse is an important but costly and time-consuming precursor to detecting trypanomes using PCR-based methods. Chapter four describes the development of a low-cost and field-friendly alternative using a novel magnetic bead protocol (MagnaExtract). The results indicate that the method could be an effective, cheaper and faster alternative to current commercially-available methods.

Finally, in chapter five the rate of rebound of tsetse following the removal of vector control was simulated using a simulation model (Tsetse Muse) and the model predictions were compared to field data obtained over 36 months from a gHAT focus in Uganda where control of tsetse, using Tiny Targets, has been halted. The simulations predicted that two years after control ceased, tsetse populations would recover by 34-73% according to assumptions regarding habitat quality. In contrast to model predictions, wild populations showed no significant rebound for up to two years after the withdrawal of Tiny Targets. In the context of northern Uganda, limited scaling back of the deployment of Tiny Targets may not lead to the rapid resumption of transmission and hence risk of gHAT.

The results of each data chapter are discussed in the context of the feasibility and benefit of xenomonitoring being integrated into a HAT surveillance system in the future.

List of publications

Original manuscripts contributing to this thesis

One of the chapters I present in this thesis has been published in a peer-reviewed academic journal. Full author contributions are listed within the article along with a summary at the beginning of the chapter.

Chapter 2- A pilot study demonstrating the identification of *Trypanosoma brucei gambiense* and *T. b. rhodesiense* in vectors using a multiplexed high-resolution melt qPCR.

Garrod G, Adams ER, Lingley JK, Saldanha I, Torr SJ, Cunningham LJ. A pilot study demonstrating the identification of *Trypanosoma brucei gambiense* and *T. b. rhodesiense* in vectors using a multiplexed high-resolution melt qPCR. *PLoS Neglected Tropical Diseases*. 2020 Nov 25;14(11):e0008308.

COVID-19 secondment

In 2020, I undertook a six-month secondment to work on the evaluation of COVID-19 diagnostics. The following publications were produced and published or submitted as a result of that secondment:

Staines HM, Kirwan DE, Clark DJ, Adams ER, Augustin …**Garrod G…** Krishna S and Planche T. IgG seroconversion and pathophysiology in severe acute respiratory syndrome coronavirus 2 infection. *Emerging infectious diseases.* 2021 Jan;27(1):85.

Byrne RL, Kay GA, Kontogianni K, Aljayyoussi G…**Garrod G**…Adams ER and Cubas-Atienzar A. Saliva alternative to upper respiratory swabs for SARS-CoV-2 diagnosis. *Emerging infectious diseases*. 2020 Nov;26(11):2769.

Adams ER, Augustin Y, Byrne RL, Clark DJ…**Garrod G**…Fitchett JRA and Krishna S, Rapid development of COVID-19 rapid diagnostics for low resource settings: accelerating delivery through transparency, responsiveness, and open collaboration. *MedRxiv*. 2020 Jan 1.

Brown L, Byrne RL, Fraser A, Owen SI…**Garrod G**…Turtle L and Adams ERA. Self-sampling of capillary blood for SARS-CoV-2 serology. *Scientific reports.* 2021 Apr 8;11(1):1-6.

Owen SI, Williams CT, **Garrod G**, Fraser AJ, Menzies S, Baldwin L, Brown L, Byrne RL, Collins AM, Cubas-Atienzar AI, de Vos M. Twelve lateral flow immunoassays (LFAs) to detect SARS-CoV-2 antibodies. *Journal of Infection*. 2021 Dec 11.

Garrod G, Owen SI, Baillie JK, Baldwin L, Brown L, Byrne RL, Cubas-Atienzar AI, Cuevas LE, Fraser AJ, Fletcher T, Goodwin L. Comparative evaluation of ten lateral flow immunoassays to detect SARS-CoV-2 antibodies. *Wellcome Open Research*. 2021 Feb 1;6(18):18.

Original manuscripts complementing this thesis

Aside from the publication in this thesis, there are publications to which I contributed to which complement its overarching theme:

Hope A, Mugenyi A, Esterhuizen J, Tirados I, Cunningham LJ, **Garrod G,** Lehane MJ, Longbottom J, Mangwiro C, Opiyo M, Stanton M, Torr SJ, Vale GA, Waiswa C, Selby R. Scaling up tsetse control to eliminate Gambian sleeping sickness in northern Uganda. 2022. Manuscript submitted for publication.

Ndung'u JM, Boulangé A, Picado A, Mugenyi A, Mortensen A, Hope A, Mollo BG…**Garrod G**… Yoni W, and Katz Z. Trypa-NO! contributes to the elimination of gambiense human African trypanosomiasis by combining tsetse control with "screen, diagnose and treat" using

innovative tools and strategies. PLOS Neglected Tropical Diseases. 2020 Nov 12;14(11):e0008738.

Original manuscripts in parallel to this thesis

The following publications were produced alongside my thesis but do not directly relate to it:

Deb R, Singh RP, Mishra PK, Hitchins L..**Garrod**… Srikantiah S, Coleman M**.** Impact of IRS: Four-years of entomological surveillance of the Indian Visceral Leishmaniases elimination programme. *PLOS neglected tropical diseases*. 2021 Aug 9;15(8):e0009101.

South A, Lees R, **Garrod G**, Carson J, Malone D, Hastings I. The role of windows of selection and windows of dominance in the evolution of insecticide resistance in human disease vectors. *Evolutionary applications.* 2020 Apr;13(4):738-51.

As Gala's supervisor, I hereby certify that the statement of contribution listed above is a fair representation of her work.

Professor Steve Torr

March 2022

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And finally, to Lewis, I can't thank you enough for your care, good humor and for making sure I was well fed and watered throughout this PhD. Now onto our Australian adventure!

Contents

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Acronyms

AAT	Animal African trypanosomiasis
BLAST	Basic local alignment search tool
CATT	Card Agglutination Test for Trypanosomiasis
COVID	Coronavirus disease
Ct	Cycle threshold
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of the Congo
ELISA	Enzyme-linked Immunosorbent assay
Ghat	Gambiense Human African trypanosomiasis
GPI-PLC	Glycosylphosphatidylinositol phospholipase C
HAT	Human African trypanosomiasis
HRM	High-resolution melt analysis
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LAMP	Loop-mediated isothermal amplification
LSTM	Liverpool School of Tropical Medicine
NDVI	Normalized difference vegetation index
NECT	Nifurtimox-eflornithine combination therapy

PCR	Polymerase chain reaction
PhHv	Phocid herpes virus
QPCR	Quantitative polymerase chain reaction
RDT	Rapid diagnostic test
rHAT	Rhodesiense Human African trypanosomiasis
SIT	Sterile insect technique
SRA	Serum resistance antigen
Tbg	*Trypanosoma brucei gambiense*
TgsGP	*T. gambiense*-specific glycoprotein
TNF	Trypanosome lytic factor
VSG	Variant surface glycoprotein
WHO	World Health Organization

1 General Introduction

1.1 Trypanosomes

Trypanosomes are protozoan parasites which infect a range of hosts including all classes of vertebrates, plants, and insects. The *Trypanosomatidae* are characterized by the presence of a single flagellum and a kinetoplast, which resulted in their naming from the Greek trypano (borer) and soma (body). The kinetoplast itself is made up of circular DNA arranged into mini and maxi circles, the number and catenation of each is dependent on the species of trypanosome.

Trypanosomes can be categorized by their method of transmission. Stercorarian trypanosomes are transmitted to hosts through the faeces of infected insects belonging to the subfamily *Triatominae* (e.g. *Trypanosoma cruzi*, the pathogen causing American trypanosomiasis transmitted by *Triatoma*) whilst salivarian trypanosomes are transmitted through the saliva of infected tsetse flies (*Glossina* spp.). The subgenera of salivarian trypanosomes namely Trypanozoon, Duttonella, Nannomonas are responsible for the majority of human and animal trypanosomiasis.

1.1.1 Lifecycle

When an infected tsetse fly feeds on a host it transmits metacyclic trypomastigotes during the bloodmeal. The parasites pass into the host's bloodstream via the capillaries and lymphatic system and become bloodstream trypomastigotes. They spread throughout the body, replicating by binary fission, and ultimately cross the blood-brain barrier to reach the cerebrospinal fluid. Bloodstream trypomastigotes can present as two forms: the rapidly replicating long slender form and the non-replicating short stumpy form. The slender form is adapted to replicate successfully within the fly and undergo antigenic variation while the non-replicating stumpy form is designed to survive within the fly host.

The developmental cycle of trypanosomes within tsetse is dependent on the species of trypanosome however all end in differentiation into infective metacyclic trypanosomes which are transmitted to the next mammalian host (Figure 1). The three subgenera of salivarian trypanosome each have a specific developmental cycle within tsetse (1)(Figure 3). The Dutonella subgenera (e.g., *T. vivax*) undertake all their development within the tsetse mouthparts whilst Nannomonas (e.g. *T. congolense*) and Trypanozoon (*T. brucei*) develop within the midgut and foregut eventually colonizing the mouthparts and salivary glands respectively(2). The entire developmental cycle ranges from several days for *T.* vivax up to three weeks for *T. brucei*.

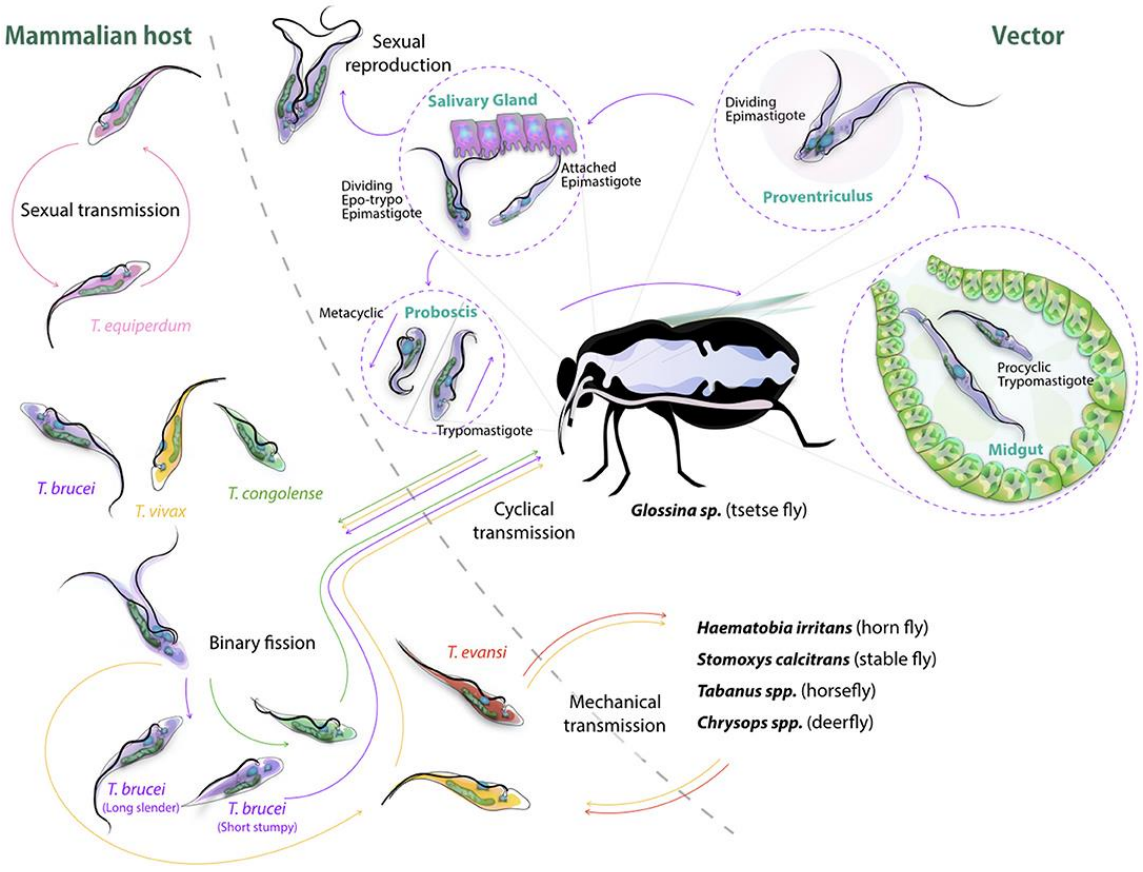

Figure 1 Transmission of salivarian trypanosomes by tsetse and other biting flies. Image taken from (2).

1.1.2 African trypanosomiasis

Human and animal African Trypanosomiasis are diseases of vertebrates caused by the trypanosomes across sub-Saharan Africa and south America. In Africa, parasites are transmitted to a host through the bite of an infected tsetse fly (*Glossina spp*.) but in South America trypanosomes are spread by biting flies such as Tabanids and *Stomoxys*. The African trypanosomiases can largely be split into two categories based on the affected vertebrate host:

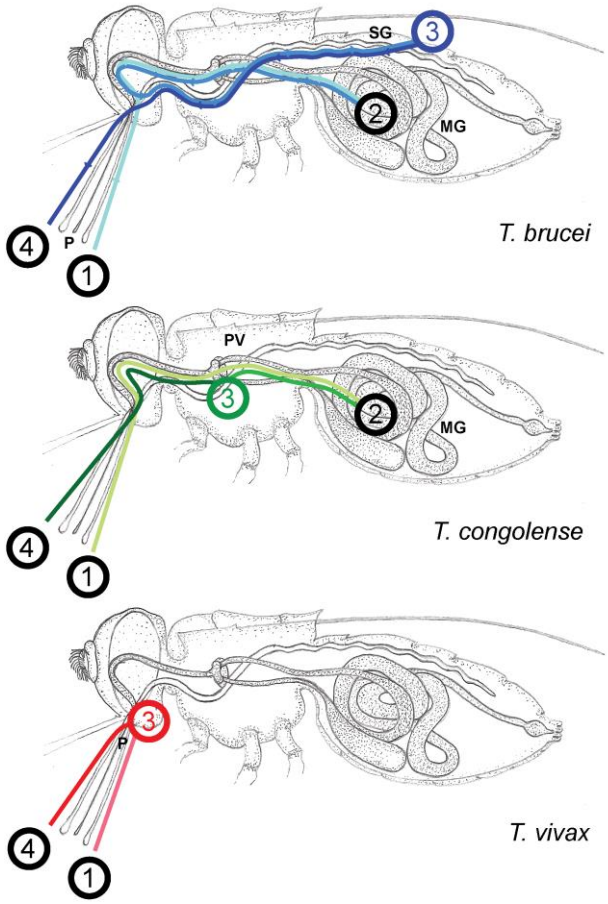

Figure 2 The development of T. brucei, T. congolense and T. vivax within the tsetse fly. Developmental cycles are split into (1); the passage of trypanosomes to the midgut (MG) followed by the development into procyclic form; anterior migration and differentiation into epimastigotes in the proventriculus (PV) (T. congolense) or salivary glands (SG) (T. brucei); the final stage is inoculation of metacyclics into a vertebrate host during feeding (4). Image taken from (1)

1.1.2.1 Animal African trypanosomiasis

Animal African trypanosomiasis (AAT) is endemic in 37 of 54 African countries and is one of the most important threats to the health of domestic livestock in sub-Saharan Africa. AAT, also known as Nagana, from the Zulu word “N’gana” translating as powerless or useless, particularly affects cattle. It has also established outside of the tsetse-belt in South

America where *T. vivax* is spread though mechanical transmission by biting flies. It is thought that the disease was introduced to South America via the transportation of Zebu cattle from Senegal to French Guyana and the Antilles (3).

A number of trypanosome species cause AAT with the most economically important species being *T. vivax* and *T. congolense*. Common symptoms in domestic animals are anorexia, listlessness, weight loss, aneamia and paralysis. AAT can result in a 50% reduction in both milk and meat yields, posing a risk to food and economic security to livestock owners (4). It has been estimated that AAT causes a total economic loss of over US $ 4.5 billion dollars per year (5–7) and greatly reduces agricultural productivity in tsetse-infested areas.

A small proportion of cattle, goats and sheep breeds are termed 'trypanotolerant', meaning they able to live in AAT endemic areas without the need for veterinary intervention(8). However, this reduced susceptibility to trypanosomiasis does not render the animals immune to infection and productivity of animals is still affected by chronic infections. Although the exploitation of trypanotolerance has been suggested as a strategy to tackle AAT, this strategy has been largely hindered by the logistical challenge of importation and the small proportion of animals displaying sufficient levels of resistance. Studies of the phylogenetic relationships between trypanosomes indicate that the salivarian trypanosomes evolved separately, forming a separate monophyletic clade of their own (9,10). Analysis of glycosomal glyceraldehyde phosphate dehydrogenase genes indicates that trypanosomes evolved from a single evolutionary origin with subsequent divergence(11). Bootstrap values obtained from analysis of salivarian trypanosome evolution indicate that the divergence of *T. vivax* occurred first, with subsequent separation of *T. brucei* and *T. congolense* (Figure 2)(12). All three species are placed within the '*T. brucei* clade' which is supported by all being mammalian parasites and undergoing

antigenic variation(11). Within *T. congolense,* Savanna and Forest subgroups are more closely associated with one another than Kilfii which is the most homogenous of the three(13). The *Nannomonas* subgenus consists of the *T. congolense* groups along with the distinct clade made up of *T. simiae* and *T. godfreyi*(13).

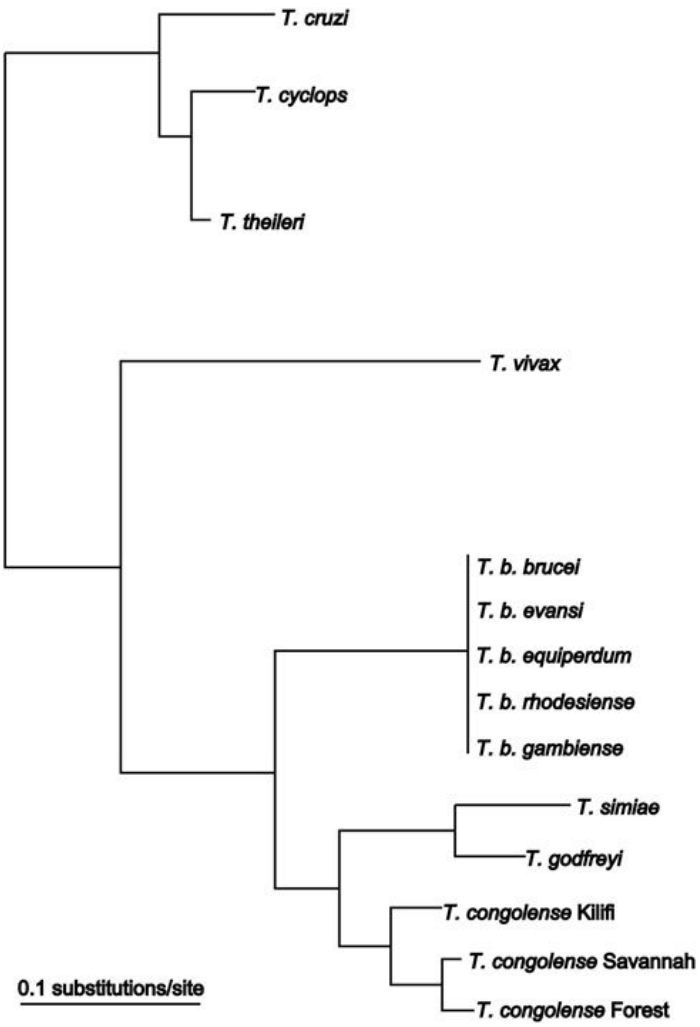

Figure 3 Phylogenetic tree of African trypanosomes based on small subunit rRNA sequences. Image taken from (10)

1.1.2.1.1 Trypanosoma brucei brucei

T. b. brucei belongs to the species complex *T. brucei s.l.* along with *T. b. gambiense* and *T. b. rhodesiense.* It is found across sub-Saharan Africa and although it does not infect humans it does cause disease in both wild and domestic animals. The pathogenicity of this sub-species appears to be dependent on genotype of the parasite amongst other factors (14). It is generally considered not to be a major contributor to the overall disease burden of AAT cases however *T. brucei brucei.* is by far the best described and most studied of the African trypanosome species. This is due to it being the most simple to study in mouse models (15,16) and the medical importance of the other two members of *T .brucei s.l.*: *T. b. gambiense* and *T. b. rhodesiense* (17). *T. b. brucei* is morphologically identical to the other two members of *T. brucei s.l.* and at present there is no diagnostic marker described for this species. As such, the only way to identify it is by a process of elimination. This is usually done by screening for the other two sub-species by detecting either of the diagnostic single-copy genes used to identify *T. b. rhodesiense* (18) or *T. b. gambiense* (19). A negative result for either gene is then used to conclude the presence of *T. b. brucei.*

1.1.2.1.2 Trypanosoma congolense

T. congolense is responsible for the majority of AAT cases. The species is made up of three sub-types which are genetically distinct: Savanna, Kilifi and Forest (20,21). Distribution of each type is dependent on the tsetse vector. The Savanna type is regarded as the main pathogen causing AAT in African cattle. It is transmitted by a number of tsetse species (*G. palpalis*, *G. morsitans and G. fusca*) and has the widest distribution across sub-Saharan Africa. The Forest group's transmission is mainly by the papalis group of tsetse resulting in its distribution being focused on riverine environments. *T. congolense* Kilifi is transmitted by the morsitans group of tsetse and is found across south-eastern Africa. Crossover in

distributions of the subtypes does occur, with co-infections with multiple *T. congolense* types widely reported(22–27).

1.1.2.1.3 Trypanosoma vivax

Trypanosoma vivax is the major pathogenic species for ruminants in Africa and South America. In South America, transmission of *T. vivax* is entirely reliant on the mechanical transmission by biting flies in the absence of tsetse. This method of transmission is enabled by the simpler, non-cyclical life cycle of *T. vivax* which lacks a procyclic stage. *T. vivax* are genetically distinct trypanosomes and belong to the subgenus *Dutonella*. The strain of *T. vivax* along with the host can result in variation in disease severity with west African *T. vivax* causing more severe disease in cattle than in East Africa where it appears to present with mild morbidity(28).

1.1.3 Human African trypanosomiasis

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a neglected tropical disease found in sub-Saharan Africa that is almost always fatal without treatment. The first recorded account of sleeping sickness was written by the English naval surgeon John Atkins in 1734. He described observing a "sleepy distemper" in local populations whilst working off the coast of Guinea. It was nearly 70 years before the second account of sleeping sickness was recorded by physician Thomas Winterbottom. He noted the characteristic swelling of the lymph glands on the neck (29), now termed Winterbottom's sign, along with the observation that Arabian slave-traders did not buy slaves with this symptom (30). Further observations of sleeping sickness-like disease were recorded throughout the 19th century however trypanosomes were not recorded in human patients until 1901 when British colonial surgeon Michael Forde found the parasites in the blood of a steamboat captain in The Gambia (31). They were initially thought to be worms but were later correctly identified as trypanosomes by Joseph Dutton in 1902 (32). Two forms of

HAT exist, each with their own distinct geographical distribution, vector, parasite and disease pathology.

Both diseases follow a two-stage pathology. The first stage, also known as the heamolymphatic stage occurs when the parasite is present in the blood. The second stage of the disease occurs once the parasites cross the blood-brain barrier and are found within the cerebrospinal fluid. This stage is identified by neurological symptoms such as changes in coordination, behaviour and sleep patterns. The disturbed sleep patterns and insomnia that patients with stage 2 HAT often present with is what gives the disease its name.

1.1.3.1 Gambiense HAT

Gambiense HAT (gHAT) is an anthroponosis found in Central and Western Africa and accounts for over 98% of all human trypanosomiasis cases (33). This form is caused by the *T. brucei* sub-species *Trypanosoma brucei gambiense*. People infected with *T. b. gambiense* can remain asymptomatic for months to years post-infection, with symptoms often presenting once the infection has significantly advanced and has reached the central nervous system. Although gHAT is largely a disease of humans, the role of animals in the transmission cycles is still widely debated(34). *T. b. gambiense* comprises two groups: Tbg1 and Tbg2 (20,35). Tbg1 accounts for the largest number of *T. b. gambiense* isolates and is resistant to trypanosome lytic factor (TNF) in human serum. Tbg2 displays more genetic heterogeneity but has only been found in Cote d'ivoire and Burkina Faso (36–38). Gambiense trypanosomes have also been found to sequester in the skin of vertebrate hosts where they remain viable and can contribute to onwards transmission (39). Pig hosts with undetectable levels of parasitemia have been shown to be infective to tsetse (40) suggesting that hosts with asymptomatic infections may act as reservoirs of disease and contribute towards transmission.

1.1.3.2 Rhodesiense HAT

Rhodesian human African trypanosomiasis (rHAT) is caused by *T. b. rhodesisense*, occurs in Southern and East Africa, This form has an acute disease pathology and can be fatal within a few months post infection without treatment. RHAT is a zoonosis with most cases occurring in wild and domestic animals with humans being the accidental hosts, therefore accounting for less than three percent of all HAT cases (41). As such, rHAT control is heavily focused on the identification and treatment of infected domestic animals. Differences in the pathology of different *T. b. rhodesiense* isolates has been noted when comparing cases from different rHAT foci (42). For example, cases reported in Uganda present as acute whilst those in the Zambian focus appear to be less virulent with reported asymptomatic cases (43–46).

1.1.4 Evasion of host immunity by trypanosomes

Both *T. b. rhodesiense* and *T. b. gambiense* are able to establish and persist in the human bloodstream despite the host's immunological response. This is achieved through the expression of specific resistance antigens which provide TNF resistance in the host immune system. Each sub-species has a specific antigen which is expressed. *T. b. rhodesiense* expresses a serum resistance gene (SRA) whilst *T. b. gambiense* developed a separate method of TNF resistance through the expression of a truncated variant surface glycoprotein (VSG) known as TgsGP (47).However, this gene is only present in *T. b. gambiense* group 1 (47,48). It is these resistance genes that are used as diagnostic markers in molecular screening tools for trypanosomiasis.

1.2 Trypanosome detection

1.2.1 Microscopy

Identification of trypanosomes through microscopic examination has been a long-standing method of screening vertebrate and invertebrate hosts. Microscopy suffers from poor and

variable sensitivity which is dependent on the skill of the microscopist, fluctuating parasitemia and is laborious to conduct. In two studies conducted within the DRC, less than 50% of microscopists in diagnostic laboratories correctly identified trypanosomes when presented with a thick and thin blood films (49,50). Infection is confirmed by the presence of trypanosomes which may be sufficient in human hosts which are commonly infected by only two species of African trypanosomes but is more complicated in tsetse and animal hosts which are infected by a number of pathogenic and non-pathogenic trypanosome species. Identification of these species is often impossible due to the morphological similarity and geographical overlap.

Historically, the distinct geographical distributions of *T. b. rhodesiense* and *T. b. gambiense* was relied upon when identifying trypanosomes to species in humans. However, with increased movement of humans and livestock along with the impact of global climate change these distributions may change (51). This would result in less reliant diagnosis if based solely on geography. This could lead to misdiagnosis in Uganda where rhodesiense and gambiense forms of the disease co-exist. Incorrect diagnosis is a risk due to the specificity of anti-trypanocidal drugs for each species and disease stage, potentially leading to inappropriate treatment.

The detection of parasites in blood samples can be done through the observation of motile trypanosomes in wet films or the staining of thin or thick films. The use of wet films is simplest however, it has poor sensitivity with a limit of detection up to 10,000 trypanosomes/mL. Thin films stained with either Giemsa or Field's stain have a similar poor sensitivity. The use of thick films can improve sensitivity somewhat and is the preferred technique when no centrifuge is available (52). To help further improve the sensitivity of microscopic examination, there are a number of methods available to concentrate the parasites in a sample namely the quantitative buffy coat, capillary tube

centrifugation and the mini-anion exchange centrifugation technique (53). The use of these methods improves the limit of detection of microscopy up to 50 parasites/mL but is limited to use in basic laboratories with electricity. Despite these limitations, most trypanosomiasis diagnoses are heavily reliant on microscopy due to its simplicity, lack of reliance on a cold chain and high specificity.

Microscopic identification of salivarian trypanosomes within tsetse often relies upon the tissue in which the parasites are seen. However, this method is limited when trypanosomes are identified within tissues such as the midgut. These tissues are involved in the development cycles of multiple species which have overlapping geographical distributions.

1.2.2 Serology

The detection of anti-trypanosomal antibodies in the blood, plasma or serum through the use of serological tools can be used as an indicator of ongoing or previous infection. The glycoproteins on the cell surface of trypanosomes (both variant and invariant surface glycoproteins) induce a strong immune response in the host. The host's specific IgG and IgM antibody response to the glycoproteins can be detected in the bloodstream and this can be targeted by serological testing (54). The specificity of these methods can be affected by the cross-reactivity between antibodies raised against other parasitic infections. A number of tests based on antibody detection have been designed for *T. b. gambiense* including rapid agglutination tests, rapid diagnostic tests and immunoassays based on immunoflouresence and enzyme-linked immunosorbent assay (ELISA). The use of each method is dependent on the environment in which they have been designed to be performed.

CATT

The Card-Agglutination Test for Trypanosomiasis (CATT) was developed in 1978 (55) and is a cheap, field-friendly diagnostic tool for gHAT which does not require a high level of

training to use. The CATT test detects the presence of antibodies against purified, freeze-dried and fixed trypanosomes expressing the variant surface glycoprotein LiTat 1.3. CATT sensitivity and specificity is high with reported values of 94% and 95% respectively(56). However, when disease prevalence is low, the positive predictive value of the test drops as a result of the increasing number of false positives for every true positive result.

	Diseased	**Non-diseased**
Test positive	True positive (TP)	False positive (FP)
Test negative	False negative (FN)	True negative (TN)

$$Sensitivity = \frac{TP}{(TP + FN)}$$

$$Specificity = \frac{TN}{(TN + FP)}$$

$$Positive\ predictive\ value = \frac{TP}{(TP + FP)}$$

$$Negative\ predictive\ value = \frac{TN}{(TN + FN)}$$

This is problematic when the CATT test is used in areas where disease prevalence of gHAT is below 5% (57–61). Additionally, false positives have been found from patients with other parasitic diseases such as malaria (62). As a result of this and the nature of available treatments, it is recommended that all CATT positive results must be followed up by parasitological examination prior to treatment (Figure 4), particularly in foci of low prevalence. The sensitivity of CATT also appears to be variable based on where it is utilized with lower sensitivities reported in West and Central Africa, potentially linked to the lack of the LiTat 1.3 gene in *T. b. gambiense* in foci such as Cameroon (63,64).

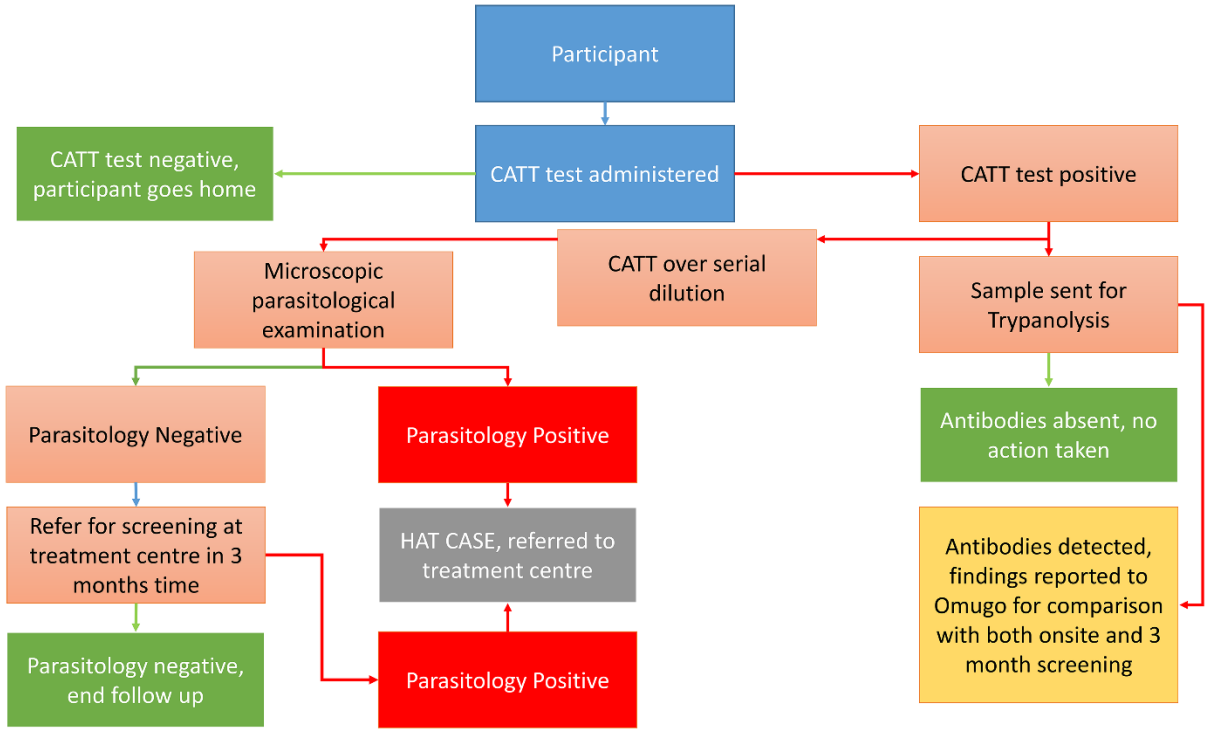

Figure 4 Gambiense HAT sampling decision tree, taken from (56)

ELISA

A laboratory alternative is the use of one of the many published trypanosome specific ELISAs (65–69). These immunoassays often utilise LiTat 1.3 or 1.5 VSG antigens which are coated onto the plate. Dilute samples are added to the plate with any anti-trypanosome antibodies binding to the antigen. A second antibody bound to a marker is added and a positive result seen by a change in colour. ELISA sensitivity and specificity is high with recorded performance of 95-100% and 97-100% respectively (65,66,69,70) . The requirement for trained personnel and a laboratory to carry out these assays has meant that the use of ELISA has largely been limited to laboratory based surveillance surveys (71).

Rapid diagnostic tests (RDTs)

The first lateral flow diagnostic test for gHAT was developed by the Foundation for Innovative New Diagnostics (FIND) and SD Bioline in 2012 and targets LiTat1.3 and LiTat1.5. Since 2012, a number of rapid diagnostic test (RDT) prototypes and variants based on these antigens have been developed (72,73), with the most recent recombinant-antigen based RDT reporting sensitivities of 98.4% in passive screening and 83.0% in active screening (74).

1.2.3 Xenodiagnosis

Most diagnostic methods for HAT are focused on the screening of human or animal hosts, often for the presence of parasites. The detection of parasites in vertebrate hosts can be challenging due to the fluctuating low level parasitaemia, particularly in *T. b. gambiense* infections. Those with low blood parasitaemia may be negative by parasitological testing, remain untreated for infection and may act as reservoir hosts for onward transmission. Xenodiagnosis involves the detection of parasites in tsetse following a bloodmeal from a suspected infected host after which they are dissected and inspected for parasites. Previous studies have utilized this method effectively to identify infected hosts carrying low level infections missed by parasitological diagnosis (75,76).

1.2.4 Molecular diagnosis

PCR

The use of polymerase chain reaction (PCR) to amplify trypanosome DNA has been widely described and utilized in human, animal and tsetse samples and therefore is used in both AAT and HAT settings. PCR amplifies short strands of DNA sequences which can be targeted using a pair of complementary oligonucleotide strands (known as primers) designed to target specific nucleotide sequences. The high sensitivity of PCR has made it a mainstay of molecular diagnostics for trypanosome detection in laboratory settings.

Additionally, different PCR assays can be used for differing diagnostic purposes. Primers can be highly specific or generic depending on the screening target, experimental design and the sample used. Species-specific primers for all the medically and veterinary important trypanosomes species have been described in the literature with the exception of *T. b. brucei* which continues to be diagnosed presumptively as a result of exclusion of the other *Trypanozoon* species.

LAMP

Loop-mediated isothermal amplification (LAMP) provides an alternative method of amplifying DNA under isothermal conditions and produces results which can be assessed visually through the production of turbidity or fluorescence. These advantages make it a more field friendly alternative to traditional PCR and is particularly suited to low-resource settings. Several LAMP assays have been described targeting the *Trypanozoon* group(77,78), *T. b. gambiense* (79) and *T. b. rhodesiense* (80). The sub-species-specific LAMP assays suffer lower sensitivity than *Trypanozoon* assays due to the reliance on single copy gene targets. Despite its utility in a field setting, LAMP is limited due to the inability to carry out high-throughput screening (81).

1.2.5 Diagnostic algorithms

To improve reliability and standardize diagnosis, a number of diagnostic algorithms have been created for gHAT to streamline and standardize mass screening protocols. Although algorithms differ, all approaches comprise mass screening, confirmatory and testing elements due to a lack of prophylactics (82). Conversely, serological screening tools are not available for rHAT. Parasitaemia in those infected tends to be high with rapid disease progression and so mass screening is not routinely employed.

1.3 Treatment

Treatment of HAT has been hampered in the past due to the toxicity of drugs used and the complexity of treatment plans. The type of treatment and drugs administered is dependent on the stage of the disease. As such, staging of disease progression has historically been the first port of call when deciding upon a treatment plan. Lumbar puncture and identification of parasites within the cerebrospinal fluid (CSF) determines whether the patient is in the first stage (haemolymphatic) or second stage (meningoencephalitic).

1.3.1 Melarsoprol

Melarsoprol is an arsenic derivative introduced as a treatment for HAT in 1949. This is a highly toxic compound causing acute encephalopathy in ~10% of patients, of which approximately half are fatal (77). As a consequence, its use is limited to the treatment of stage 2 rHAT.

1.3.2 Suramin

Suramin was developed in Germany in the 1920s and first used to treat HAT in 1922(83). It is effective at treating the first stage of both gHAT and rHAT and has additional use as a microfilaricide against *Brugia pahangi* and *Onchocerca* spp. Suramin is also used as a veterinary drug against *T. evansi* in camels, and *T. evansi* and *T. equiperdum* in equines (84). The treatment regime consists of multiple intravenous injections administered every 3-7 days for four weeks. Suramin is a slow acting trypanocide with parasites targeted within the blood and lymph nodes 12-36 hours post administration.

1.3.3 Pentamidine

For those in the first stage of *gambiense* HAT (no trypanosomes in the CSF along with ≤5 white blood cells [WBC] per µl. Pentamidine is recommended. Pentamidine is an antimicrobial agent also used in the treatment of other parasitic infections including

visceral and cutaneous leishmaniasis. Treatment involves daily deep intramuscular injections for a week and is generally well tolerated, however minor adverse reactions are common (85). Cure rates of patients with first stage HAT using pentamidine are high, with rates of 95% reported (86).

1.3.4 Eflornithine

Eflornithine was approved by the US Food and Drug Administration in 1990 for the treatment of gHAT. Eflornithine was mainly used in instances of melarsoprol failure of *T. b. gambiense* infections. A major drawback of eflornithine is the treatment protocol which is complex and is difficult to carry out in resource limited settings. Due to the drug's short half-life, treatment involves slow infusions at six hourly intervals for 14 days.

1.3.5 Nifurtimox-eflornithine therapy (NECT)

In 2009, the Nifurtimox-eflornithine combination therapy (NECT) was added to the WHO Essential Medicine LIST (EML). Nifurtimox was originally registered for the treatment of American Trypanosomiasis (Chagas disease) in the 1960s. As a result of two large scale clinical drug-combination trials, NECT was found to be effective at treating second stage gHAT in addition to being well tolerated. NECT is simpler to administer with half as many major adverse-related events reported when compared to eflornithine monotherapy (87). Treatment involves 14 intravenous infusions of eflornithine for one week and oral nifurtimox three times a day for ten days. Due to the large volumes of eflornithine and saline required for administrationthe requirement of patient hospitalization and nursing staff the logistics of NECT treatment are challenging(88). In 2013, NECT became the first line treatment for second stage gHAT(89).

1.3.6 Fexinidazole

Fexinidazole is the first oral treatment for HAT and in 2018 The European Medicine's Agency Committee for Medicinal Products for Human Use gave a positive opinion for its

use against stage 1 and stage 2 gHAT. The effectiveness against both stages removes the need for staging, and as a result, lumbar puncture is no longer needed. Fexinidazole is administered once daily for ten days.This consists of an initial four day loading dose followed by six days at a higher dosage. The ten day treatment duration poses challenges to treating remote and isolated communities, however the potential of fexinidazole to play a key role in eradication of HAT has resulted in the DRC adopting this treatment regime as standard(90).

1.3.7 Acoziborole

The most recent development within the world of HAT therapeutics comes in the emergence of acoziborole. The anti-trypanocidal activity of the antimicrobial agent was first reported in 2010 (91) and was then followed up by Drugs for Neglected Diseases Initiative. This drug is particularly promising as it has the potential to be administered orally, at the point of diagnosis.

1.4 Tsetse

1.4.1 Tsetse groups and their habitats

Tsetse or Glossinidae are dipteran flies which are vectors of African trypanosomes, transmitting the parasites between infected and uninfected hosts. Tsetse are found exclusively in sub-Saharan Africa apart from two recorded instances in the Arabian peninsula in 1903 and 1910 (92). Their distribution is bounded by the Sahara Desert to the north and the temperate regions of southern Africa in the south. This distribution also reflects the distribution of human and animal trypanosomiasis with the exception of AAT in South America. All tsetse are obligate blood feeders, with both sexes feeding on blood mainly from vertebrate hosts with some species feeding on reptiles. Tsetse are made up of 30 different species, of which six transmit the majority of African trypanosomiasis: *Glossina morsitans morsitans, G. m. centralis, G. pallidipes, G. palpalis*

palpalis, G. fuscipes fuscipes, and *G. tachinoides* (81,82). The 'tsetse belt' of Africa covers an area of 10 million km^2 however the habitats in which flies exist within this belt are highly specific. These habitats generally segregate the tsetse species into the three groups of tsetse: savannah, forest and riverine. The Savanna group (*Morsitans*) is found in the grass lands and savannah woodlands of West, East and Southern Africa(95). The Forest group (*Fusca*) is associated with a range of forests spanning Western Africa whilst the riverine group (*Palpalis*) is distributed along the river and lake systems of Central and West Africa (Figure 5). The distribution of each tsetse group reflects the type of HAT that they transmit with gambiense HAT transmitted by the *Palpalis* group and the *Morsitans* group acting as vectors of rhodesiense HAT.

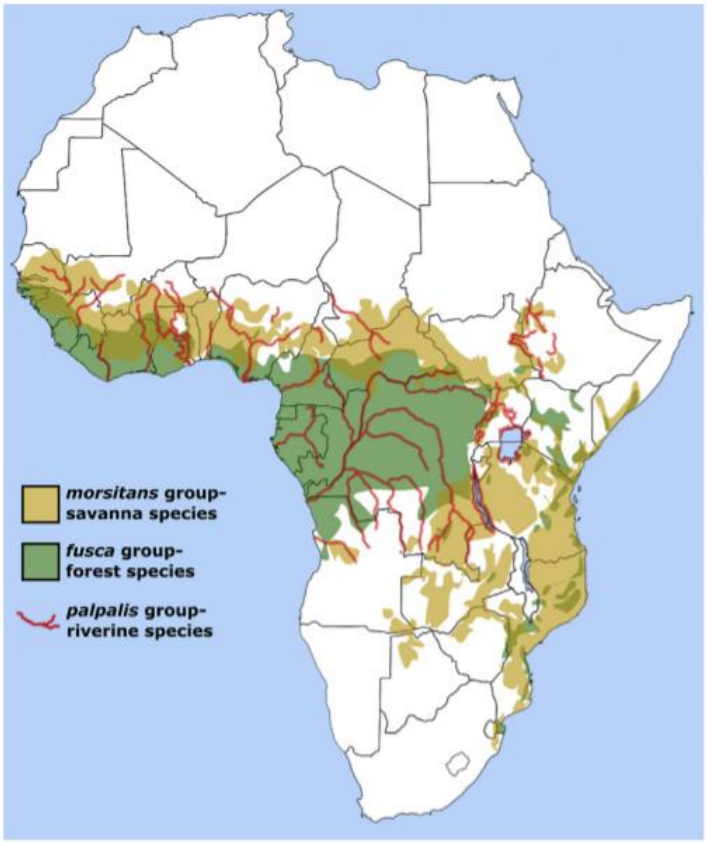

Figure 5 Distribution of the three tsetse groups in Africa: fusca, morsitans and palpalis. Image from (90)

1.4.2 Tsetse biology

Tsetse are unusual amongst insects in that they reproduce by adenotrophic viviparity. This form of reproduction is only found within the superfamily Hippoboscoidea and plays a key role in the success of tsetse control. Female tsetse mate when they are approximately five days old, producing a single egg which develops into a larva within her uterus. The larva is reliant on the mother during this stage to provide all nutrition for development via milk glands. After nine days she gives birth to a L3 larva which burrows into the loose soil onto which it has been deposited. Underground, the larva pupates and develops into an adult fly (Figure 6).

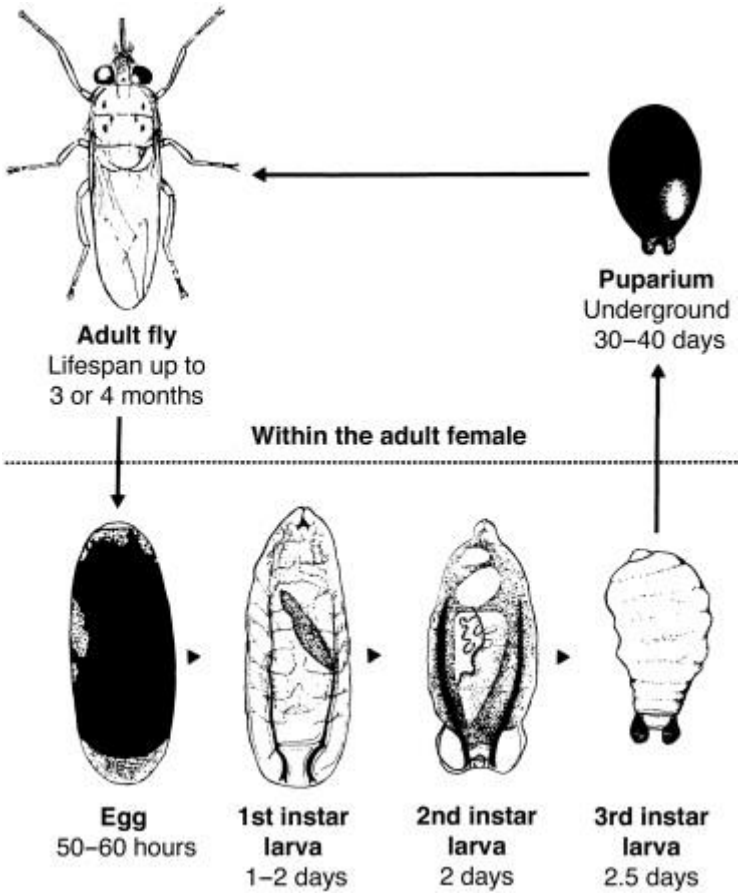

Figure 6 Life cycle of the tsetse fly. Image taken from (91)

The speed of development is temperature dependent but takes, on average, 30 days at 24°C (96). Female tsetse are fully fertile upon emergence from the soil whilst males reach fertility at three days post emergence (97). Over the course of her lifetime a female tsetse is likely to produce four offspring (96). The low reproduction rate of tsetse means that with a modest daily mortality rate of 4% an entire population can be eliminated (98). The successful development of trypanosomes within the tsetse involves a series of complex interactions between both parasite and host. As tsetse age, they appear to become more refractory to infection by *T. brucei* and *T. congolense* (99,100), with susceptibility to parasites being highest at the first bloodmeal (101–104).

Due to tsetse's reliance on blood for their nutritional needs, effective locating of sources of bloodmeals is crucial. Tsetse locate their hosts through a combination of visual and olfactory cues depending on the environment in which they inhabit. It is this that is exploited in tsetse trapping and control with the type and density of targets and traps dependent on the tsetse group to be controlled.

1.4.3 Tsetse control

Bush clearing

The exploitation of tsetse's sensitivity to habitat fragmentation was one of the first methods of HAT vector control employed in Africa(105). Clearance of all woody vegetation around areas of man-fly contact for riverine tsetse and the elimination of shrubs and trees for Savanna flies was utilized in regions of Nigeria, Ghana, Uganda and Sudan(106–109). Although this method can be effective at dramatically reducing tsetse numbers the control is threatened by reinvasion from neighboring areas and the practice is no longer considered acceptable due to its large ecological impact. Although no longer used, habitat destruction and its impact on tsetse numbers remains a common side effect of farming and the building of larger settlements in historically tsetse dense areas.

Host destruction

In 1865 Charles and David Livingstone stated that 'the destruction of all game by the advance of civilization is the only chance of getting rid of tsetse'. This viewpoint gained traction and resulted in eradication campaigns largely reliant on hunting including the successful eradication campaign on Principe island, historic Southern Rhodesia (now Zimbabwe) (110), Uganda (111), South Africa (112), Zambia(113) and Mozambique (114) amongst others. Despite being widely applied in Africa, the strategy was largely ineffective at eradicating tsetse with domestic animals often replacing wild hosts as a bloodmeal source. Furthermore, as the understanding of the importance of biodiversity grew, this

method was seen as no longer acceptable and the advent of insecticides largely ended host destruction as a control strategy.

Ground and aerial spraying

The spraying of tsetse resting sites with insecticides can be deployed in two forms: ground and aerial spraying. Ground spraying is the targeted application of persistent organochlorides such as DDT to trees and bushes using knapsack pressurized sprayers. The persistence of the insecticides used results in tsetse control for several months following application. This method has been utilized in larger control operations through the use of mist blowers and foggers to apply insecticides (96,115). Ground spraying was effectively used to control ~10 000 km^2 in Zimbabwe until 1991(116), at which point the ecological impacts of this method became widely publicized (117,118). The residual activity of the insecticides used is both the key to its success and the main drawback of this method. Knock-on effects on non-target organisms and bioaccumulation within the food chain along with the logistical challenges of deploying this technique mean it is no longer widely utilized as a control strategy.

Aerial spraying was developed in the 1970s and involved the deposition of insecticides in the form of a mist from planes. It can be utilized to target large areas of challenging terrain (96). In the 1940s, this strategy was used in Kwazulu-Natal, South Africa and successfully eliminated *Glossina pallidipes* Austen (112). Tsetse control in the Okavango Delta of Botswana has also utilized aerial spraying with 8000km^2 sprayed with deltamethrin in 2001. This strategy only controls adult tsetse and as a result multiple rounds are required to continue control of emerging tsetse from the soil. Insecticide spraying only controls tsetse in the specific area being targeted and so reinvasion of flies from neighboring areas which border the control area challenge the effectiveness of this technique.

Insecticide treated cattle

Applying insecticides to livestock results in 'live baits' which attract host-seeking tsetse while they attempt to blood feed (119). To reduce costs, application of the insecticide can be focused on the legs and belly of animals where flies are most likely to land. When flies do land to take a bloodmeal they are exposed to the lethal insecticide applied to the livestock's hair. This method is particularly useful in areas in which livestock are present in high densities and numbers and can be further fortified with the use of insecticide treated targets (120). Furthermore, additional impact of this method on other pests of livestock such as ticks helps reduce the prevalence of other vector-borne diseases such as East Coast fever (121).

Sterile Insect Technique

Sterile insect technique (SIT) involves the rearing and release of large numbers of irradiated sterile males into an already suppressed and ideally isolated population. The released males outnumber the natural male population and mate with virgin female flies. As a female tsetse mates only once, she will not produce any offspring for the remainder of her life. SIT has been used successfully to eliminate *G. austeni* from Unguja Island, Zanzibar in the 1990s (122), however, its extreme costs of ~$758 per km (123) along with the requirement for repeated releases make SIT an infeasible option for most trypanosomiasis endemic countries.

Traps and targets

The control of tsetse through the use of traps was first carried out in 1906 in Sao Tome and Principe through the use of sticky black overalls which were worn by workers along with vegetation clearing and elimination of pig hosts (124). Following the observation that tsetse respond to visual cues, Harris designed the first 'modern' tsetse trap in 1930 to control *G. pallidipes* in Zululand, South Africa. Iterations of this trap were designed and

then deployed across Africa (125–128). The performance of these traps in controlling tsetse was variable depending on the tsetse species to be controlled (126) and they were largely used for sampling rather than control(129,130).

Trap design has largely been built upon the observation that *Glossina* are heavily reliant on the visual cues such as shapes, colours and movement for host location. The importance of visual stimuli on host recognition was key to the design of biconical trap designed by Challier and Laveissiere in the 1970s (131,132). It was from this original trap design that most modern tsetse traps are based, including the Vavoua and pyramidal traps (133,134). The ability of tsetse to differentiate between colours and their specific attraction to a shade of blue known as 'phthalogen blue' was an important discovery in the optimization of tsetse traps and targets (135,136). The combination of blue and black panels was highly effective at attracting flies and induced a landing response.

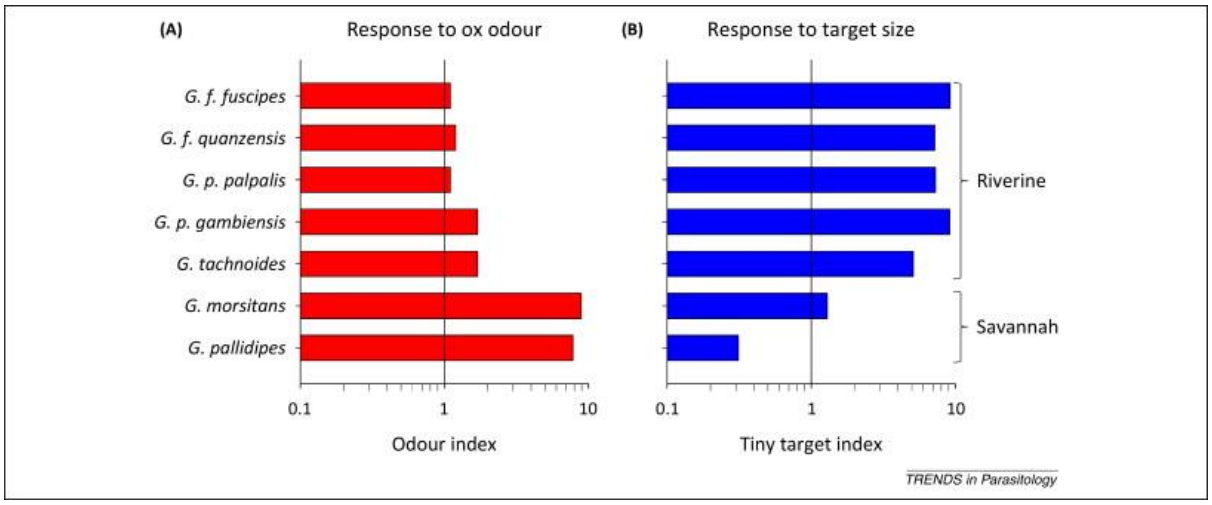

Figure 7 Responses of riverine and savannah tsetse to ox odour (A) and visual targets (B). Image taken from (138)

In the 1970s, studies conducted in Zimbabwe using a combination of electrocuting devices along with stationary hosts indicated that the addition of host odours dramatically increased the number of Savanna tsetse attracted to baits(105). Vale *et al* conducted a

defining set of experiments which demonstrated the variable attractiveness of odours from a range of host animals(137) and as a result of these studies, octenol and acetone were isolated as highly attractive odours for tsetse(139–141). A range of attractants developed to bait traps and targets have since been created for a number of tsetse species(142). In the 1980s, control of Savanna tsetse was concentrated on the use of odour enhanced targets which act as artificial baits. Riverine flies do not rely on odour cue to the extent that savannah tsetse do (Figure 7) and so their control is centered solely around the use of visual stimuli (143).

The effectiveness of visual cue size also differs between riverine and savannah flies. Studies investigating the impact of reducing target sizes showed that by reducing overall surface area of targets by $1/16^{th}$ reduced catches of savannah species by ~97% whilst riverine fly catches only dropped by 50%. This resulted in the development of 'tiny-targets' which have since become the mainstay of riverine tsetse control whilst Savannah species control continues using large targets (Figure 8) (144,145). Additionally, shape and size also impacts attractiveness, with larger, horizontal rectangles attracting more tsetse than smaller vertical ones (137,146).

Figure 8 Targets used for the control of riverine and savannah tsetse. (A) A 'Tiny target' 25×25 cm in size consisting of blue and black netting panels impregnated with insecticide. (B) Odor baited horizontal-oblong target (1.8 x 1m) made up of two blue panels with a central impregnated black panel. Image taken from (138)

1.5 Thesis objectives

The WHO targeted elimination of gHAT as a public health problem by 2020 (fewer than 2,000 cases reported per year). This goal was achieved in 2017 and sustained in the years since. In 2020, 663 cases of HAT were reported globally, with gambiense HAT accounting for 565 of these(147). To date, Togo and Côte d'Ivoire have received officially validated elimination with three countries having submitted their dossier of elimination for validation and a further three in the process of preparing their dossier. In 2020, the WHO released a roadmap outlining elimination targets for both forms of human African trypanosomiasis. Rhodesiense HAT targets were set at eight countries achieving elimination as a public health problem by 2030. Due to the anthroponotic nature of gambiense HAT along with the progress made to date the elimination (interruption of

transmission) of gHAT was scheduled for 2030 (147). With fewer cases reported for HAT and the upcoming elimination targets it is both increasingly important and challenging to detect and report all remaining cases and areas of transmission. As efforts to screen at-risk populations are bolstered (147) in addition to fewer positive cases, the costs per positive human or fly detected will increase along with the amount of time and effort required. As elimination is approached and case numbers decline, the reliability of diagnostic tools such as RDTs will drop with decreasing positive predictive values and the rate of false positive rates of tests outnumbering that of true positives (148). Reliance on screen and treat strategies will no longer be viable following elimination and there is a need for new methodologies that are sustainable in the long-term.

Tsetse control has played a key role in the achievement of elimination targets but to date much of the surveillance effort has been focused on the human population. Trapping and screening tsetse fly vectors has the potential to be significantly simpler and cheaper than screening humans and so has the potential to play an important role in future monitoring and control strategies. This thesis examines the feasibility of using xenomonitoring as a tool for HAT control through the development of novel molecular techniques which enable the rapid screening of tsetse for human and animal infective trypanosomes in a multiplex format (81). To further enable high-throughput screening, I evaluate and adapt a low-cost rapid DNA extraction method for tsetse extraction. Finally, I examine the feasibility of a xenomonitoring tool following the cessation of vector control. I do this through the analysis of tsetse population dynamics in a gHAT focus in Uganda where tsetse control is currently being withdrawn. Using these data, I also aim to assess the risk of HAT resurgence and the implications for tsetse control in the future.

Overall objective

The overall aim of the thesis is to develop and test the utility of a high throughput xenomonitoring system to detect and quantify the levels of transmission of the pathogens causing HAT. Such a system could enable national programs develop systems to assess whether local transmission of HAT has been eliminated.

Research questions and hypotheses

To achieve this overall objective I:-

1. **Development of a qPCR-based assay for the detection of human African trypanosomes**

Developed and assessed whether a novel HRM qPCR can be used to detect and distinguish the subspecies of *Trypanosoma brucei* causing gHAT and rHAT. The hypothesis was that a qPCR can be developed which, in comparison to standard PCR methods, provides a more efficient and rapid means for screening wild-caught tsetse for the subspecies of *T. brucei* but with similar or better sensitivity and specificity than current methods.

2. **Development of a qPCR -based assay for the detection of animal African trypanosomes**

To accompany (1), I also developed and assessed a novel HRM qPCR to detect and distinguish the species of *Trypanosoma* causing HAT (*T. brucei*) and AAT (*T. congolense, T. vivax*). The hypothesis was that a qPCR can be developed which, in comparison to standard PCR methods, provide a more efficient and rapid means for screening wild-caught tsetse for the three species of *Trypanosoma* causing HAT (*T. brucei*) or AAT (*T. vivax, T. congolense*). Combining the qPCR methods for *T. brucei* and *Trypanosoma* would provide a two-stage system for rapidly and reliably screening tsetse for three species and two subspecies of *Trypanosoma*.

3. **Examination of a low-cost magnetic bead method to extract trypanosome DNA from tsetse as an alternative to column-based extraction methods**

To improve the speed, practicability and cost-effectiveness of extracting DNA, I optimized and adapted a novel method using a magnetic bead extraction method and tested the hypothesis that this method is cheaper, easier and quicker than the standard column-based method whilst being no less effective.

4. **Evaluate the impact of withdrawing vector control on a tsetse population in a historic HAT focus in northwest Uganda**

Evaluated the impact of the scale-back of tsetse control in the Maracha district of northern Uganda, an area where cases of gHAT occurred prior to 2010 but screening of the human population and over 5 years of tsetse control, using Tiny Targets, has apparently eliminated transmission. I tested three linked hypotheses associated with the scale back of tsetse control. First, I tested whether there was significant increase in the catches of tsetse from monitoring traps. Second, I examined whether there was a significant change in age structure as resulting in an apparent rise in the average age of the tsetse population. Finally, I used a combination of classical (dissection) and PCR-based methods to assess whether there was significant increase in the proportion of tsetse infected with *Trypanosoma* species

2 A pilot study demonstrating the identification of *Trypanosoma brucei gambiense* and *T. b. rhodesiense* in vectors using a multiplexed high-resolution melt qPCR.

2.1 Acknowledgement of work done:

Gala Garrod, Emily R. Adams, Stephen J. Torr and Lucas J. Cunningham were responsible for study conception and study design. Jessica K. Lingley, Isabel Saldanha and Gala Garrod carried out DNA extraction and PCR assays presented in this paper. Gala Garrod was responsible for qPCR assay design, sample screening, data analysis, manuscript writing and publication. Emily R. Adams, Stephen J. Torr and Lucas J. Cunningham were responsible for study supervision.

2.2 Introduction

Recent global efforts to control human African trypanosomiasis (HAT) have successfully reduced disease transmission. Between 2012 and 2020, cases of HAT dropped from over 7000 to 565 (149). Disease incidence is now far more focal, with the Democratic Republic of Congo accounting for 84% of all cases reported in 2016 (150). The WHO targeted the elimination of HAT as a public health problem (defined as <1 case per 10,000 inhabitants per year, averaged over five years) by 2020 (Malawi Ministry of Health, unpublished data). In 2020, Togo and Côte d'Ivoire were validated (defined as the process of documenting elimination as a public health problem (151)) by the WHO as the first two countries to reach this milestone (152). With the success of control efforts to date, the WHO has targeted the interruption of transmission of gHAT for 2030. Due to the role of animal reservoir hosts in rHAT transmission, elimination targets are more conservative for this form of the disease. The WHO has targeted eight rHAT endemic countries to eliminate the disease as a public health by 2030 (153). Although rHAT makes up less than 5% of all HAT cases (41), there have been outbreaks in countries such as Malawi, which recorded an increase in case numbers from an average of ~30 cases per year to 90 in 2019 (154). Of the recorded cases, 91% were reported to be in the second stage of disease (155). These data and elimination targets highlight the need for rapid and real-time surveillance tools to detect increases in transmission and validate elimination. Detection of transmission could in turn be used to guide active screening and potentially catch cases before they progress to the heamolymphatic stage of disease.

The WHO roadmap for neglected tropical diseases mentions the need for a "high-throughput test to assess elimination and post-elimination surveillance on samples in a reference laboratory". At present, all emphasis is on the detection of parasites in humans. This usually involves the active screening of the vertebrate host, which is costly and labour

intensive, issues that only become even more apparent when disease incidence is very low. Even with a passive screening strategy, the reliance on rapid diagnostic tests as screening tools is problematic. With low case numbers, the specificity of these tests drops, resulting in a higher incidence of false positives (156). Due to the risk of side effects due to treatment, every positive rapid test result is referred to the nearest facility able to conduct parasitological confirmation (157). Patients that arepositive by rapid test but negative for parasites are retested every three months with rapid tests until a negative serological result is achieved. Any further positive rapid test results must be followed up once more for parasitological testing. This procedure is both logistically and economically challenging and risks loss to follow up of potentially positive individuals. Furthermore, no rapid tests are currently available for the screening of rHAT.

Screening the vector for parasites, known as xenomonitoring, has been utilized effectively in the Lymphatic Filariasis elimination program and is listed as a integral component of the WHO's 'Guidelines for Stopping Mass Drug Administration and Verifying Elimination of Human Onchocerciasis' (158). The addition of xenomonitoring to complement the screening from the human population could reduce costs and produce transmission data in real time, particularly as gHAT can by asymptomatic for months or even years in the human host. This real-time data could be of particular importance in the lead up to elimination, monitoring for potential recrudescence and screening for hitherto unrecognised foci of gHAT. Current molecular tools for the detection of human pathogenic sub-species of *T. brucei s. l.* rely on PCR and gel electrophoresis. At present, there are no qPCR-based tools described for the detection of *T. brucei gambiense* and *T. b. rhodesiense*. A closed-tube PCR produces a clear and easy to interpret read-out, reduces risks of contamination and has the additional potential for quantification of the pathogen of interest. In the manuscript presented in this chapter, I describe the design of a qPCR-based

screening tool which detects tsetse for the presence of human pathogenic trypanosomes. Furthermore, the assay allows for the identification of samples with sufficient genomic material for the detection of single-copy genes.

2.3 Chapter 2 summary

A multiplexed qPCR based high resolution melt assay was designed based on previously described gene targets for *T. brucei rhodesiense* and *T. b. gambiense.* A further primer set was designed and included which indicates if samples have sufficient genetic material for the successful detection of the sub-species-specific gene targets. The assay's sensitivity was found to be at least as sensitive as previously designed and widely used PCR assays along with 100% specificity when challenged with non-target trypanosome DNA. Assay performance was evaluated on 96 tsetse caught from a rHAT focus in Tanzania. The assay successfully identified three flies to be infected with *T. b. rhodesiense* with no false positives recorded. Evaluation of this assay's performance in a *T. b. gambiense* focus is needed to evaluate its use in gHAT xenomonitoring.

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

A pilot study demonstrating the identification of *Trypanosoma brucei gambiense* and *T. b. rhodesiense* in vectors using a multiplexed high-resolution melt qPCR

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Abstract

Human African Trypanosomiasis (HAT) is a potentially fatal parasitic infection caused by the trypanosome sub-species *Trypanosoma brucei gambiense* and *T. b. rhodesiense* transmitted by tsetse flies. Currently, global HAT case numbers are reaching less than 1 case per 10,000 people in many disease foci. As such, there is a need for simple screening tools and strategies to replace active screening of the human population which can be maintained post-elimination for Gambian HAT and long-term for Rhodesian HAT. Here, we describe the proof of principle application of a novel high-resolution melt assay for the xenomonitoring of *Trypanosoma brucei gambiense* and *T. b. rhodesiense* in tsetse. Both novel and previously described primers which target species-specific single copy genes were used as part of a multiplex qPCR. An additional primer set was included in the multiplex to determine if samples had sufficient genomic material for detecting genes present in low copy number. The assay was evaluated on 96 wild-caught tsetse previously identified to be positive for *T. brucei s. l.* of which two were known to be positive for *T. b. rhodesiense*. The assay was found to be highly specific with no cross-reactivity with non-target trypanosome species and the assay limit of detection was 10^4 tryps/mL. The qPCR successfully identified three *T. b. rhodesiense* positive flies, in agreement with the reference species-specific PCRs. This assay provides an alternative to running multiple PCRs when screening for pathogenic sub-species of *T. brucei s. l.* and produces results in less than 2 hours, avoiding gel electrophoresis and subjective analysis. This method could provide a component of a simple and efficient method of screening large numbers of tsetse flies in known HAT foci or in areas at risk of recrudescence or threatened by the changing distribution of both forms of HAT.

Author summary

With global cases of Human African Trypanosomiasis (HAT) reaching pre-elimination numbers, identifying areas of trypanosome transmission becomes increasingly important.

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With decreasing numbers of cases, the cost of identifying each positive case increases. Screening for trypanosomes in tsetse flies instead of in the human host allows for a faster, more cost-effective and efficient method of detecting areas in which parasite transmission is on-going. Molecular tools such as PCR play a critical role in molecular identification of parasites in vectors. However, at present, multiple PCRs are required to confirm the presence or absence of human infective trypanosomes. Here we present a novel assay which allows for the screening of both species of trypanosomes responsible for HAT in one assay. This method eliminates the need for multiple assays per sample, produces results which are easier to interpret, and reduces the risk of contamination of samples. We found the assay to be as sensitive as the current gold-standard PCR when evaluated on a subset of wild tsetse flies. With further field evaluation, this method could provide an alternative monitoring method for HAT, which can be used leading up to elimination and maintained once elimination has been achieved.

Introduction

Human African Trypanosomiasis (HAT) is a potentially fatal disease caused by subspecies of *Trypanosoma brucei s. l.* transmitted by the bite of an infected tsetse fly (*Glossina* spp). HAT consists of two forms of the disease, each with its own distinct parasite, vectors, disease pathology, treatment and geographical distribution. Gambian HAT (gHAT), caused by *Trypanosoma brucei gambiense*, is a largely anthroponotic disease found across central and west Africa and accounts for the large majority of HAT cases (>97%) [1]. Gambian HAT can remain asymptomatic for months to years with symptoms often presenting once the infection has significantly advanced. Conversely, Rhodesian HAT (rHAT), caused by *Trypanosoma brucei rhodesiense*, is a zoonosis with occasional human infection, and represents less than 3% of all HAT cases. The World Health Organisation (WHO) has targeted the elimination of HAT as a public health problem by 2020, defined as less than 1 new case per 10, 000 inhabitants in at least 90% of endemic foci and fewer than 2000 cases reported globally [2]. Due to the zoonotic nature of rHAT, this WHO target is applicable to gHAT only. With gHAT on the brink of elimination and rHAT persisting in several foci across East and Southern Africa [3], it is crucial to identify any remaining active cases, foci of transmission and areas of resurgence. The epidemiology of the two forms of HAT differ greatly and therefore monitoring and screening strategies also differ. Monitoring gHAT is largely reliant on the screening of the at-risk human population and treatment of cases [4]. Accurate estimates of disease prevalence require high rates of coverage, which can be difficult to achieve, particularly in areas affected by conflict and political instability [5] and where prevalence approaches <1 case per 10,000. As a result, there has been emphasis on the development of rapid diagnostic tests (RDTs) and field-friendly screening tools [6–10]. In comparison to gHAT, there has been little progress or investment into the development of a screening tool for rHAT with current emphasis on passive case detection and control of the vector population. A reliance on passive detection results in a delay in the identification and treatment of infected individuals, both of which are crucial for the control of disease transmission.

With declining numbers of cases, active screening programmes are no longer cost-effective [11] and there is a need for a monitoring tool which can be maintained sustainably for rHAT and post-elimination for gHAT. Xenomonitoring, the screening of vectors for the presence of parasites, provides a potential alternative to host sampling. This method has already been successfully utilised as a surveillance tool within the Lymphatic Filariasis elimination programme

[12–14]. Vectors are often routinely collected as part of vector control programmes and as such, making use of this material may prove to be more economical than active screening of either human or animal populations. Additionally, screening vectors for infection is often less time-consuming and with efficient processing, can provide a view of disease transmission in real-time. Microscopy has been traditionally used for vector screening due to its low cost, high specificity and ease of use in-field. However, the sensitivity of microscopy is variable, it is labour intensive and morphological identification of *T. brucei gambiense* and *T. b. rhodesiense* trypanosomes is not possible [15,16].

The development of molecular tools has provided alternatives to traditional screening methods. PCR is widely used for the detection of trypanosome DNA, with highly sensitive assays developed for *T. brucei s. l.* [17] which includes *T. b. gambiense* and *T. b. rhodesiense* along with the animal trypanosome *T. b. brucei*. Successful amplification indicates the presence of one of the members of *T. brucei* s. *l.* but does not identify which member of *T. brucei s. l.* is present. Identification of *T. b. gambiense* and *T. b. rhodesiense* is reliant on the detection of specific single-copy genes (TgsGP [18] and SRA [19,20] respectively) for each subspecies. For detection of these low copy genes, the presence of sufficient genetic material is crucial. A negative result may indicate the absence of the target species or simply that insufficient DNA is present. To differentiate between these two scenarios, primers have been designed to screen for other single-copy genes [21], namely the single-copy phospholipase-C (PLC) gene, present in all members of *T. brucei s. l.* [22–25]. Samples found to have sufficient DNA can then be screened using primers specific for *T. brucei s. l.* subspecies.

High-resolution melt analysis (HRM) is a post-qPCR analysis method which can be used to detect heterogeneity within nucleotide sequences and has previously been used for the detection of other parasite species [26–28]. A fluorescent dye is added to the PCR reaction which intercalates into double stranded DNA. Following amplification, the amplicon is heated gradually causing the strands to separate. Separation of the double strands releases the incorporated dye causing a drop in fluorescence. The rate of DNA strand disassociation and the temperature at which it separates (Tm) is dependent on the amplicon's nucleotide sequence. Different sequences will have different melting temperatures which can then be used as a diagnostic identifier. HRM is a closed tube process resulting in a reduced risk of contamination and produces results in approximately two hours circumventing gel electrophoresis, making it a faster, more specific alternative to traditional PCR. Multiplexing allows for the screening of a number of targets simultaneously, making sample processing more efficient.

Objectives

We aim to develop a qPCR HRM assay designed to distinguish between the two human infective sub-species of *T. brucei s. l.* To improve the reliability of the assay primers were included to determine if sufficient DNA is present for the sub-species primers to work. To meet this objective we first designed and identified suitable primers that are compatible with the HRM qPCR, followed by assay optimisation and evaluation of sensitivity and specificity. Finally, the assay was compared to the current gold standard PCR using field samples available from other studies (S1 Fig).

Methods

Ethics statement

Ethical approval for this work was obtained from the Commission for Science and Technology (Costech) in Tanzania (permit number 2016-33-NA-2014-233). The samples from Tanzania were collected under the auspices of a BBSRC funded project ("Life on the edge: tackling

human African trypanosomiasis on the edge of wilderness areas", BB/L019035/1; COSTECH research permit number 2014-280-NA-2014-223).

Primers

In order to be compatible with a HRM qPCR assay, primers that produced an amplicon of 150–350 base pairs with distinct melt temperatures were sought. Based on the literature, novel *T. b. rhodesiense* primers were derived from the sequence of a sub-species-specific serum-resistance-associated (SRA) protein gene (accession number AF097331.1). The primers for *T. b. gambiense*, previously designed and published by Radwanska *et al.* [29] were found to be compatible with the HRM qPCR method. These primers target the sub-species-specific glycoprotein (TgsGP: accession number AJ277951). The third primer set was designed to identify the presence of sufficient genetic material. These primers amplify a single-copy phospholipase-C (PLC) gene found in all members of the Trypanozoon group [22–25].

Assay optimisation

To optimise the new HRM assay, the specificity of the multiplexed assay was evaluated using DNA from a range of non-target trypanosome species: *Trypanosoma congolense* Savannah (Gam2), *T. congolense* Forest (ANR3), *T. congolense* Kilifi (WG84), *T. simiae* (TV008), *T. godfreyi* (Ken7), *T. vivax* (Y486) and *T. grayi* (ANR4). Additionally, cross-reactivity of *T. b. gambiense* and *T. b. rhodesiense* primers against non-target *T. brucei s. l.* sub-species (i. e. *T. b. rhodesiense*, *T. b. brucei* and *T. b. gambiense*) DNA was tested. Each species' DNA was run in triplicate along with negative water controls and target species positive controls.

The analytical sensitivity of the assay was assessed using a tenfold dilution series of *T. b. gambiense* and *T. b. rhodesiense* DNA obtained from culture which ranged from an estimated 10^6 to 10^1 tryps/mL, with each concentration run in duplicate. Quantification of DNA in terms of trypanosomes was calculated using concentrations of DNA as read by a Qubit 2.0 fluorometer (Invitrogen) and an assumed approximate DNA quantity of 0.1pg per trypanosome [30]. TgsGP and SRA PCR were run alongside on the same dilution series for a direct comparison of sensitivities [21,29].

Screening of field samples

A subsample of wild-caught tsetse were selected to validate the HRM performance on field samples. The flies had been captured in 2015–2016 in Tanzania as part of the study reported by Lord *et al* [31]. Full methods are described in Lord *et al* [31], but in brief, a total of 5,986 tsetse were trapped using odour-baited Nzi traps deployed at sites in Grumeti and Ikorongo wildlife reserve, and Serengeti National Park. Captured flies were stored individually in 100% ethanol at room temperature and brought to LSTM for analysis. DNA extraction was carried out using Genejet DNA purification kit (Thermo K0721) according to the manufacturer's instructions. Flies were screened for *T. brucei s. l.* and *T. congolense* Savannah using TBR [17] and TCS PCR [32] respectively. Both primer sets target satellite repeat sequences specific to each species. Flies positive for *T. brucei s. l.* were then screened for *T. b. rhodesiense* using SRA PCR.

PCR

A subsample of 96 flies positive by TBR PCR were used in this study, of which two had been previously found to be positive for *T. b. rhodesiense* [31]. The species composition of the tsetse was 69.8% *Glossina pallidipes* (67/96) and 30.2% *G. swynnertoni* (29/96). Of the 96 *T. brucei s.*

l. positive flies, 11.5% (11/96) were also found to be positive for *T. congolense* Savannah. Flies were re-screened using TBR [17] and SRA PCR [21] to reconfirm the presence and integrity of parasite DNA. TBR PCR conditions were as follows: initial denaturation at 95˚C for 10 minutes, 40 cycles of 95˚C for 30 seconds, 63˚C for 90 seconds and 72˚C for 70 seconds followed by an extension step at 72˚C for 10 minutes. SRA PCR conditions were: initial denaturation at 95˚C for 10 minutes, 40 cycles of 94˚C for 30 seconds, 63˚C for 90 seconds and 72˚C for 70 seconds followed by an extension step at 72˚C for 10 minutes. Additionally, flies were screened for *T. b. gambiense* using TgsGP PCR [29] using the following conditions: denaturation at 95˚C for 10 minutes, 40 cycles of 95˚C for 30 seconds, 63˚C for 90 seconds and 72˚C for 70 seconds followed a 10 minute extension at 72˚C.

HRM qPCR

All 96 flies were screened using the novel multiplexed HRM qPCR. HRM reactions were run in a total volume of 12.5μl consisting of 2.5μl DNA template, 6.25 μl HRM Master Mix (Thermo-start ABgene, Rochester, New York, USA), 3.25 μl sterile DNase/RNase free water (Sigma, ST. Louis, USA) and 400nM of all forward and reverse primers were included in a multiplex. Reactions were carried out on a Rotor-Gene 6000 real-time PCR machine (Qiagen RGQ system). The following protocol was followed: denaturation at 95˚C for 5 minutes followed by 40 cycles and denaturation for 10 seconds at 95˚C per cycle, annealing and extension for 30 seconds at 58˚C, and final extension for 30 seconds at 72˚C. The melting step ran from 75˚C to 90˚C with a temperature increase of 0.1˚C every 2 seconds. A threshold was set at 10% of the maximum normalized fluorescence (dF/dT) of the highest peak, with all peaks that both occurred at the diagnostic temperature (Tm) and crossed this threshold classified as positive.

Results

Primers

One primer-pair per target was selected based on successful amplification, distinct Tm and production of a clear peak. Product sizes for each amplicon ranged from 134–319 base pairs (Table 1) with peak temperatures ranging from 79.2˚C to 87.5˚C. To allow for automated calling of peaks, bin widths of 1.5˚C (0.75˚C either side of diagnostic Tm) were set for each target (Fig 1).

Table 1. HRM and PCR primers used for identification of single-copy gene targets of *T. b. rhodesiense*, *T. b. gambiense* and *T. brucei s. l.* HRM primers were run in multiplex whilst PCR primers were run in singleplex. *This primer set was run in multiplex in HRM and in singleplex in PCR.

Primer	Species	Primer sequence 5'-3'	Product size (bp)	Product Tm (˚C)	Reference	Assay
PLC1	Trypanozoon	CAGTGTTGCGCTTAAATCCA	319	79.1	This study	HRM
PLC2		CCCGCCAATACTGACATCTT				
TbRh1	*T. b. rhodesiense*	GAAGCGGAAGCAAGAATGAC	134	84.2	This study	HRM
TbRh2		GGCGCAAGACTTGTAAGAGC				
TgsGP1	*T. b. gambiense*	GCTGCTGTGTTCGGAGAGC	308	87.5	[29]	HRM and PCR*
TGsGP2		GCCATCGTGCTTGCCGCTC				
657	Trypanozoon	CGCTTTGTTGAGGAGCTGCAA GCA	324	-	[21]	PCR
658		TGCCACCGCAAAGTCGTTATT TCG				
SRA 02	*T. b. rhodesiense*	AGCCAAAACCAGTGGGCA	669	-	[21]	PCR
SRA 03		TAGCGCTGTCCTGTAGACGCT				

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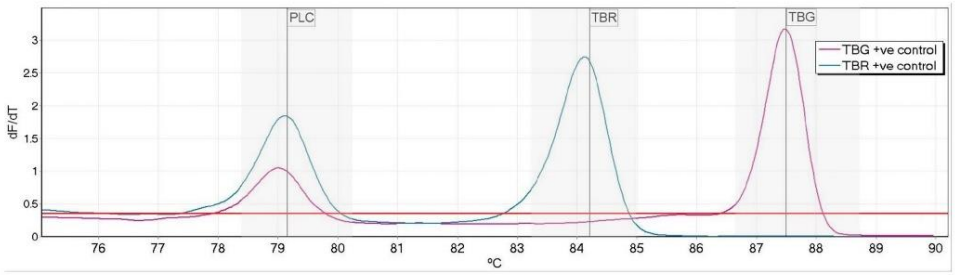

Fig 1. High resolution melt profile of phospholipase-C (PLC), *T. b. rhodesiense* (TBR) and *T. b. gambiense* (TBG) targets. Normalized fluorescence dF/dt is plotted against degrees ˚C (deg.) The melt temperatures for each peak were 79.1˚C for PLC, 84.2˚C for *T. b. rhodesiense and* 87.5˚C for *T. b. gambiense*. The positivity threshold is shown in red and target specific bins indicated in grey.

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Analytical specificity and sensitivity

No non-specific amplification was seen when the assay was challenged with a range of non-target trypanosome species, namely *T. congolense* (Savannah, Kilifi and Forest subgroups), *T. vivax*, *T. simiae*, *T. simiae* Tsavo, *T. godfreyi* and *T. grayi*. The limit of detection was found to be an estimated 10^4 trypanosomes/mL for *T. b. gambiense* and *T. b. rhodesiense* using purified DNA from culture (S4 Fig). When the performance of the HRM was compared to TgsGP and SRA PCR on the same DNA dilution series, the HRM was as sensitive at detecting *T. b. gambiense* DNA and tenfold more sensitive for *T. b. rhodesiense* DNA (S2 and S3 Figs).

Screening of field samples

All flies (96/96) were positive by TBR PCR when initially rescreened. Using the multiplexed HRM, PLC positive control peaks were produced by 43 samples (44.8%), indicating sufficient target DNA for identification of either *T. b. gambiense* or *T. b. rhodesiense*. PLC PCR identified 19 samples (19.8%) with sufficient DNA quantity (Table 2). All samples which were positive by PLC PCR were also positive by HRM. None of the flies positive for *T. congolense* Savannah produced any non-specific amplification. Three flies (3.1%) were identified as positive for *T. b. rhodesiense* DNA both by HRM and PCR (Table 2), all of which also produced PLC peaks and were positive by PLC PCR (Fig 2). No flies were found to be positive for *T. b. gambiense* through either PCR or HRM. Flies that were positive for PLC but negative for *T. b. gambiense* or *T. b. rhodesiense* were considered to be infected with livestock trypanosome *T. b. brucei*.

Discussion

Here we describe a novel high-resolution melt analysis for the detection and differentiation of *T. b. rhodesiense* and *T. b. gambiense*. This multiplexed assay screens for both pathogenic trypanosome species simultaneously with the addition of a third primer set to identify the

Table 2. Breakdown of results of HRM and PCR on 96 field caught tsetse.

		HRM			PCR		
Tsetse species	**Total screened (N)**	**PLC positive (%)**	**SRA positive (%)**	**TgsGP positive (%)**	**PLC positive (%)**	**SRA positive (%)**	**TgsGP positive**
G. pallidipes	67	34 (50.7)	2 (3.0)	0	15 (22.4)	2 (3.0)	0
G. swynnertoni	29	9 (31.0)	1 (3.4)	0	4 (13.8)	1 (3.4)	0

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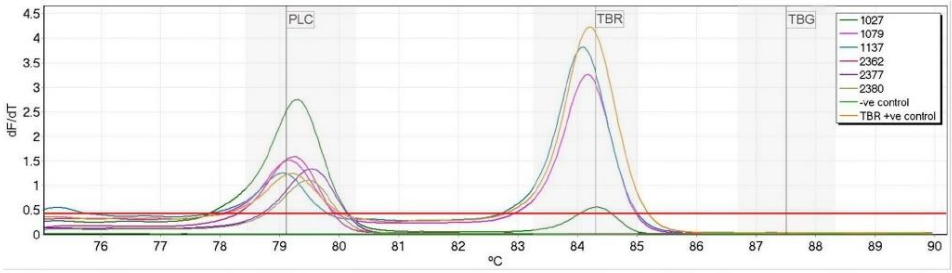

Fig 2. Melt profile of field samples. Three samples were identified as *T. b. rhodesiense* due to the production of both PLC and TBR peaks (samples:1027, 1079 and 1137). Samples which only produced PLC peaks were considered to be *T. b. brucei* (samples: 2362,2377 and 2380). The TBR positive control was *T. b. rhodesiense* DNA and the negative control used was nuclease free water. Normalized fluorescence dF/dt is plotted against degrees °C (deg.) with threshold indicated in red and species-specific bins shown in grey.

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presence of sufficient DNA to detect single-copy genes, acting as a positive control. The assay demonstrated high specificity with no cross-reaction with other non-target trypanosome species also transmitted by tsetse. The limit of detection of the HRM was lower than those reported in the literature for TgsGP [29] and SRA PCR [21]. However, when the three assays were compared directly against a dilution series of DNA, HRM was found to be as sensitive as TgsGP PCR and tenfold more sensitive than SRA PCR. The assay identified three wild caught tsetse flies to be positive for *T. b. rhodesiense* DNA. The three *T. b. rhodesiense* positives included two flies that had been previously identified by Lord *et al* [31]. All three flies including the additional fly that was identified by the HRM were positive by SRA PCR. However, the Ct value of the additional SRA positive was 30.5 in comparison to the two previously recorded positives which had Ct values of 21.1 and 21.8. This may indicate a lower DNA quantity and may represent a sample at the threshold of PCR detection. Of the 96 flies screened, 44.8% were positive for the universal-Trypanozoon PLC gene by HRM, indicating that they contained sufficient DNA for single-copy gene detection and therefore the detection of the SRA or TgsGP targets; if present. The remaining 55.2% of tsetse that were negative for the single-copy PLC gene can be assumed to have insufficient DNA present for the detection of the sub-species targets, we can therefore not rule these out as being negative for either *T. b. gambiense* or *T. b. rhodesiense*. Similar results have been reported in previous studies [33] and such a result is unsurprising when comparing the copy numbers, with TBR primers targeting a ~10,000 copy region and the sub-species primers targeting a single-copy gene, and presents an ongoing challenge. The two methods also differed in the number of flies positive for the single-copy PLC gene with the HRM method identifying a higher number of flies, indicating a greater sensitivity. This is likely due to the difficulty in identifying faint bands on a gel compared to the ability to better quantify a positive result using qPCR. When the Ct values of flies positive by HRM and PCR were compared to those that were only positive by HRM, the Cts of those positive by HRM only were slightly higher suggesting a potential failure of the PCR due to lower quantities of DNA in these samples (S5 Fig).

The method has three advantages over traditional PCR methods. First, the HRM time to result of $\leq$2 hours is faster than PCR followed by gel electrophoresis which can take over three hours for product amplification and visualisation. Second, this is a closed-tube assay which reduces contamination risk. Finally, it does not require interpretation of gel electrophoresis results. Through the use of detection bins, sample processing can also be automated, further

speeding up and simplifying data analysis. The simple and fast nature of this method indicates it could be suitable for the high-throughput processing of tsetse. With prevalence of *T. b. gambiense* in tsetse from HAT foci predicted to be as low as 1 in 10^5 [34], there is a need for a xeno-monitoring tool which can be applied to large numbers of samples. At present, this method is not suitable to a low-resources setting because it requires a qPCR machine, however with further optimisation this technique could be applicable to more field-friendly technologies such as the Magnetic Induction Cycler (Mic) (Bio molecular Systems). This method has the potential to provide the basis of a real-time trypanosome transmission monitoring platform, enabling timely reactive measures by disease control programmes. Furthermore, with the risk of traditionally distinct geographical distributions of both Rhodesian and Gambian HAT changing due to the movement of livestock [35], human migration [36] and climate change [37–40], it may become increasingly important to screen simultaneously for both trypanosome species. The HRM allows for this and removes the risk of presumptive screening based on historic disease distributions.

Study limitations

The authors acknowledge that the reliance of low copy genes for target identification is a limiting factor of the described diagnostic assay. However, at present these single copy genes are the only identifiers of members of the *T. brucei s. l.* and so pose a challenge to any diagnostic method based on these targets. This study was also challenged by a small number (96) of field caught flies from a rHAT focus and none from a gHAT focus where tsetse infected with *T. b. gambiense* might be present. Consequently, assay performance on field samples could only be evaluated for *T. b. rhodesiense*. Due to trypanosome prevalence in HAT foci predicted to be very low [34], obtaining sufficient field samples for any assay validation will be difficult. As control efforts such as active screening and treating the human population, combined with tsetse control, the numbers of infected tsetse will likely decline even further [41–44]. The authors are currently in planning to validate this method in a gHAT focus. Additionally, the field samples had only been screened for the presence of *T. brucei s. l.* and *T. congolense* Savannah. Studies of flies caught in this area suggest that these flies would have likely been infected with other trypanosome species [45,46] however this cannot be confirmed. Further validation using field caught flies with known infections with other non-target species would provide further evidence of the specificity of this method. Another limitation of this study was the comparison of only two methods for the identification of *T. b. rhodesiense* when there are alternative molecular methods available for HAT detection such as *Trypanozoon* sub-species specific LAMP [47,48] assays, which have demonstrated a high degree of sensitivity. Comparison between a wider range of methods would help assess HRM's potential as a screening tool for human trypanosomes. A final limitation of this assay, as with all assays targeting DNA, is the inability to differentiate between a tsetse with an active infection or one with a transient one. Similarly, the assay is unable to determine whether a fly has a mature infection and is therefore capable of transmitting the disease. By using RNA as a target it may be possible to identify an active infection and determine what stage of infection it is; either mature or immature. Detailed analyses of RNA transcripts would be required to overcome this challenge.

In order to overcome these limitations follow-up studies will be conducted in order to more fully validate the HRM assay against larger field samples for both *T. b. gambiense* and *T. b. rhodesiense*.

In summary, we describe the development of a novel HRM assay for the detection and discrimination of human African trypanosomes in tsetse flies. The assay also incorporates an internal control, identifying samples with sufficient genomic material. The closed tube nature

of the assay in addition to the relatively fast time and potential for automated calling lends itself to use in high throughput xenomonitoring surveillance campaigns for HAT.

Supporting information

S1 Fig. Molecular analysis workflow of field caught flies.
(TIF)

S2 Fig. Gel image showing the results of TgsGP PCR on a serial dilution of *T. b. gambiense* DNA from 10^6 tryps/mL to 10^1 tryps/mL. Marker (M) is 100bp (Invitrogen).
(TIF)

S3 Fig. Gel image showing the results of SRA PCR on a dilution series of *T. b. rhodesiense* DNA on concentrations from 10^6 tryps/ml to 10^1 tryps/mL. Marker (M) is 100bp (Invitrogen).
(TIF)

S4 Fig. Limit of detection of multiplexed HRM qPCR. DNA concentrations for *T. b. gambiense* and *T. b. rhodesiense* range from 10^6 to 10^1 tryps/mL. Positivity threshold is shown in red and target specific bins indicated in grey.
(TIF)

S5 Fig. Ct values of flies positive by HRM only and both HRM qPCR and PCR.
(TIF)

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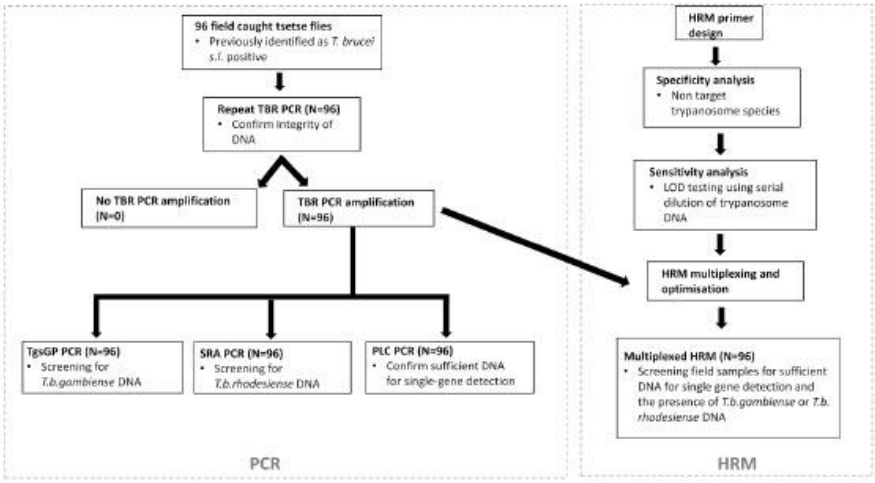
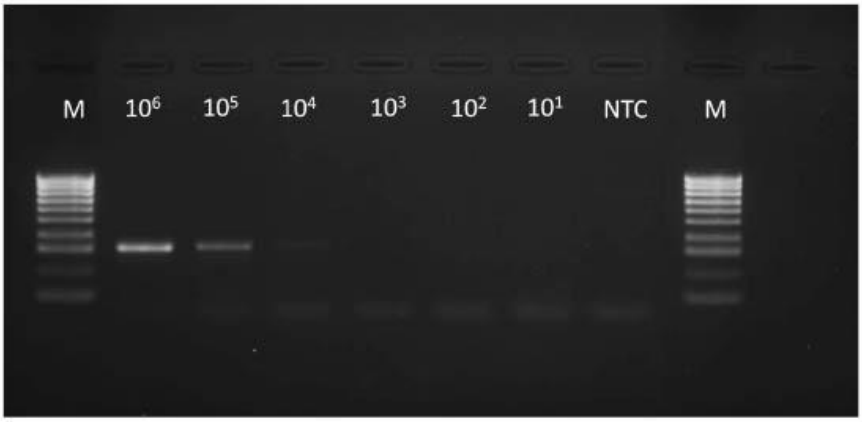
Supporting information

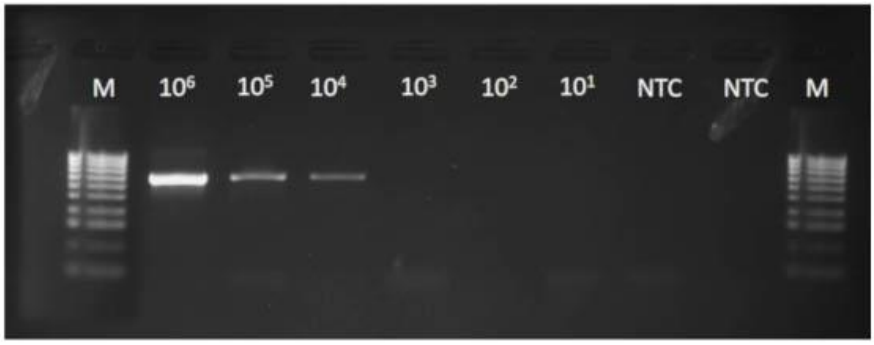


S1 Fig. Molecular analysis workflow of field caught flies.

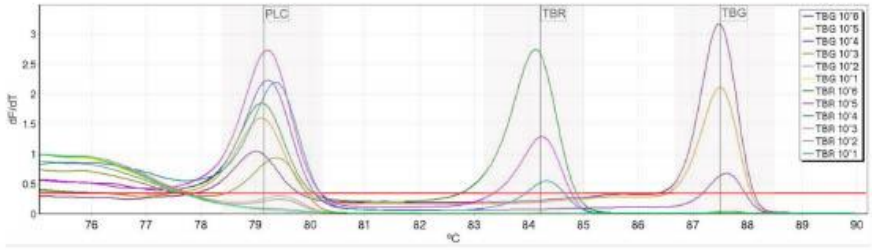


S2 Fig. Gel image showing the results of SRA PCR on a dilution series of T.b. rhodesiense DNA on concentrations from 10^6 tryps/ml to 10^1 tryps/mL. Marker (M) is 100bp (Invitrogen)

Supporting information



S3 Fig. Gel image showing the results of TgsGP PCR on a serial dilution of T.b. gambiense DNA from 10^6 tryps/mL to 10^1 tryps/mL. Marker (M) is 100bp (Invitrogen)



S4 Fig. Limit of detection of multiplexed HRM qPCR. DNA concentrations for *T.b.gambiense* and *T.b.rhodesiense* range from 10^6 to 10^1 tryps/mL.

Positivity threshold is shown in red and target specific bins indicated in grey

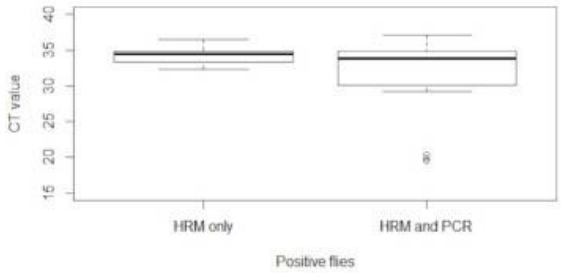
S5 Table 1 HRM performance on 96 field samples against traditional species-specific PCR.

		PCR	
		Positive (%)	**Negative (%)**
HRM	**Positive (%)**	3/96 (3.1)	0/96 (0)
	Negative (%)	0/96 (0)	93/96 (96.9)

Supporting information

S6 Table 2 Sensitivity, specificity, positive and negative predictive values for HRM qPCR

Sensitivity (%) [95% CI]	Specificity (%) [95% CI]
100 [29.2-100]	100 [96.1-100]



S7 Fig 5 Ct values of flies positive by HRM only and both HRM qPCR and PCR

3 Development and evaluation of a qPCR multiplex for animal African trypanosomes

3.1 Abstract

Introduction Surveillance of animal African trypanosomiasis is limited, relying on rarely conducted surveys of livestock. Instead, presumptive treatment of animals with trypanocides is performed routinely. With increasing risk of drug resistance and lack of cost-effective surveillance tools there is an increasing need for a screening tool which can monitor transmission pathogenic trypanosomes of medical and veterinary purposes. I designed a xenomonitoring tool which screens tsetse for the main pathogenic species of AAT which can be used in conjunction with the assay described in chapter 2 providing a cost-effective monitoring tool for both AAT and HAT.

Methods Three primer sets for *Trypanosoma brucei*, *T. vivax* and *T. congolense* were selected and multiplexed into one optimized qPCR assay. An additional primer pair targeting the Phocine herpesvirus was incorporated as an internal positive control. Specificity of the assay was tested using a panel of non-target trypanosome species (*T. grayi*, *T.godfreyi, T simiae, T. simiae* Tsavo and *T. theileri*) and tsetse DNA. The assay sensitivity was evaluated using a dilution series of DNA. Initial validation was carried out using laboratory-infected tsetse which were screened using the qPCR and traditional species-specific PCR. qPCR performance was tested on field-caught tsetse from both Tanzania and Uganda.

Results When the assay was challenged with the specificity panel, no cross-reactivity was recorded. Sensitivity of the assay was found to be at least as good as species-specific PCR for all three species. When assay performance was tested using laboratory flies, sensitivity was >97% sensitive with 100% specificity for both *T. b.brucei* and *T. congolense* Savanna. Although specificity remained 100%, sensitivity of the assay dropped for field-caught tsetse (71.1%) with some improvements in sensitivity seen when primers were run in singleplex.

Conclusions The multiplexed assay described allows for rapid screening of flies for the three main pathogenic species of AAT and was designed to be used in conjunction with the assay described in chapter 2. Despite high specificity and sensitivity of the assay in initial validation, the sensitivity of the assay dropped when tested on field samples. In order to be a useful xenodiagnostic tool, this assay would require further optimization and possible primer redesign to improve its utility for field use.

3.2 Introduction

Animal African trypanosomiasis (AAT), also known as Nagana is a haemoparasitic disease of wild animals and livestock mainly affecting cattle, sheep and goats across the tsetse belt of sub-Saharan Africa. AAT is largely caused by three species of trypanosome: *T. vivax*, *T. congolense s.l*. (consisting of three groups: Forest, Savanna and Kilifi) and *T. brucei s.l.* Animal trypanosomiasis also occurs outside of the tsetse belt where *T. vivax* and *T. evansi* are transmitted mechanically by biting flies and through the sexual transmission of *T. equiperdum*. AAT has large direct and indirect impacts on agricultural productivity in AAT endemic regions with reductions in meat and milk yields, reduced power of draught animals and household income. Across sub-Saharan Africa, livestock is the main source of livelihood for 40% of households, of which 309 million individuals live below US$2 per day (159). This reliance on the productivity of livestock is threatened by the estimated US$1-1.2 billion AAT related losses per year (160).

Diagnosis of nagana is largely based through observation of symptoms such as weakness, weight loss, lethargy, anemia and abortion(161). However these symptoms vary between host animals and trypanosome species and can be confused with the symptoms of tick-borne diseases. Animals that do not die as a result of infection often maintain fluctuating low-level parasitemia for months to years, providing a reservoir from which further transmission can occur. Parasitological confirmation of infection is not routine, with symptomatic animals often being presumptively treated for infection using trypanocidal drugs. With the use of these drugs remaining the major method of AAT control utilized by livestock owners in many AAT endemic countries (116,162), there is increasing risk of drug resistance, with 21 African countries already reporting treatment failure (163). The assessment of AAT prevalence has historically relied on the screening of livestock blood using microscopy either through examination of thick and thin films (164) or screening of

the buffy coat (165,166). These techniques are labour intensive and can suffer from low sensitivity. Serological techniques have also been employed for a number of pathogenic trypanosome species of veterinary importance (167–169), however these methods are unable to differentiate between past and ongoing infection and so do not provide clear data on prevalence at the time of screening. The development of a xenomonitoring based surveillance tool which provides data on parasite transmission could enable more focused and timely AAT control strategies whilst keeping costs low.

Molecular tools for the detection of trypanosomes have largely centered around the use of PCR, with a large number of assays described either targeting trypanosomes generically or a specific species of interest. One of the main challenges of screening for trypanosomes of veterinary importance is the potential presence of other trypanosome species increasing the risk of cross-reaction. These non-target species may include less pathogenic trypanosomes or non-pathogenic species such as *T. theileri*. To reduce this risk the screening process can be limited to the species of interest using species-specific primers. Although highly specific, the use of these primers can become cumbersome and time consuming when screening for a number of species of interest. Alternatively, generic primers produce data on all circulating trypanosomes, which can be of particular interest when investigating the diversity of parasites in an area. Despite a number of trypanosomes species having already been described, there are a large number which remain unidentified, the identification of which can be enabled by the use of generic primers. When analysing generic primer data, it is these unidentified species which can confuse the classification of amplified products and can produce a large amount of additional data that is not of interest when screening for a specific species. Even when species of interest are well described, their identification through the sizing of PCR products can be inaccurate and challenging, particularly if DNA quality or quantity is poor. Furthermore, traditional

PCR followed by imaging using gel electrophoresis is liable to contamination and the interpretation of gels can be subjective.

At the time of writing, the development of qPCR based screening tools for AAT is limited to two studies. One describes a multiplex real-time PCR assay targeting the ITS-1 regions of *T. brucei s.l.* , *T. vivax* and *T. congolense* in cattle (170) which was evaluated in Burkina Faso. The second describes two separate assays one targeting *T. congolense* Savanna and the other *T. b. brucei* in tsetse (171). However, this assay's performance remains untested in wild tsetse populations.

In this chapter I describe a novel qPCR-based multiplexed assay which has been optimized for screening tsetse for the three main pathogenic species of trypanosomes. I aim to build upon the previous two assays by targeting higher copy-number regions in order to improve sensitivity, optimizing for xenomonitoring purposes and validating on wild tsetse. High resolution melt analysis (HRM), is used post-PCR which has the ability to detect heterogeneity within nucleotide sequences. A fluorescent dye is added to the PCR reaction which intercalates into double stranded DNA. Following amplification, the amplicon is heated gradually causing the strands to separate. Separation of the double strands releases the incorporated dye causing a drop in fluorescence. The rate of DNA strand disassociation and the temperature at which it separates (Tm) is dependent on the nucleotide sequence. Different sequences will have different melting temperatures, and it is this temperature that is used as a diagnostic identifier. HRM is a closed tube process resulting in a reduced risk of contamination and produces results in approximately 2 hours making it a faster alternative to nested PCR which can take over five hours. A multiplexed HRM allows for the highly sensitive detection of a number of targets offering the potential of a high-throughput and less costly assay. The assay's performance was evaluated on laboratory infected tsetse and on wild caught flies from two AAT foci in Tanzania and Uganda. This assay was

designed for potential use within a one health context. Flies could be initially screened using this panel which indicates the presence of veterinary pathogens (*T. vivax* and T. *congolense*) in addition to *T. brucei s.l.* which is both of veterinary and medical importance. The assay described in chapter two could then be used to screen any *T. brucei s.l.* positives to help identify samples to sub-species, enabling a more integrated method of surveillance for both forms of the disease

3.3 Materials and Methods

3.3.1 Primer design

Species-specific primers were designed for the amplification of three animal and human trypanosomes: *T. brucei s.l.*, *T. congolense s. l.*, and *T. vivax*. Primers were designed for *Trypanosoma brucei s. l.* based on a 10,000 copy satellite repeat sequence (172) (K00392.1). *T. congolense s. l.* primers target a *T. congolense s.l.* generic sequence found in the 18s ribosomal region (U22315.1). Nucleotide sequences were obtained from the NCBI database and aligned using the CLUSTALW algorithm. Sequences of non-target trypanosome species that are often found circulating in tsetse were included in the alignment to ensure specificity of primers. The genetic heterogeneity of *T. vivax* is a challenge for primer design and so it was decided to utilize previously designed primers (173,174). These primers target a sequence encoding the Tv27 antigen which has been reported in a number of *T. vivax* isolates screened across Africa(175), increasing the likelihood of detecting any isolates that may be encountered. Primers were designed to produce an amplicon of between 100-300 base pairs with melting temperatures ranging between 70-93°C. The Tm of each amplicon was predicted using OligoAnalyzer 3. 1 software (https://www.idtdna.com/calc/analyzer). The specificity of candidates was initially checked using a NCBI Primer-Blast (176) search against the genomes of tsetse endosymbionts *Wigglesworthia glossinida* (CP003315.1), *Sodalis glossinidus* (AP008232.1),

Wolbachia (AE017196.1) and the ITS/18s regions of *Glossina morsitans* (GQ255906.1), *Glossina fuscipes fuscipes* (HQ387132.1), *Glossina palpalis palpalis* (KF597298.1), *Homo sapiens* (KY962518.1), *Trypanosoma theileri* (KY412803.1) and *Bodo saltans*-a free-living relative of trypanosomes (AY028449.1). To be distinguishable, each of the three products was designed to have a distinct melt temperature with sufficient allowances for shifts in temperature due to genetic variability within wild trypanosome populations. The predicted melt curve profile of each product was investigated through the use of uMELT Melting Curve Predictions Software

3.3.2 Internal positive control

To indicate successful DNA extraction and amplification by qPCR, Phocid herpesvirus (PhHv) was used as an internal positive control. Primers were designed to target the PhHv Glycoprotein B gene (Z68147.1)(177).

3.3.3 Primer efficiency

Primer efficiency was examined using a dilution series of target DNA with each dilution run in triplicate. An eight-fold dilution series was created ranging from 1ng/ul to $1x10^{-7}$ng/ul, the results of which were used to produce a standard curve for each primer set with primers run at a concentration of 400nm. To calculate the amplification efficiency the slope of each standard curve was input into a qPCR efficiency calculator (Thermo Fisher qPCR Efficiency Calculator | Thermo Fisher Scientific - UK). For *T. congolense s. l.* a mean efficiency value was taken of the three readings for each subgroup.

3.3.4 Specificity

Following initial selection based on Tm and production of a single peak, the specificity of each primer sets was evaluated against all stocks of non-target trypanosome species kept at the Liverpool School of Tropical Medicine (LSTM). The specificity panel used included a

range of pathogenic and non-pathogenic trypanosome species along with non-infected tsetse DNA obtained from LSTM insectaries (Table 1). DNA controls were run in sets of technical triplicates.

Table 1 Trypanosome and tsetse DNA specificity panel

DNA type	Species	Strain	Country of origin	Year of isolation	Host	DNA source
Trypanosome	*T. brucei brucei*	M248	Kenya	1981	Sheep	Parasite culture
	T. brucei gambiense	Eliane	Côte d'Ivoire	1952	Human	
	T. brucei rhodesiense	Unknown				
	T. vivax	Y486	Nigeria	1976	Cattle	
	T. congolense Forest	ANR3	The Gambia	1988	Fly - *G. p. palpalis*	
	T. congolense Savanna	Gam2	The Gambia	1977	Cow	
	T. congolense Kilifi	WG84	Kenya	1981	Goat	
	T. simiae	TV008	The Gambia	~1980	Fly - *G. morsitans*	
	T. simiae Tsavo	114	Tanzania	2000	Fly - *G. pallidipes*	
	T. godfreyi	Ken7	The Gambia	1988	Fly - *G. morsitans*	
	T. grayi	ANR4	The Gambia	1988	Fly - *G. palpalis gambiensis*	
	T. theileri	N/A	Uganda	2014	Fly - G. *f. fuscipes*	Field sample
Tsetse	*G. m. morsitans*	N/A	2020	N/A	N/A	LSTM

3.3.5 Limit of detection

The analytical sensitivity of the assay was assessed using a tenfold dilution series of *T. congolense* Forest, *T. congolense* Kilifi, *T. congolense* Savanna, *T. brucei brucei* and *T. vivax* DNA. The dilution series ran over eight orders of magnitude from $1x10^5$ to $1x10^{-2}$ tryps/mL, with each concentration run in duplicate. Quantification of DNA in terms of trypanosomes was calculated using concentrations of DNA as read by a Qubit 2.0 fluorometer (Invitrogen) and an assumed approximate DNA quantity of 0.1pg per parasite (178).

Primer performance was tested over a range of annealing temperatures between 50-65°C, with each set tested individually. All candidates were initially screened in a HRM singleplex, after which, primers were combined in duplex with another primer set. Following this, pairs were combined in a stepwise manner to investigate their performance in a multiplex. The use of multiple primer pairs in one assay runs the risk of non-specific amplification due to mispairing. If non-specific amplification occurred, the assay was optimised through adjustments to the annealing temperature, time and cycle number. The multiplexed assay was run on multiple Rotor-Gene machines to confirm consistency of performance across systems.

3.3.6 Laboratory infected tsetse

Performance of the qPCR was first evaluated using laboratory-reared *G. m. morsitans.* Flies were split into three groups, each consisting of a total of 50 flies:

i) Bloodmeal spiked with *Trypanosoma brucei brucei* GFP J10

ii) Bloodmeal spiked with *Trypanosoma congolense* Savanna

iii) Control bloodmeal

Each group was fed on 5mL defibrinated horse blood. Spiked bloodmeals were created by the addition of 200µl of thawed trypanosome stabilate at a concentration of 10^5 trypanosomes/mL. Blood (TCS Biosciences Ltd) was heated to 37 ˚C and flies were fed through a silicone membrane. To allow for infection to establish, flies were killed seven days post-bloodmeal and the midguts of flies fed spiked blood were screened for infection using a compound microscope. Dissecting needles and forceps were cleaned using bleach and water between dissections and a fresh slide used per fly. Each fly was stored in an individual Eppendorf tube filled with 100% ethanol and all flies were stored regardless of infection status. Control flies were stored directly without dissection.

3.3.7 Wild caught tsetse

A total of 2,221 tsetse were caught in six districts (Arua, Maracha, Yumbe, Koboko, Moyo and Adjumani) in West Nile, Uganda using pyramidal traps. All flies were stored individually in Eppendorf tubes filled with 100% ethanol. Once flies were transported to LSTM they were screened for the presence of *T. congolense s.l.*, *T. brucei* and *T. vivax* DNA using species-specific PCR primers listed in Table 2*.* All *T brucei s. l.* positive flies were re-screened with GPI-PLC PCR to identify those with sufficient genomic material for the amplification of *T. b. gambiense* and *T. b. rhodesiense* specific single-copy genes (SRA and TgsGP) (18). Screening for *T. b. rhodesiense* was conducted using a GPI-PLC and SRA primer multiplex (18) whilst TgsGP PCR for *T. b. gambiense* detection had to be conducted as an additional PCR. Alongside this protocol, the same flies were screened using the qPCR methodologies outlined in this chapter and chapter two (Figure 10). Samples were screened using the veterinary panel initially, and any identified to be *T. brucei s. l.* positive were then screened using the HAT qPCR (chapter two). Overall, this reduced the number of molecular assays conducted from four/five (depending on whether GPI-PLC/SRA multiplex or TgsGP PCR was used) to two.

Table 2 Species-specific PCR primers

Primer set	Target	Sequence	Ref
TBR	*T. brucei s.l.*	CGAATGAATATTAAACAATGCGCAGT	(179)
		AGAACCATTTATTAGCTTTGTTGC	
TVM	*T. vivax*	TCGCTACCACAGTCGCAATCGTCGTCTCAAGG	(180)
		CAGCTCGGCGAAGGCCACTTGGCTGGGGTG	(174)
TCF	*T. congolense* Forest	GGACACGCCAGAAGGTACTT	(181)
		GTTCTCGCACCAAATCCAAC	
TCS	*T. congolense* Savanna	GGACAAACAAATCCCGGGCACA	(179)
		GGACACGCCAGAAGGTACTT	
TgsGP	*T. b. gambiense*	GCTGCTGTGTTCGGAGAGC	(182)
		GCCATCGTGCTTGCCGCTC	
SRA	*T. b. rhodesiense*	CCATGGCCTTTGACGAAGAGCCCG	(183)
		CTCGAGTTTGCTTTTCTGTATTTTTCCC	
GPI-PLC	*T. brucei s.l.*	CTTTGTTGAGGAGCTGCA	
		CACCGCAAAGTCGTTATT	

Due to limited number of trypanosome positive flies from Uganda, an additional complementary set of samples from an area with higher levels of *Trypanosoma* infection were included to evaluate the qPCR assay. These samples were *G. pallidipes* and *G. swynnertoni* captured from Simanjiro district and Tarangire National Park in Tanzania in August 2019, methods fully outlined in (184). Flies were screened using species-specific PCRs for *T. vivax* and *T. brucei s.l.* and *T. congolense* Savanna due to this being the *T. congolense* group present in this region (Table 2). A subset of 1,116 flies were selected for assay validation to encompass a range of positives from all three species, along with a

number of negative flies (n=978). Tsetse field sites are demonstrated in Figure 9. The levels of agreement between the qPCR and species-specific PCRs were calculated using Cohen's Kappa statistic (185), and graded according to the methodology presented by Landis and Koch (1977)(186).

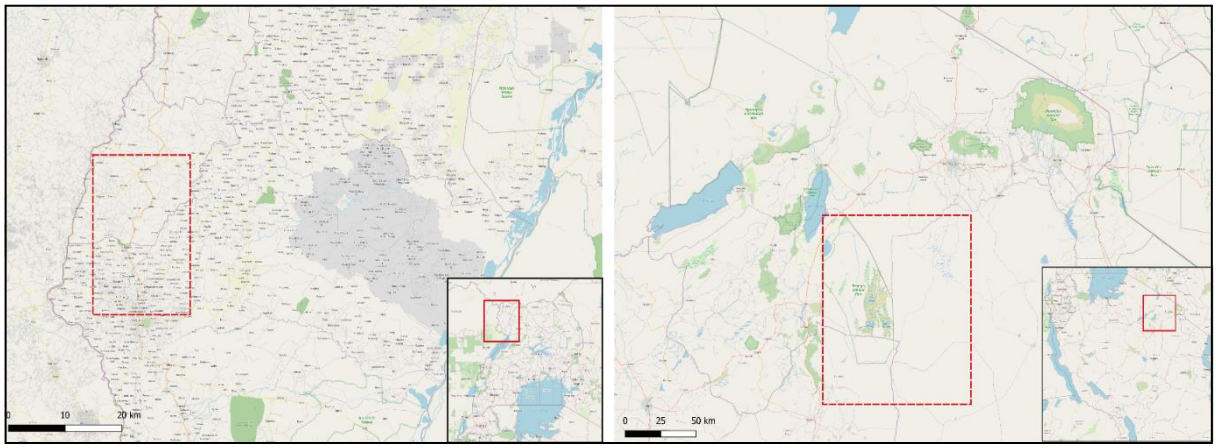


Figure 9 Location of tsetse trapping areas in Uganda (left) and Tanzania (right)

3.3.8 DNA extraction

All flies, both laboratory and wild caught, were extracted using the same method. DNA was extracted using the Thermo Scientific GeneJET Genomic DNA Purification Kit and following the manufacturer's Mammalian Tissue and Rodent Tail Genomic DNA Purification Protocol. Flies were removed from ethanol, transferred into a 96 well plate and placed onto a heat block set to 56°C for 3-4 hours. Once the ethanol had evaporated, the flies were placed into individual microtubes with a one steel ball per fly and 20µl proteinase K and 180 µl of Digestion solution were added to each tube. Tubes were sealed, vortexed and placed into a tissuelyser for 2 minutes at intensity level 3. Following tissue lysis, tubes were centrifuged at 1500g for 30 seconds. Samples were then incubated overnight at 56°C. The following morning samples were removed from the incubator and centrifuged at 1500 for 30 seconds. Each sample was transferred to a new, labelled Eppendorf tube and 20 µl RNase A solution added. Samples were vortexed then left to incubate at room temperature for 10

minutes. 200µl Lysis solution added and samples vortexed for 15 seconds until a homogenous solution was obtained. 400µl 100% molecular grade ethanol was added and samples vortexed and centrifuged briefly. The lysate was transferred to a GeneJET Genomic DNA Purification Column inserted into a new collection tube. Columns were centrifuged at 6000 g for 1 minute. The GeneJet column was placed into a new 2mL collection tube and the flow through discarded. 500µl of Wash Buffer 1 was added and each sample centrifuged for 1 minute at 8000 x g. The flow through was discarded and the purification column was placed back into the same collection tube. Then, 500µl of Wash Buffer 2 was added and samples centrifuged for 3 minutes at maximum speed (~12000 x g). If any residual solution was seen in the columns, the collection tubes were emptied and the re-spun for 1 minute. The collection tubes were discarded and the GeneJET columns transferred to sterile 1.5 microcentrifuge tubes. 200µl of Elution Buffer was added to the center of the column membrane to elute genomic DNA. Samples were incubated at room temperature for 2 minutes and then centrifuged at 8000 x g for 1 minute. The resulting 200 µl of extracted DNA was transferred to a sterile PCR plate.

3.3.9 Species-specific PCR

Tsetse samples were screened for *T. vivax*, *T. brucei s.l.* and *T. congolense* Forest/Savanna using species-specific PCR (Table 2). PCR reactions were performed using a total reaction volume of 25µl consisting of 12.5 µl DreamTaq (Bioline),0.5 µl of forward and reverse primer (25mmol), 6.5 µl of nuclease free water and 5 µl of DNA template. PCR conditions were as follows: initial denaturation at 95˚C for 5 mins followed by 35 cycles of 94˚C for 15 s, 54˚C for 15 s and 72˚C for 10 s with a final extension at 72˚C for 5 mins.

3.3.10 Effect of multiplexing on sensitivity

To investigate the impact of multiplexing on the assay's sensitivity all positive samples from Uganda and Tanzania were re-screened in singleplex along with a subset of negative

flies (N=21). Flies were screened in singleplex for the trypanosome species they had previously been found to be positive for using PCR. Differences in sensitivity between multiplex and singleplex formats of the assay were evaluated using McNemar's Chi-squared test (https://epitools.ausvet.com.au/mcnemar) at the 5% significance level.

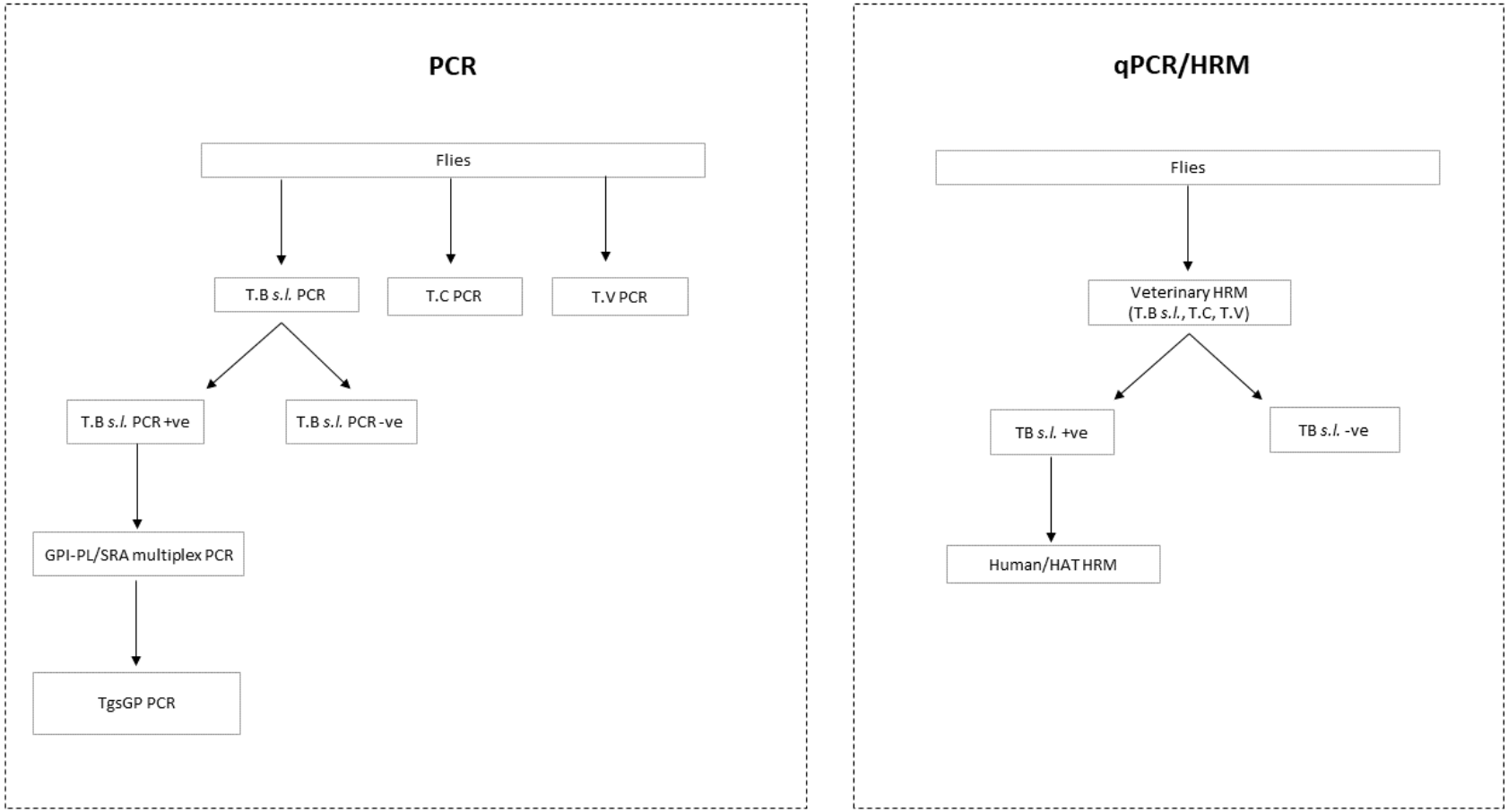


Figure 10 Workflow for screening wild tsetse for trypanosome infection.

3.4 Results

3.4.1 Primer design and selection

Primer candidates were screened using target trypanosome DNA as template and selected based on positive amplification, distinct melt temperature (°C) and peak height fluorescence. In order to be able to differentiate between each species, each primer set had to have a unique diagnostic melt peak which was initially predicted using uMelt software (Figure 11).Primers chosen for the qPCR multiplex are shown in Table 3.

Table 3 qPCR species-specific primers

Primer	**Species**	**Primer sequence 5'-3'**	**Ref**
TB2 Forward	*T. brucei s.l.*	GCGCAGTTAACGCTATTATACACA	This study
TB2 Reverse		AAGAACAGCGTTGCAAACTT	This study
TC Forward	*T. congolense s.l.*	AGAAACACGGGAGCGGTC	This study
TC Reverse		GAGGCCATAATCTCCCAAGGA	This study
TVM Forward	*T. vivax*	TCGCTACCACAGTCGCAATCGTCGTCTCAAGG	(180)
TVM Reverse		CAGCTCGGCGAAGGCCACTTGGCTGGGGTG	(174)
HV5 Forward	Phocid herpesvirus type 1	CGTGAGGTGTGTACATTGGC	This study
HV5 Reverse		GCCTCTGTTGCTTCACGTTT	

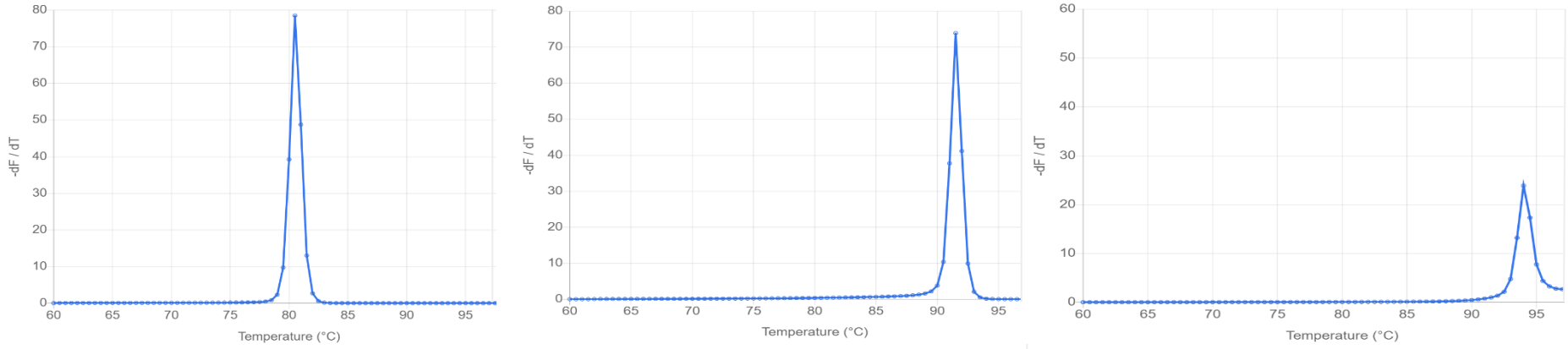


Figure 11 Predicted melt curves for (left to right) T. brucei s.l., T. congolense s.l. and T. vivax

When primers were multiplexed, a range of annealing times and temperatures were assessed to reduce any non-specific binding. Overall, primers performed best at an annealing temperature of 63° C and annealing time of 50 seconds.

3.4.2 Primer efficiency

Efficiencies were found to be highly similar between trypanosome primer sets with the R^2 range being between 0.93-1.07 (Table 4, Figures 11-15). The Phocine herpesvirus-1 primers were twice as efficient, and so, the concentrations at which they were run in the multiplex were adjusted to reflect this. This aimed to reduce the risk of out-competition of the trypanosome primers, particularly in samples with low concentration of trypanosome DNA. A dilution series of the PhHv primers was run with concentrations ranging from 04µM to 0.02uM (0.04,0.20,0.10,0.04,0.02). The dilution that produced a Ct value of 33-34 was selected for the final assay as this was unlikely to outcompete with the trypanosome primers but still produce a clear, positive control signal. A fixed amount of 1ul PhHv DNA at this concentration was added to each sample prior to qPCR.

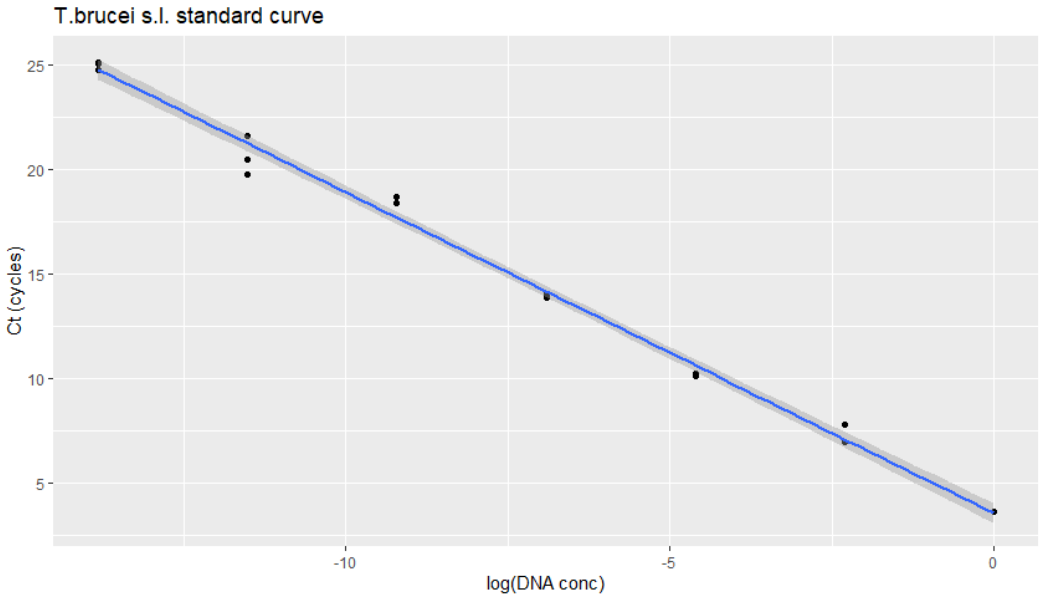


Figure 12 T. brucei primer efficiency curve

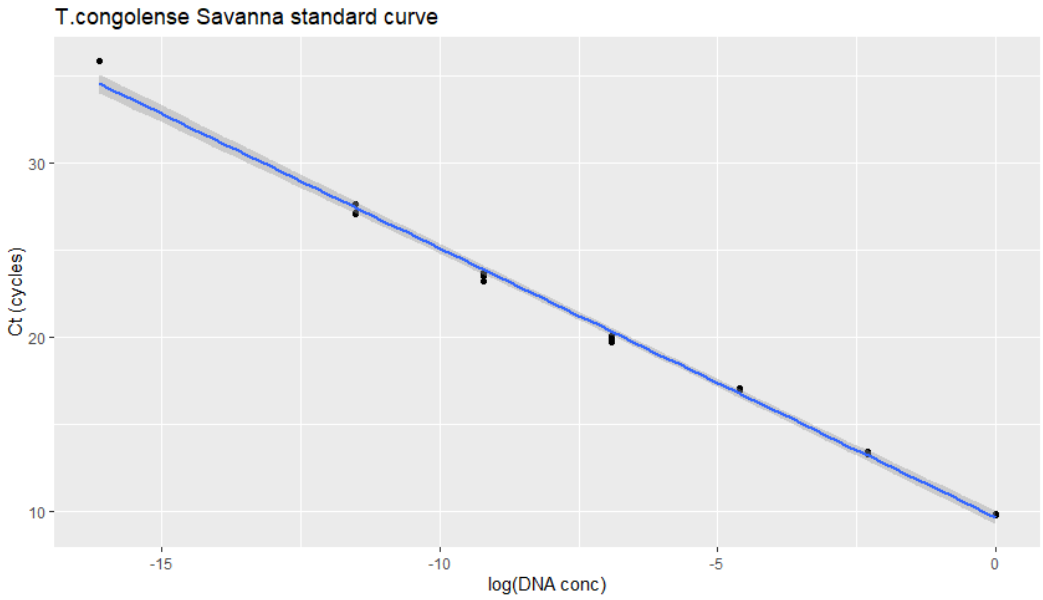


Figure 13 T. congolense Savanna primer efficiency curve

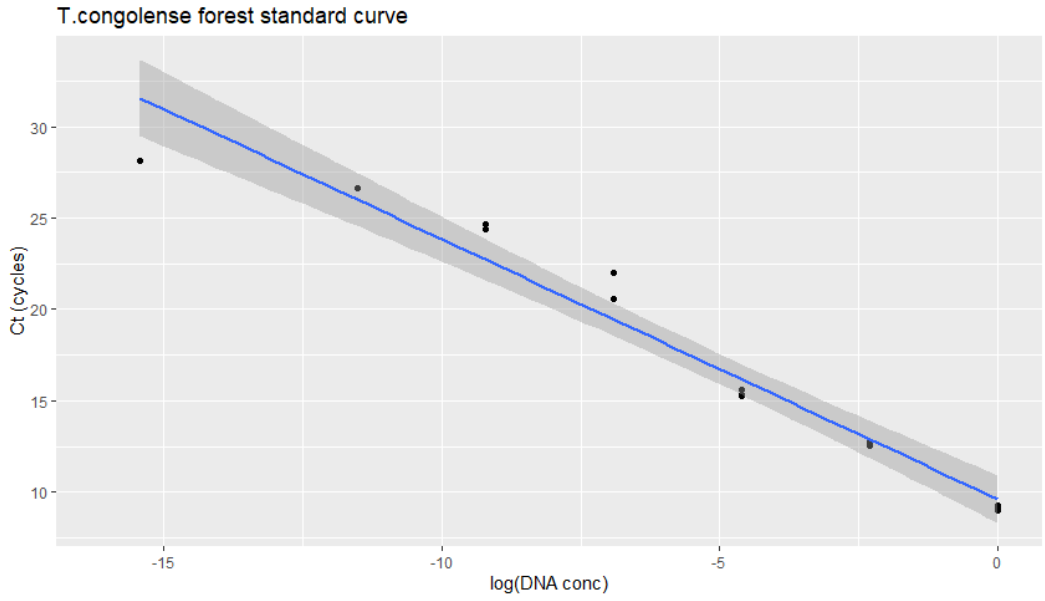


Figure 14 T. congolense Forest primer efficiency curve

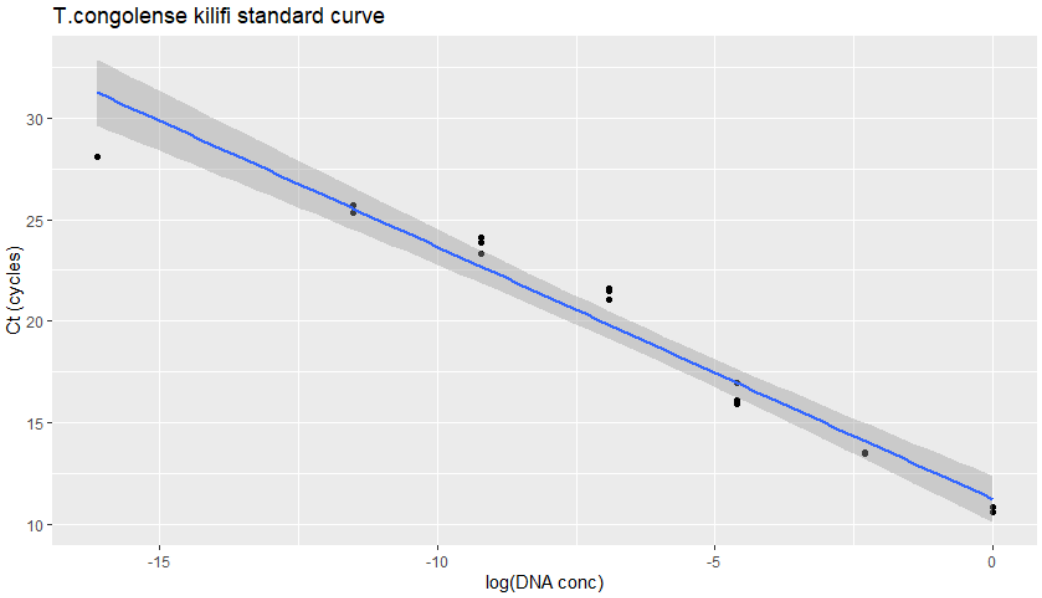


Figure 15 T. congolense Kilifi primer efficiency curve

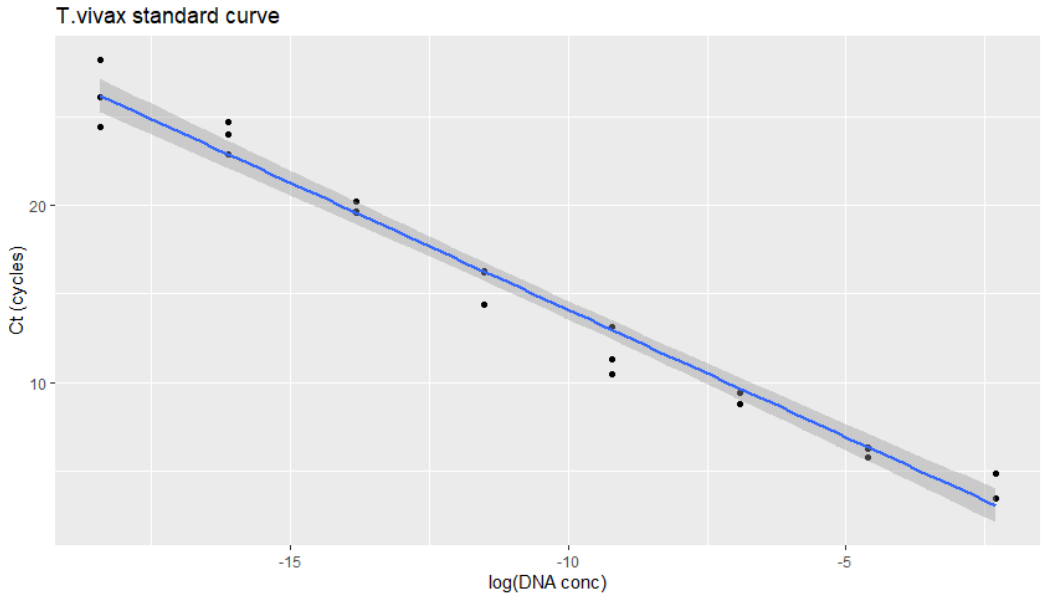


Figure 16 T. vivax primer efficiency curve

Table 4 Primer efficiency for each primer included in the qPCR multiplex. TC primer efficiency is taken as an average of the primer performance against Savanna, Forest and Kilifi subtypes.

Primer set	**Target**	**Efficiency (%)**	**R^2**
TB2	*T. brucei s.l.*	0.92	0.994
TVM	*T. vivax*	0.99	0.98
PhHv	Phocine herpesvirus-1	2.00	0.97
TC	*T. congolense s.l.*	(0.91-1.24) μ= 1.04	(0.95-1.08) μ=1.00

3.4.3 Specificity

All primers were screened for cross-reactivity against the specificity panel. Specificity of qPCR primers was found to be very high with no cross-reactivity seen against any non-target DNA.

3.4.4 Limit of detection

Each of the three trypanosome primers were found to be highly sensitive in multiplex with each being able to detect up to the genomic equivalent of one trypanosome per mL (Table 5). The TC primers were found to be one order of magnitude more sensitive, detecting 0.1 trypanosome per mL.

Table 5 The limit of detection for each of the trypanosome qPCR primer sets

Trypanosome primer set	**ng/ul**	**Tryps/mL**
TB	0.000001	1
TC	0.0000001	0.1
TVM	0.000001	1

3.4.5 HRM assay

The final, optimized high resolution melt qPCR reactions were performed using a total reaction volume of 25 µl consisting of 5 µl DNA, 12.5µl HRM Master Mix (Thermo-start ABgene, Rochester New York USA), 3.25 µl sterile DNase/RNasefree water (Sigma, St. Louis, USA) and 0.5 µl of both forward and reverse trypanosome primers (concentration of 400nM). Phocine herpesvirus primers were run at a lower concentration of 100nM to mitigate out-competition with trypanosome primers. Reactions were carried out on a Rotor-Gene 6000 real-time PCR machine (Qiagen RGQ system). The following protocol was followed: denaturation at 95 °C for 5 minutes followed by 40 cycles of denaturation for 10 seconds at 95°C per cycle, annealing and extension for 30 seconds at 55°C, and final extension for 2 seconds at 72°C. The melting step ran from 65°C to 95°C with a temperature increase of 0.1°C every 2 seconds. The read out of the assay showing the diagnostic peaks of each of the three trypanosome species along with the internal positive control is demonstrated in Figure 17.

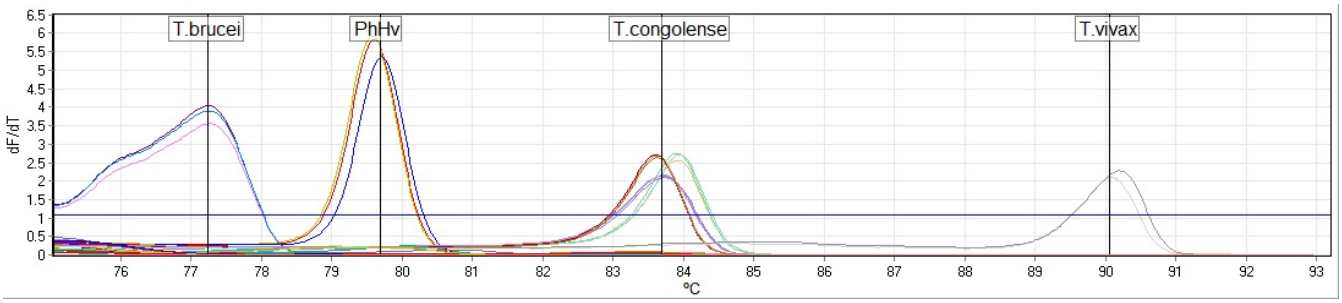


Figure 17 High-resolution melt peaks for each target trypanosome species (T. brucei s.l., T. congolense s.l. and T. vivax)

3.4.6 Laboratory flies

Sensitivity and specificity of the qPCR when assessed using laboratory infected flies was found to be high. *T. congolense* Savanna sensitivity was found to be 100% [95% CI 83.89-100] with a specificity of 89.66% [95% CI 72.65-97.81] (Table 6). *T. brucei* sensitivity was 97.73% [95% CI 87.98-99.94] with 100% specificity [95% CI 54.07-100] (Table 7).

Table 6 qPCR HRM and PCR results for flies fed T. congolense Savanna bloodmeals

T. congolense Savanna	**PCR**	
qPCR HRM	+ve	-ve
+ve	21	3
-ve	0	26

Table 7 qPCR HRM and PCR results for flies fed T. brucei brucei bloodmeals

T. brucei s.l	**PCR**	
qPCR HRM	+ve	-ve
+ve	43	0
-ve	1	6

3.4.7 Wild caught flies

Uganda

Following initial screening using species-specific PCR, the prevalence of trypanosome infection in the wild-caught flies from West Nile was as follows: *T. brucei s.l.* 0.18% *T. congolense* Forest 0.23%, *T. vivax* 2.84% and one fly (0.05%) co-infected with *T. vivax* and *T. congolense* Forest (Table 8). Sensitivity varied greatly between trypanosome species, ranging from 16.67% for *T. congolense* Forest to 89.06% for *T. vivax*. The confidence intervals of the observed sensitivities reflect the very low number of trypanosome positive flies collected (Table 9). No false positive results were recorded for any of the 2,221 flies screened.

Table 8 Number of flies caught in Uganda positive by species-specific PCR for trypanosomes

Species	**N positive**	**%**
T. brucei s.l.	4	0.18
T. congolense Forest	5	0.23
T. vivax	63	2.84
T. congolense Forest *+T. brucei s.l.*	0	0
T. congolense Forest *+ T. vivax*	1	0.05
T. brucei + T. vivax	0	0
T. congolense Forest + T. vivax + T. brucei s.l.	0	0

Table 9 Sensitivity and specificity of the qPCR multiplex when evaluated on Ugandan tsetse

Species	**HRM +ve/PCR +ve (n)**	**Sensitivity (%) [95% CI]**	**Specificity (%) [95% CI]**
T. brucei s.l.	3/4	75.00 [19.41-99.37]	100 [99.83-100.00]
T. congolense Forest	1/6	16.67 [0.42-64.12]	100 [99.83-100.00]
T. vivax	57/64	89.06 [78.75-95.49]	100 [99.83-100.00]

Tanzania

A subset of 1,116 flies were selected based on PCR positivity (N=138) (Table 10) along with a 978 PCR negative flies made up from the remaining flies on each plate. The sensitivity of the qPCR assay for *T. brucei s.l.* and *T. vivax* was similar when validated on the Ugandan and Tanzanian datasets (Table 9 and Table 11). The sensitivity of the assay for *T. congolense* was higher in the Tanzanian dataset (70.27% versus 16.67%) however this is likely due to the low number of positives found in the Ugandan flies. The specificity of the assay was 100% across all three species in both sample sets with no false positives.

Table 10 Number of flies caught in Tanzania positive by species-specific PCR for trypanosomes

Species	**N positive**	**%**
T. brucei s.l.	50	4.48
T. congolense Savanna	25	2.24
T. vivax	49	4.39
T. congolense Savanna + T. brucei s.l.	8	0.72
T. congolense Savanna + T. vivax	3	0.27
T. brucei + T. vivax	2	0.18
T. congolense Savanna + T. vivax + T. brucei s.l.	1	0.09

Table 11 Sensitivity and specificity of qPCR multiplex when evaluated on wild-caught infected and uninfected tsetse from Tanzania

Species	**qPCR +ve/PCR +ve (n)**	**Sensitivity (%) [95% CI]**	**Specificity (%) [95% CI]**
T. brucei s.l.	40/61	76.92 [63.16-87.47]	100 [99.56-100]
T. congolense Savanna	26/37	70.27 [53.02-84.13]	100 [99.66-100]
T. vivax	46/55	83.64 [71.20-92.23]	100 [99.65-100]

3.4.8 Combined sensitivities

When the results of both datasets were combined to give overall sensitivity and specificity data for the qPCR assay sensitivity was found to be fairly low ranging from 61.36% for *T. congolense* to 85.83% for *T. vivax* (Table 12). Specificity remained high at 100% for all species. None of the flies were positive for SRA of TgsGP using PCR or qPCR HRM. Using the HAT panel, all flies that were positive by GPI-PLC only were considered to be *T. brucei brucei* (n=61).

Table 12 Overall sensitivity, specificity data, percentage agreement and Cohen's Kappa for the multiplex qPCR when both Ugandan and Tanzanian sample sets were combined.

Species	**HRM +ve/PCR +ve (n)**	**Sensitivity (%) [95% CI]**	**Specificity (%) [95% CI]**	**Agreement (%) and Kappa [95% CI]**
T. brucei s.l.	43/65	66.15 [53.35-77.43]	100 [99.89-100]	99.34 and 0.79 [0.71-0.87]
T. congolense	27/43	62.79 [46.73-77.02]	100 [99.89-100.00]	99.52 and 0.77 [0.66-0.88]
T. vivax	103/119	86.55 [79.09-92.12]	100 [99.98-100]	99.52 and 0.93 [0.89-0.96]

The percentage agreement between species-specific PCR and the multiplex qPCR were found to be high with Cohen's kappa values ranging from substantial agreement for *T. congolense* (0.77) and *T. brucei s.l.* (0.79), to almost perfect agreement for *T. vivax* (0.93) (Table 12).

3.4.9 Effect of multiplexing on sensitivity

Sensitivity of the assay was found to be improved by running the primers in singleplex (Table 13). McNemar's chi-square test was used to compare the sensitivity and specificity of the multiplex assay versus the singleplex assays. There was a significant difference

(<0.05) between the performance of the two assay versions when screening for all three species of trypanosomes.

Table 13 Sensitivity of singleplex assay for T. brucei s.l., T. congolense s.l. and T. vivax

Species	**PCR +ve (n)**	**Singleplex**	
		Sensitivity (%) [95% CI]	**Specificity (%) [95% CI]**
T. brucei s.l.	65	65.57 [52.31-77.27]	100 [99.65-100]
T. congolense s.l.	43	78.95 [92.98-90.45]	100 [69.15-100]
T. vivax	119	90.80 [78.97-96.80]	100 [69.15-100]

3.5 Discussion

In this chapter, I describe the development and validation of a multiplexed qPCR-based assay for the detection of the three main pathogenic species of animal African trypanosomiasis. Trypanosome primers were designed to target high copy-number regions in each species' genome and produce distinct melt profiles which can be used to differentiate between amplified products. The incorporation of an internal positive control allows for reliable interpretation of negative results. Assay optimization included the balancing of primer efficiencies along with assessing run conditions in which all primers performed well. The limit of detection was found to be 1 trypanosome/mL for *T. brucei* and *T. vivax* and an order of magnitude more sensitive for *T. congolense*. When compared to species-specific primers targeting the same regions, the qPCR TB2 primers were as sensitive as the TBR primers described by Moser *et al* (179) at detecting *T. brucei s.l*. The

assay's LOD for *T. congolense* was found to be higher (0.1 tryps/mL) when compared to the assay described by Ahmed *et al* (187) which has a reported limit of detection of 40 parasites/mL(171).

The specificity of the assay was challenged by a large specificity panel encompassing DNA from all the major pathogenic and non-pathogenic species of trypanosome that would likely be encountered when sampling tsetse. The assay performed well, with 100% specificity. It is important to highlight that the amplification of trypanosome DNA from a tsetse does not necessarily indicate a fly is infected. The presence of trypanosome DNA could be from a recent bloodmeal from an infected host or remnants of a cleared infection(81) . As a consequence, screening tsetse for trypanosome DNA does not indicate how many flies have viable infections, but nevertheless, this can still provide data on the presence and prevalence of trypanosomes in an area. This could be of particular use in an elimination setting and could help guide control operation decisions.

Initial evaluation of the assay's performance was carried out on laboratory infected tsetse fed bloodmeals spiked with *T. b. brucei* and *T. congolense* Savanna cultures. This helped to evaluate the ability of the assay to perform in the presence of tsetse DNA, mammalian blood and other potential inhibitors. The qPCR effectively differentiated between infected and control flies with high sensitivity and specificity across both trypanosome infected groups as well as controls.

Following validation on laboratory flies, the assay was tested on wild-caught tsetse from Uganda and Tanzanian AAT foci where *T. brucei s.l., T. congolense s.l.* and *T. vivax* are endemic. Furthermore, both areas are foci of human African trypanosomiasis with Tanzania experiencing ongoing transmission of *T. b. rhodesiense* and northwestern Uganda being a historic focus of *T. b. gambiense.* The presence of the trypanosomes of interest in

addition to different tsetse species, bloodmeal hosts and trapping methods allowed for the performance of the assay to be evaluated in two very different xenomonitoring contexts.

Unlike its performance in the laboratory flies, the qPCR assay's sensitivity was found to be much lower with drops in sensitivity by 31.58% and 37.21% for *T. brucei* and *T. congolense* respectively. These drop in sensitivity may be a result of the laboratory flies feeding on bloodmeals with much higher parasite loads than wild flies, resulting in DNA quantity in laboratory flies being present in higher concentrations. The number of parasites in wild flies is unknown and so it can be challenging to validate assays in the laboratory with parasite numbers similar to those likely to be seen in the field. Field flies are likely to contain smaller amounts of trypanosomes DNA which may be missed by the assay.

A second potential aspect of the assay which may be hindering its performance is the multiplexing of multiple primer sets in one test. Although multiplexed assays are appealing due to the ability to screen for multiple targets simultaneously, they can suffer from lower sensitivity. When the performance of the qPCR assay was compared to a singleplexed version for each species, improvements in sensitivities of 16.16% and 4.25% were recorded for *T. congolense* and *T. vivax* respectively. The sensitivity of *T. brucei* primers remained largely the same with multiplexed sensitivity found to be slightly higher than in singleplex at 66.15% compared to 65.57%. The poor sensitivity of the qPCR assay is particularly puzzling when looking at the performance of the *T. vivax* primers. The same primers were used in the qPCR and in the PCR, suggesting similar performance should be expected, particularly when comparing the singleplex qPCR to the traditional PCR assay. Although the *T. vivax* primers were the most sensitive of the three trypanosome primers, their performance in qPCR was much lower than in a standard PCR format. This may be due to differences in the qPCR HRM and PCR mastermix, as it has been found that even

differences in the supplier of commercial PCR mixes can result in significant differences in assay sensitivity (188).

In contrast to the differences in sensitivity, the qPCR assay described in this chapter was highly specific with no false positive results recorded. The high specificity of any assay used to make control or intervention decisions for AAT will be of the upmost importance, particularly if the interventions are costly or labour intensive such as the mass treatment of animals.

The results of this study suggest this assay performs better in a singleplex format, however, still does not perform as well as traditional species-specific PCR. Future development on the work described may incorporate the use of a probe-based approach. The integration of probes can provide improvements to assay sensitivity whilst maintaining a high level of specificity, however, this is done at an additional overall cost per sample.

With crossover between human and animal trypanosomiasis, in terms of pathogen, vector and environments the use of a 'one health' approach could benefit the control of both diseases. There is a need to improve surveillance through the development of novel monitoring and surveillance tools to assist with directing and focussing intervention strategies. This will be of particular importance going forward with rates of trypanocide resistance likely to increase, changing distributions of disease with climate change (189) and elimination targets.

4 A simple and more efficient alternative for DNA extraction for HAT monitoring

4.1 Abstract

Introduction DNA extraction from individual flies is time consuming and costly, particularly when using commercial kits. These costs become increasingly prohibitive in low-resource settings when there is a need to examine tens of thousands of tsetse due to extremely low trypanosome infection rates. Furthermore, due to the potential for flies to be carrying small numbers of trypanosomes, it is crucial that extraction methods are able to effectively and efficiently extract low DNA volumes; molecular methods such as qPCR rely on high DNA quality and yield. I investigated the potential of a novel magnetic-bead based method (MagnaExtract) for high-throughput extraction which could be used in remote and low-resource settings in conjunction with a PCR-based xenomonitoring tool.

Methods The MagnaExtract protocol was optimized by adapting the volume of beads added and the addition of an overnight lysis step. The performance of the optimized protocol was assessed using laboratory-reared tsetse spiked with trypanosome DNA. The performance in terms of DNA yield and quality were compared to a column-based kit (Qiagen DNeasy). Additionally, the dilution series of spiked laboratory flies was used to attempt to quantify the number of trypanosomes present in wild infected tsetse.

Results Initial comparison of the DNeasy and MagnaExtract protocols showed that the magnetic bead method produced significantly lower yields of DNA with lower purity. The addition of an overnight lysis step and doubling the volume of beads used during the extraction produced DNA yields of comparable quality and quantity to DNeasy that amplified well using qPCR. Using a serial dilution of trypanosomes, the infection rates of wild flies were found to range between 10^3 - 10^4 trypanosomes/mL in tsetse infected with *T. brucei*, *T. congolense* and T*. vivax* from Uganda. This is the first estimate of parasitemia in wild flies and provides evidence for the potential of pooling.

Conclusions DNA extraction kits are often complicated and costly, limiting their use in the field and make them inappropriate for a xenomonitoring system for remote and/or low-resource settings. In this chapter, I find that MagnaExtract provides a viable, low-cost, simple and more time-efficient method of screening a large number of flies. This method could be adapted for use in the field with efforts required to make homogenizing of samples more field friendly. Further work examining the pooling limits of flies would help bolster the initial indications that pooling would be a viable and cost-saving strategy.

4.2 Introduction

One of the main challenges facing the implementation of a xenomonitoring strategy for trypanosomiasis is the requirement to screen a large number of flies. With estimates of the number of flies carrying *T. b. gambiense* parasites being as low as one in 10,000 (190), there is a clear need to screen tens of thousands of flies to get as accurate an estimate of prevalence as possible. As such, a fast and efficient method to process flies is required. In previous chapters, I develop molecular tools to improve efficiency of screening. However, a remaining bottleneck is the extraction of DNA from flies. The method of DNA extraction can have important ramifications for downstream molecular processing. The analytical sensitivity of trypanosome detection using PCR has been shown to be directly affected by the extraction method used (191). With the number of trypanosomes within wild flies unknown, it is essential that DNA extraction is able to produce DNA yields of the highest quality possible.

The most widely used DNA extraction method from tsetse requires the use of silica-based columns and/or use of a centrifuge. The centrifuge draws the sample through the silica membrane, nucleic acids bind to this membrane while the remainder of the sample passes through (Figure 18). The use of column-based extraction incurs high costs, particularly when large numbers of samples are processed. Additionally, processing time is relatively long at approximately 1.5 hours following sample preparation (192).

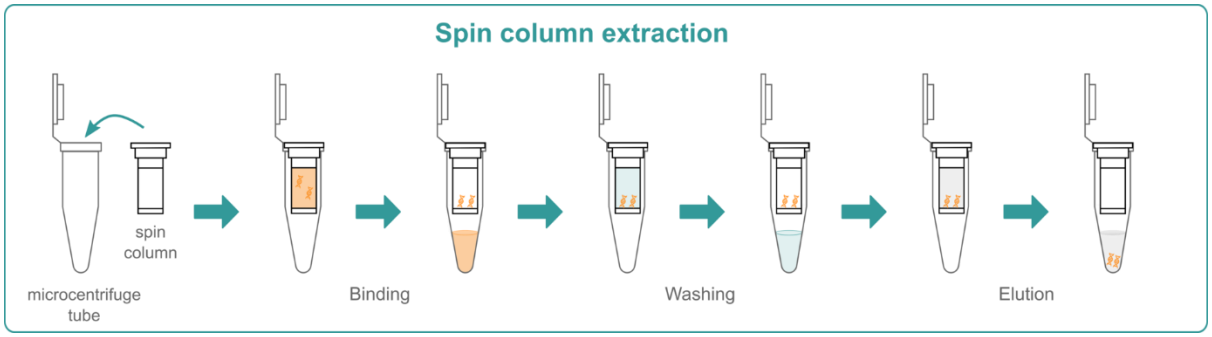


Figure 18 Column based DNA extraction. Source: Integra Biosciences

One approach that may offer a faster and cheaper method of extraction is the use of magnetic beads. The potential for their use in the purification of nucleic acids was first described in the 1990s(193,194) . There have been a number of commercialized kits developed since, however many are prohibitively expensive. The magnetic beads used in DNA extraction are 20-30nm in size made from iron oxides which demonstrate superparamagnetic properties when in the presence of an external magnet. As a result, the beads can perform solid-phase reversible immobilization, meaning bound nucleic acids can be removed from the beads following the removal of the magnetic field. Once the DNA is bound to the beads an external magnetic source is used to attract the beads to one side. Once the beads are in place and no longer mobile, they are washed several times. Following washing, the magnetic field is removed, and the beads are eluted in elution buffer, releasing the purified DNA (Figure 19). Furthermore, magnetic bead protocols do not require centrifugation, enabling scale-up without the addition of more equipment.

Figure 19 DNA extraction using magnetic particles. Source: Cytiva Life Sciences

MagnaExtract (192) is low-cost novel magnetic bead-based extraction method for which the beads can be made up easily by the user. This method has been evaluated for the molecular detection of antimicrobial resistance genes in freshwater samples in Malawi. It was found to yield greater DNA from samples when compared to the Qiagen DNeasy kit and performed as well as a commercially available magnetic bead-based kit (192).

Here, I describe the application of an existing low-cost magnetic bead-based methodology to extract DNA from tsetse. I further optimize the protocol to improve the methods for trypanosome detection and quantify the performance of the extraction in terms of overall DNA yield, quality and the limit of detection.

Data available on the parasitemia of animals and humans is scanty, however no data is available on the parasite load of tsetse. Studies of parasitaemia of animals experimentally infected with *T. congolense* found parasite loads of up to 10,000/mL parasites in waterbuck and oryx and 1,000,000 /mL in cattle and eland(195). Parasitaemia in cattle appears to fluctuate prior to becoming a chronic infection and so the number of parasites reflect this(196). Parasite loads in humans with gambiense HAT range between 100 and 10,000 parasites/mL (53). To date, studies of wild tsetse classify flies as either infected or

uninfected with no data on parasitemia (197–199). Here, we use Ct values produced using serial dilutions of known concentration of trypanosome stabilates to infer parasite numbers in wild caught tsetse from Tanzania.

4.3 Materials and Methods

Initial proof of principle studies were carried out using laboratory reared *G. m. morsitans* fed bloodmeals made up by spiking 200µl thawed *T. brucei brucei* GFP J10 to five mL of defibrinated horse blood. Blood was heated to 37°C and teneral flies were fed through a silicon membrane. Seven days post-feeding, flies were killed and stored whole at -20°C. Ten flies were extracted using Qiagen DNeasy (protocol outlined in chapters two and three) and ten using MagnaExtract following the protocol outlined in Byrne *at al* (192). Flies were thawed at room temperature prior to extraction and placed in individual 1.5mL collection tubes using forceps which were cleaned between each fly using 10% bleach to prevent contamination. Flies from both groups were homogenized by adding a stainless-steel ball bearing into each collection tube along with 180µ ATL buffer and 20µl Proteinase K. Tubes were then sealed and samples lysed in a TissueLyser for 20 seconds at 15Hz.The total homogenate (200µl) was split into two aliquots of 100µl, one for each extraction method. In the first instance, MagnaExtract samples were processed directly from this point whilst DNeasy samples were incubated at 56°C overnight as per protocol.

Briefly, the MagnaExtract samples were then processed by placing samples onto a heat block set at 95°C for 10 minutes after which they were vortexed and centrifuged at 8000RPM for five minutes. The supernatant was retained and the pellet discarded. 100µl of the supernatant was added to 100µl AMPure (Beckman Coulter, USA) magnetic beads and incubated at room temperature for five minutes. The solution was pelleted using a magnetic rack and the supernatant discarded. The beads were washed with 500µl 70% ethanol, and the solution pelleted once more using a magnetic rack. DNA was eluted in 30µl

nuclease-free water and carefully removed from the pelleted magnetic beads. Extracted DNA was stored at -20°C until needed for analysis.

4.3.1 Optimisation

Following initial proof of concept extractions on 10 samples, the following investigative experiments were conducted to optimize the protocol for tsetse:

1. The addition of an overnight lysis step to improve cell lysis. Flies were homogenized as previously described, placed in a hybridization oven set to a temperature of 56°C and an oscillator-rotor intensity of 5 for overnight incubation.
2. A range of volumes of magnetic beads were added to investigate whether DNA yield was dependent on the volume added during extraction. A sub-sample of *T. brucei brucei* infected flies (n=10) were extracted using two times, four times and eight times the volume of beads stated in the original protocol (referred to below as MagnaExtract 2x, 4x and 8x).

During each experiment, all MagnaExtract samples were matched with a sample produced by extracting DNA from a fly using the DNeasy method (n=10). Throughout, all protocols were assessed based on overall DNA yield (ng/ul) and DNA purity. DNA purity scorings were calculated using absorbance ratios at specific wavelengths, specifically 260, 280 and 230 m using a NanoPhotometer NP80 (Implen, Germany). This was first described by Warburg and Christian (200) in 1942 and is based on the Beer-Lambert Law which states:

$$OD = eCb$$

Where the extinction coefficient (e), the sample concentration (C) and optical pathlength (b) are used to calculate the optical density (OD). Two ratios are used to determine DNA purity: A260/A280 and A260/A230. An A260/A280 value of ~1.8 is regarded as an indication of a pure DNA sample, a value > 2 is regarded as high whilst a value of under 1.7 is considered low. When examining the ratio of A260/A230, a value of between 2.0-2.2 is ideal. Low ratios may be indicative of the presence of residual phenol, guanidine or carbohydrates.

4.3.2 Evaluation of optimized extraction

DNA extraction efficiency, the limit of detection and amplification success using qPCR was evaluated by spiking known numbers of trypanosomes into samples of uninfected laboratory reared *G. m. morsitans* 48 hours post-bloodmeal. Flies were stored individually and spiked with a dilution series of either *T. b. brucei* or *T. congolense* Savanna stabilates from cultures grown at LSTM. The concentration of parasites in each stabilate was quantified using a hemacytometer. A coverslip was placed over the counting chambers and each chamber charged with 10µl of stabilate. The total number of trypanosomes (alive and dead) overlying four x 1 mm^2 areas of the counting chambers was recorded. The counting of trypanosomes was repeated for a total of two replicates and the average calculated. The concentration of trypanosomes per µl was deduced using the following formula:

$$Trypanosomes\ per\ \mu l = \frac{total\ trypanosomes\ in\ 4mm^2}{4}$$

A dilution series was created ranging in concentration from 100,000 trypanosomes/mL to 1 trypanosome/mL. Ten flies per dilution were spiked with 200µl of respective dilutions.

Flies were homogenized and two aliquots of homogenate produced, one for DNA extraction using the Qiagen DNeasy, the other for the optimized MagnaExtract protocol developed by the previous optimization experiments. Having known numbers of trypanosomes in a sample produced further data which allowed the evaluation of whether qPCR Ct values correlated to the number of trypanosomes in a fly. These data could potentially inform whether pooling of flies during DNA extraction would be possible (based on a 1:10 dilution leading to an increase in Ct value by 3.3 cycles). Finally, the Ct values obtained through the serial dilution experiments were used to estimate the natural parasitaemia of flies previously found to be *T. brucei s. l.* positive from Tanzania. The sample collection is outlined in chapter three.

4.3.3 Statistical analysis

Data handling, analysis and testing for significance were performed using R (3.5.5) (R, 2020) Kruskal-Wallis one way analysis of variance with Dunn's post-hoc test was performed to identify differences in DNA yields using each extraction method.

4.4 Results

4.4.1 DNA yield

Unaltered protocols

Comparison of the mean DNA yield between the DNeasy and original MagnaExtract protocols found that the DNeasy method produced yields over 15 times larger than those produced by MagnaExtract (Table 14). The yield using DNeasy ranged from 0.65-254.05 ng/ul whilst Magnaextract yields were significantly lower and demonstrated a smaller range of 0.5-34.65 ng/ul (Table 14,Figure 20).

Table 14 Minimum, mean, median and maximum DNA yields for DNeasy versus unaltered MagnaExtract, MagnaExtract with an overnight lysis step (MagnaExtract over), MagnaExtract with two, four and eight times the volume of beads in addition to overnight incubation. Magnaextract: unmodified MagnaExtract, Magnaextract_2x: MagnaExtract protocol with overnight lysis and 2 times beads Magnaextract_4x: MagnaExtract protocol with overnight lysis and 4 times beads, MagnaExtra_8x: MagnaExtract protocol with overnight lysis and 8 times beads and Magnaextract_over: Magnaextract protocol with overnight incubation.

Method	**DNA yield (ng/µl)**			
	Minimum	Mean	Median	Maximum
DNeasy	0.65	156.79	125.98	254.05
MagnaExtract	0.50	10.20	10.10	27.35
MagnaExtract over	0.00	129.27	31.00	668.60
MagnaExtract over 2x	29.40	164.70	211.91	647.00
MagnaExtract over 4x	1.50	75.70	92.60	240.50
MagnaExtract over 8x	6.10	72.80	87.03	201.80

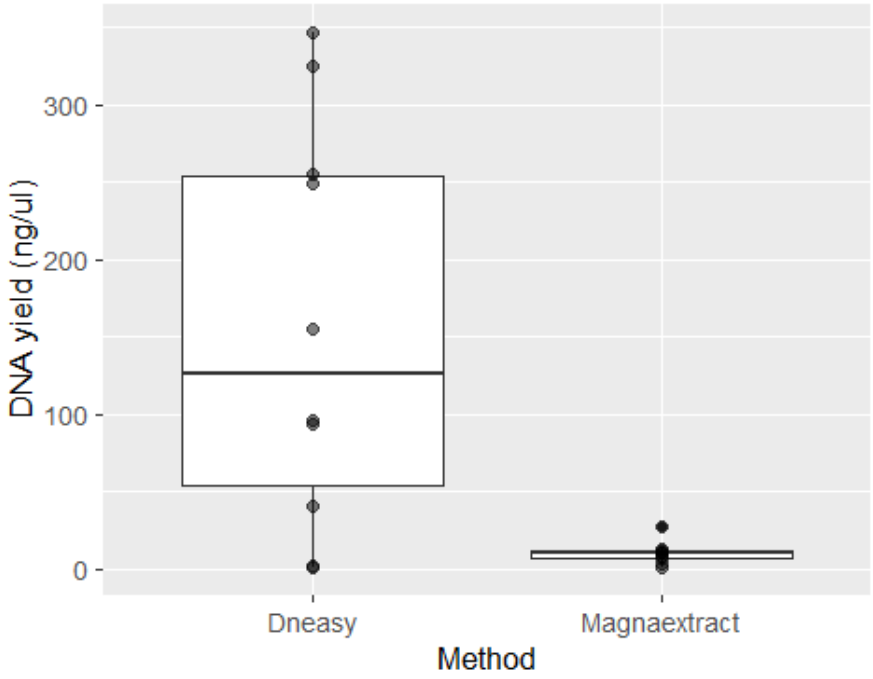


Figure 20 Overall DNA yield (ng/ul) for 10 flies using either DNeasy or unaltered Magnaextract methods

Addition of overnight incubation

The addition of an overnight incubation in ATL buffer and Proteinase K solution was found to increase mean MagnaExtract yields from 10.20 to 129.27ng/µl. This difference was evaluated using Tukey multiple pairwise-comparisons and was not found to be significant (p= 0.342)

Volume of magnetic beads

Altering the volume of beads added during the extraction process had an effect on DNA yield with 2x, 4x and 8x the amount of beads stated in the original protocol producing mean yields of 211.91, 75.70 and 87.03ng/µl respectively (Figure 21). The addition of twice as many beads (200µl) resulted in significantly higher DNA yields than the unaltered MagnaExtract protocol (p=0.04). Mean DNA yields for MagnaExtract with twice as many beads where higher those extracted using DNeasy (211.91 ng/µl and 151.08ng/µl

respectively). On the ten matched flies the range of DNA yield was larger for MagnaExtract (43.65-647.00ng/µl) than DNeasy (37.30-268.95ng/µl).

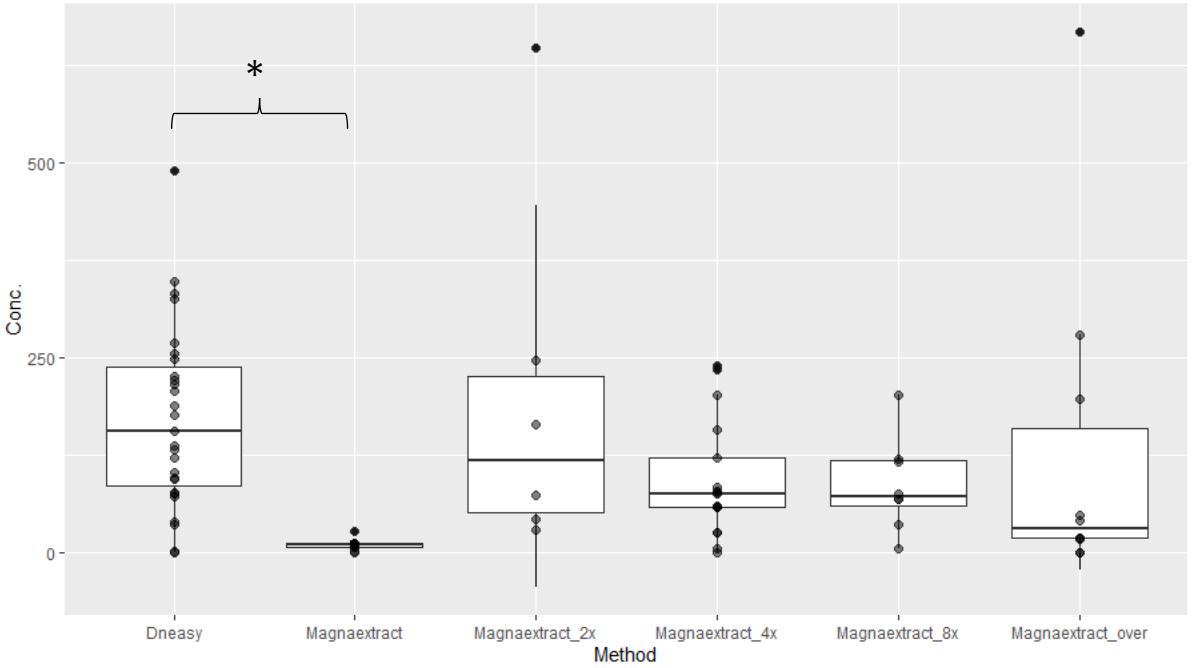


Figure 21 DNA yield for samples extracted using MagnaExtract and DNeasy protocols. Dneasy :all samples extracted using unmodified DNeasy protocol (n=50), Magnaextract: unmodified MagnaExtract, Magnaextract_2x: MagnaExtract protocol with overnight lysis and 2 times beads Magnaextract_4x: MagnaExtract protocol with overnight lysis and 4 times beads, MagnaExtra_8x: MagnaExtract protocol with overnight lysis and 8 times beads and Magnaextract_over: Magnaextract protocol with overnight incubation. All MagnaExtract extractions were carried out using 10 flies per group.

4.4.2 DNA purity

The purity of DNA extracts were not found to vary greatly between methods based on the purity ratios (Table 15 and Table 16). Observation of mean A260/A280 purity scores found that only DNeasy and Magnaextract 2x produced samples of ideal and high quality respectively. The largest range of purity scores was found using MagnaExtract 4x whilst the smallest was in the MagnaExtract 2x (Table 15). No method produced samples within

the desired range of a A260/A230 ratio of 2-2.2 (Table 16), however, MagnaExtract 4x produced samples with a mean ratio of 2.11.

Table 15 A60/A280 ratios for samples extracted using each method

	A260/A280 ratio			
Method	**Minimum**	**Mean**	**Median**	**Maximum**
DNeasy	0	1.83	2.23	2.31
Magnaextract	-7.5	0.75	1.5	2.83
Magnaextract over	0	1.57	1.98	2.25
Magnaextract 2x	1.64	2.1	2.15	2.6
Magnaextract 4x	-15	0.93	2.06	2.31
Magnaextract 8x	0.62	2.05	2.22	2.47

Table 16 A260/A230 ratios for tsetse samples extracted using each method

Method	**Minimum**	**Mean**	**Median**	**Maximum**
DNeasy	-17.54	1.65	2.97	7.09
MagnaExtract	-59.25	-5.92	-0.13	0.87
MagnaExtract over	-3.41	2.4	0	22.65
MagnaExtract 2x	-2.54	2.46	1.58	12.15
MagnaExtract 4x	-766	2.11	2.73	9.29
MagnaExtract 8x	-2.71	4.98	2.29	28.64

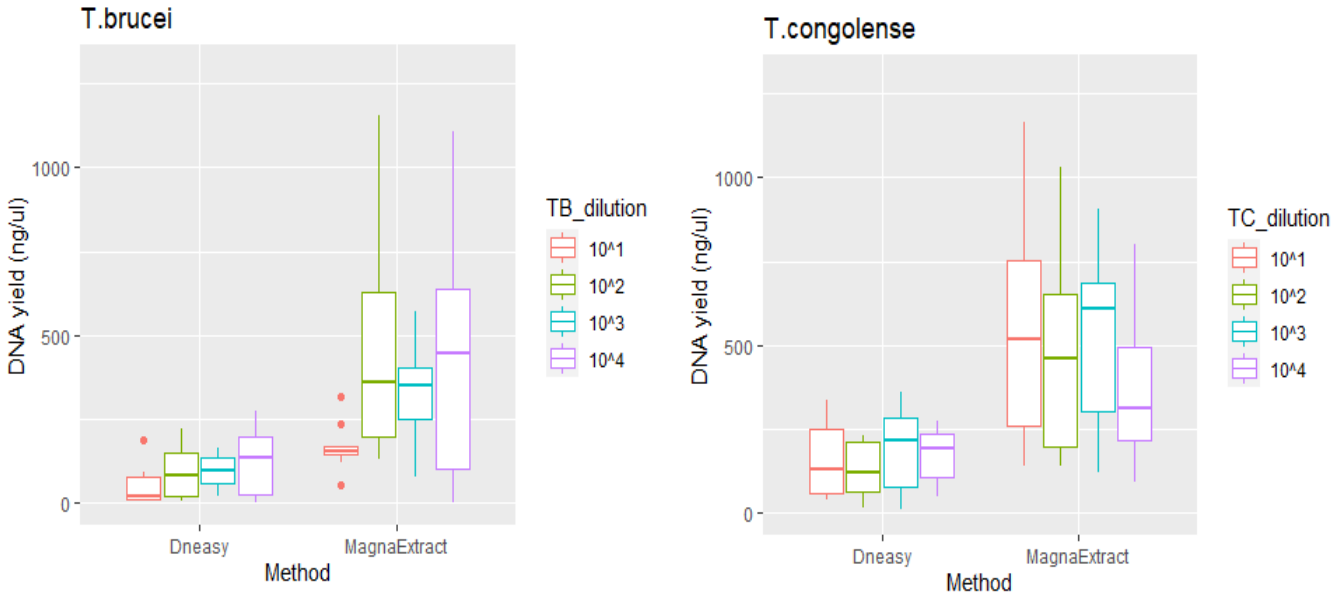


Figure 22 DNA yield (ng/ul) for flies spiked with T. b. brucei (left) or T. congolense Savanna (right) stabilates.

4.4.3 Evaluation of optimized extraction

Overall, DNA yield was higher for the 2x bead method with overnight incubation when compared to DNeasy on both set of trypanosome spiked flies and so this method was taken forward as the optimized methodology (Figure 22).This yield includes both DNA from any trypanosomes present and from the fly carcass. All DNA extracts were suitable for qPCR with all methods amplifying successfully using either *T. b. brucei* or *T. congolense* Savanna species-specific primers. Both methods successfully extracted samples which contained 10 trypanosomes/mL and consequently all samples amplified well with species-specific qPCR primers described in chapter 3 (Table 17).

Table 17 Species-specific qPCR primers for T. brucei s.l., T. vivax and T. congolense s.l.

Primer	**Species**	**Primer sequence 5'-3'**
TB2 Forward	*T. brucei s.l.*	GCGCAGTTAACGCTATTATACACA
TB2 Reverse		AAGAACAGCGTTGCAAACTT
TC Forward	*T. congolense s.l.*	AGAAACACGGGAGCGGTC
TC Reverse		GAGGCCATAATCTCCCAAGGA
TVM Forward	*T. vivax*	TCGCTACCACAGTCGCAATCGTCGTCTCAAGG
TVM Reward		CAGCTCGGCGAAGGCCACTTGGCTGGGGTG

Table 18 Ct values for tsetse spiked with known numbers of trypanosomes

Trypanosome	Dilution	DNeasy			MagnaExtract		
		Mean Ct	**Min-max Ct**	**Range**	**Mean Ct**	**Min-max Ct**	**Range**
T. congolense Savanna	10^4 tryps/mL	**25.332**	23.87-26.27	2.4	**23.469**	22.23-24.51	2.28
	10^3 tryps/mL	**28.462**	27.69-28.97	1.28	**27.403**	24.68-33.04	8.36
	10^2 tryps/mL	**32.196**	30.42-33.31	2.89	**30.784**	28.11-33.08	4.97
	10^1 tryps/mL	**36.278**	34.01-39.97	5.96	**33.743**	31.65-35.72	4.07
T. brucei brucei	10^4 tryps/mL	**24.262**	21.79-31.93	10.14	**27.24**	23.51-34.96	11.41
	10^3 tryps/mL	**26.958**	25.83-27.86	2.03	**29.404**	26.59-36.91	10.32
	10^2 tryps/mL	**32.782**	29.85-33.55	3.70	**31.824**	29.89-32.78	2.89
	10^1 tryps/mL	**35.606**	33.33-38.50	5.17	**35.5425**	33.26-37.50	4.24

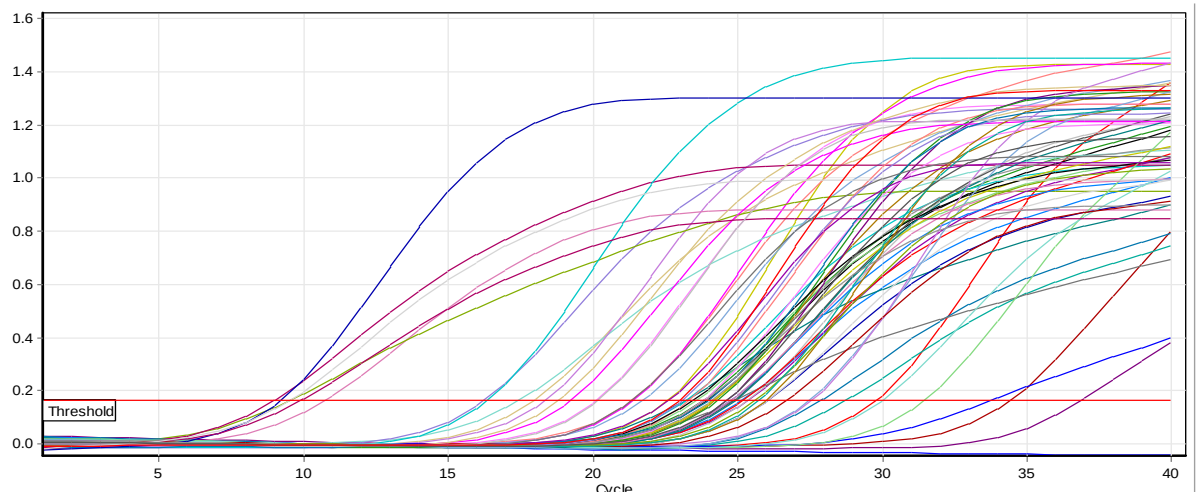


Figure 23 Amplification curves of T. brucei s.l. positive wild tsetse

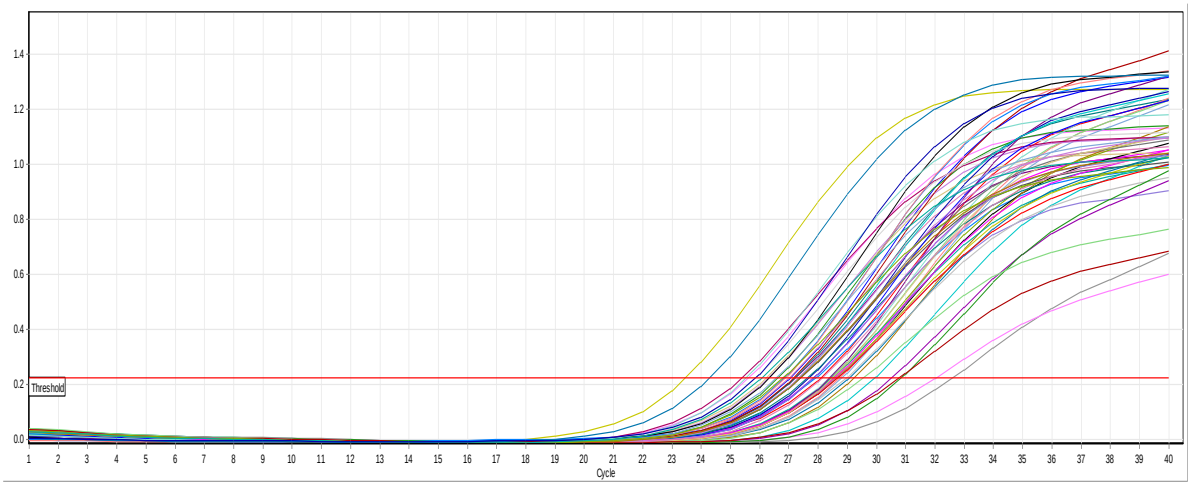


Figure 24 Amplification curves of T. vivax positive wild tsetse

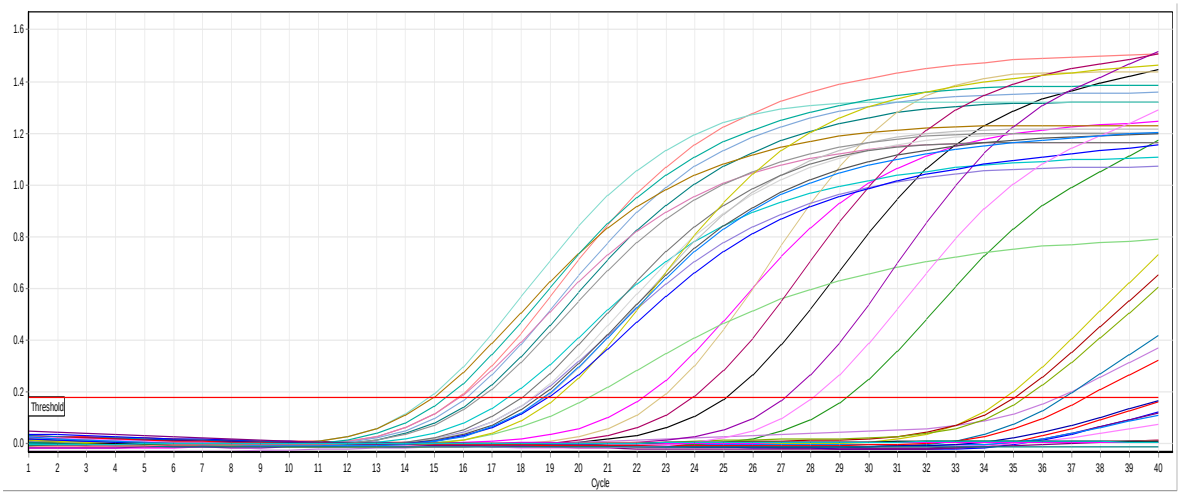


Figure 25 Amplification curves of T. congolense s.l. positive wild tsetse

The range of Ct values for each dilution showed more variation in MagnaExtract samples when compared to those extracted using Dneasy (Table 18). Mean Ct values obtained for each dilution were generally lower for MagnaExtract with no variation between both methods over 3 cycles. All dilution series amplified successfully indicating that both DNeasy and MagnaExtract could be effectively used in samples with trypanosomes numbers as low as 10 parasites/mL.

When these Ct values were applied to extrapolate the number of trypanosomes in wild flies from Tanzania the ranges indicated large variation in terms of number of trypanosomes present (Figure 23,Figure 24,Figure 25). The range for *T. brucei s.l.* infected flies was particularly broad, spanning over 27 cycles (Figure 23) with highest number of parasites implied by a Ct value of 9.01 with lowest to 36.88 cycles (Table 19). Flies positive for *T. congolense s.l.* recorded Ct values spanning 15 to 37.74 cycles,with a mean of 23.01 cycles. Tsetse positive by qPCR for *T.* vivax showed the smallest range of Ct values and the lowest mean number of cycles of 27.88 . Using mean Ct values as a proxy indicator of trypanosome numbers, this suggest that in wild flies found carrying *T. brucei s.l.* and *T. congolense* parasites the average number of trypanosomes is approximately 10^4 (Table 19). Flies positive for *T. vivax* had a mean number of 10^3 parasites with the positive samples showing the smallest amount of variation. The Ct values obtained using this subset of flies suggest that pooling flies in pool sizes up to ten individuals could be a potential strategy going forwards when screening large numbers of wild caught tsetse.

Table 19 Ct values of trypanosome positive flies from Tanzania

Species	**Mean Ct**	**Estimated numbers of trypanosomes based on mean Ct**	**Ct range**
T. brucei s.l.	22.99	10^4	9.01-36.88
T. congolense Savanna	23.01	10^4	15.00-37.74
T. vivax	27.88	10^3	23.51-32.62

4.5 Discussion

In this chapter, the applicability of MagnaExtract as an alternative DNA extraction method to column-based kits in the context of African trypanosomiasis xenomonitoring was examined. A major benefit of utilizing xenomonitoring as part of a disease control strategy is that tsetse are simple to collect and process and the presence of the disease in local vectors indicates that the transmission of the trypanosomiasis in the area is ongoing. With increasingly low prevalence of wild tsetse carrying human or animal trypanosomes as a result of long-term disease control (198,201), the number of flies needing to be processed to achieve sensible estimates of transmission is becoming both logistically and economically challenging. Therefore, there is incentive to investigate more high throughput, cost-effective and field friendly alternatives for DNA extraction.

DNA extraction methods based on magnetic bead technology provide multiple advantages over column-based methods, namely the simplicity of the DNA concentration stage which does not require costly spin columns, centrifuges and wash buffers. This results in a faster processing time and a simpler and potentially field-friendly protocol. Furthermore the use of MagnaExtract, compared to column-based extraction kits is more economical costing £1.43 per sample in comparison to the Qiagen DNeasy blood and tissue kit cost of £2.77

(192). In the context of HAT xenomonitoring, extraction of DNA from tsetse is the most costly element of vector screening with high-throughput qPCR remaining relatively inexpensive once qPCR machinery purchase costs have been omitted. Initial analysis of the MagnaExtract protocol with no alterations found that it performed poorly in comparison to DNeasy. With the further optimization which included the addition of an overnight lysis step and doubling of the magnetic bead volumes resulted in yields that were comparable if not better than DNeasy. The purity of samples extracted using DNeasy 2x based on A260/280 ratios indicated that extracted DNA had lower levels of contaminating phenols and proteins. Higher volumes of beads (400µl and 800µl) produced lower yields, perhaps due to the beads reaching a point of saturation and therefore being unable to bind to any remaining DNA.

DNA from trypanosomes extracted using this novel method successfully amplified during qPCR. MagnaExtract was able to amplify with subsequent successful detection of low numbers of trypanosomes, with positive detection at 1 parasite/mL. Following the overnight lysis, the modified MagnaExtract protocol presented here takes 30 minutes to carry out following the lysis step in comparison to 2-3 hours required for the DNeasy method.

The data presented here represent the first attempt at quantifying the number of trypanosomes in wild tsetse. Ct values were used as a proxy for trypanosome number and indicated flies carry a large range of parasites with mean number of parasites at around 10^3 to 10^4 /mL. A study by Gibson *et al* (1997) investigating the cycles of differentiation and multiplication of *T. brucei* in tsetse indicated that following a bloodmeal the numbers of parasites increases for the first three days. Following this, the number of trypanosomes either continued to increase and remained at a high level or began to drop leading to some flies having undetectable parasitaemias within five days post-bloodmeal (202). If this

phenomenon is to be extrapolated into wild populations this fluctuation may explain the wide range of trypanosomes found within field samples. The presence of trypanosome DNA does not mean flies are infectious and so it is important to bare this in mind and this data cannot be used to directly infer active transmission of disease.

The optimized MagnaExtract protocol described in this chapter offers a potential alternative to column-based extraction kits when screening trypanosomes in tsetse. Furthermore, this method only requires a mini-centrifuge and a magnetic stand, making this a potential option for high throughput DNA extraction that can be conducted in the field. One hurdle that continues to face the faster extraction of DNA from tsetse particularly in the field, is the need to homogenize the carcass of the flies in order to release any trypanosomes. The large mass of tsetse and the relatively large amount of force required to sufficiently macerate the samples, particularly at scale requires a mechanical tissue lyser. Laboratory pestles, grinders and mills are available, however the individual homogenising of flies is often unrealistic with very low infection rates in vectors. Future evaluation of alternatives to homogenizing large numbers of flies is required and would provide a significant step towards enabling a high throughput system for screening tsetse.

5 Evaluating the rate and risk of tsetse population rebound following the withdrawal of vector control

5.1 Abstract

Introduction Following elimination of gHAT, focus will shift to the long-term withdrawal of vector control. Tiny Targets are highly effective at controlling tsetse populations and their withdrawal or the interruption of scheduled deployment has the potential to result in a rapid rebound of fly populations and potentially risk a resurgence of disease. The risk of rebound and the implications for HAT transmission are investigated using a combination of deterministic model simulations and empirical field data in areas of northern Uganda where deployment of Tiny Targets has been halted

Methods Rebound of tsetse in two scenarios was modeled using the simulation model 'Tsetse Muse'. One scenario assumed even habitat quality, and the other with increasingly poor habitat across the transect as might be expected in the upper reaches of a river system. Simulations consisted of one year of vector control followed by two years of no target deployment. To test the model predictions, I also analysed catches from monitoring traps deployed in areas where Tiny Targets were withdrawn after eight years of control in Maracha district in 2019. Abundance, age structure and trypanosome infection rates were assessed from tsetse caught in monitoring traps operated for 12 months when targets were present and up to 24 months after they had been withdrawn. Previously collected trial data was also used to investigate the effect of interruptions in vector control deployment on tsetse.

Results Simulated rebound was fastest in the even habitat scenario with fly densities reaching up to 73% of pre-intervention values two years after vector control ceased. Rebound was slower in the uneven habitat with densities only rebounding to 29% of their prior value. In both scenarios, rebound was fastest on the downstream edge due to the population being bolstered by reinvading flies. Field data showed that not deploying targets for a single round of Tiny Target deployment did not result in a significant increase in tsetse numbers. Similarly, full withdrawal of control did not result in a statistically significant increase in tsetse numbers after two years. No difference was seen in mean age of flies or trypanosome infection rates.

Conclusions On the one hand**,** the results indicates that the risk of rebound is less than previous predictions. Fly numbers, age and infection rates remained consistently low following two years of withdrawal, suggesting a low risk of gHAT transmission. These findings indicate that conservative and strategic withdrawal of control may not result in a rapid rebound of flies and increased risk of disease. On the other hand, opportunities for xenomonitoring rebounding tsetse populations may be less than previously anticipated.

5.1.1 Contributions

The analyses within this chapter use a number of different tsetse trap datasets. The first is from traps deployed following my design in Maracha and Arua between 2019-2021. Historic data from traps deployed in Maracha by the LSTM Tsetse Ecology and Control Group (TEC Group) and district entomologists in Uganda were used to provide additional data on long-term population dynamics. Data on the temporary interruption to deployment were produced by members of the TEC group in LSTM and are included in a recent publication currently under submission. In this chapter, I designed and conducted all the data analyses.

5.2 Introduction

The history of human African trypanosomiasis is characterised by resurgent epidemics, often associated with civil unrest, economic instability and the removal of disease control programs leading to rapid increases in transmission. The target of eliminating gHAT as a public health problem by 2020 was achieved by Togo and Côte d'Ivoire with countries such as Uganda on the brink of WHO validated elimination in 2022. The interruption of gHAT transmission is now targeted for 2030(153). As such, the focus now turns to the maintenance of disease elimination and the new set of challenges this poses. With highly reduced number of cases, the funding for disease control will likely be stretched despite the current funding for elimination targets already being "far from sufficient"(203).

Gradual withdrawal of vector control is likely to play a key element in the post-elimination era. With reduced vector control the population of tsetse will begin to recover. The rate at which this happens will dictate the degree of HAT resurgence risk. With human migration from HAT endemic areas to historic foci being a key threat to the maintenance of elimination, understanding the risk of Tiny Target withdrawal is critical. Tsetse are highly

mobile travelling up to ~1km per day (204), this degree of movement has important implications for the risks of population rebound. Flies are likely to migrate into areas once targets are removed, helping the recovery of local populations. This has implications for areas with continued control as neighbouring populations of flies can penetrate 5km into a area with ongoing vector control(205,206).Models of tsetse population dynamics predict that a tsetse population will rebound within three years with reinvasion (207,208).

However, the removal of targets and rebound of vector populations could also provide potential new opportunities to implement a xenomonitoring surveillance strategy. Infection rates in tsetse populations, even in areas where incidence of gHAT is relatively high, are low (<0.1%, (190)). The difficulty of sampling sufficient numbers of tsetse to provide a meaningful measure of transmission is made even more difficult when tsetse control is ongoing. With the effectiveness of Tiny Targets such that populations of tsetse are reduced by 90%, collecting sufficient numbers of flies to screen for infection is extremely difficult to achieve. However, as vector control is scaled back, tsetse numbers will increase and xenomonitoring of a rebounding vector population may provide a cost-effective strategy for quantifying disease risk. Accordingly, this chapter describes the analysis of tsetse populations where vector control has been interrupted or withdrawn.

West Nile in Uganda is a historic focus of gambiense sleeping sickness which has experienced long term vector control for the last decade. Here, *G. fuscipes fuscipes,* the local vector of *T. b. gambiense*, has been controlled using deployment of Tiny Targets since 2011 where they were initially introduced into two districts (Arua and Maracha) to assess their impact on vector populations at scale (207). Following the successful reduction of tsetse populations to less than 10% of their previous value, Tiny Target deployment was expanded in 2014 across five districts and further expanded in 2017 to cover a total area of 3900km^2 and seven districts (Figure 26) (209).

The location of tsetse control has been dictated by the occurrence of gambiense HAT cases or as a result of potential increased risk of transmission such as the movement of ~1 million South Sudanese refugees across the Ugandan border in 2016. The withdrawal of Tiny Targets is based on an area experiencing at least five full years of control along with no new HAT cases reported in the last five years. Based on these criteria being met and maintained, the overall area of control in Maracha district was scheduled to be reduced from 170km^2 in 2019 to 0km^2 in 2020 onwards (Figure 27).

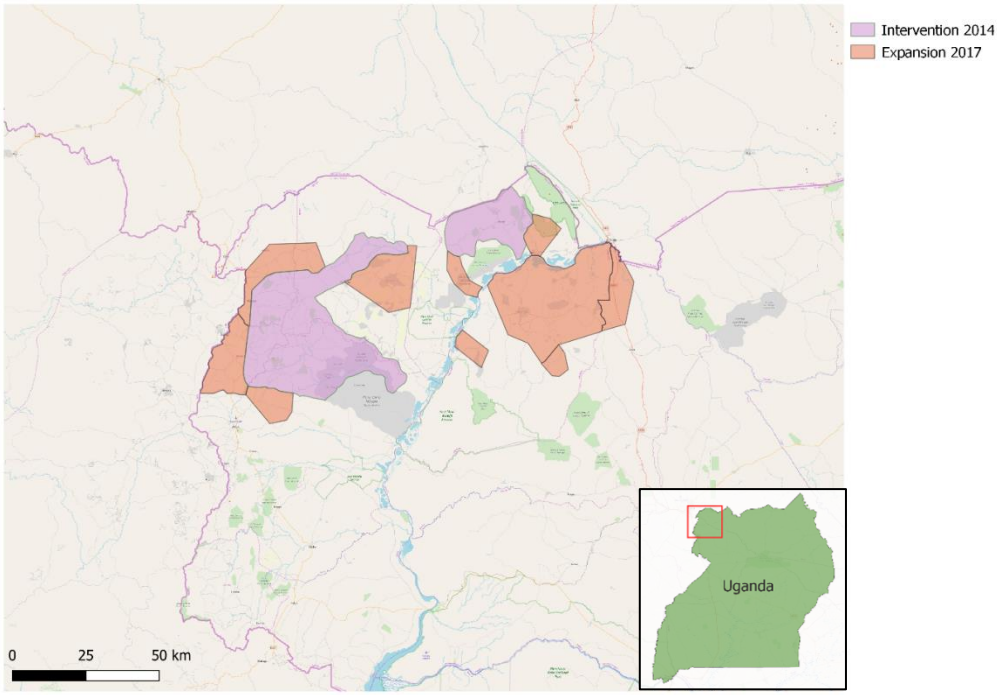


Figure 26 Map of West Nile indicating areas of tsetse control, with initial deployment in 2014 and expansion in 2017

In this chapter I simulate the population dynamics of tsetse following the withdrawal of targets and contrast this with longitudinal field data from tsetse traps. With the field data collected, risk of rebound was assessed by the number of tsetse caught per trap, the trypanosome infection rate as assessed using both dissection and PCR and the age of tsetse based on ovarian category.

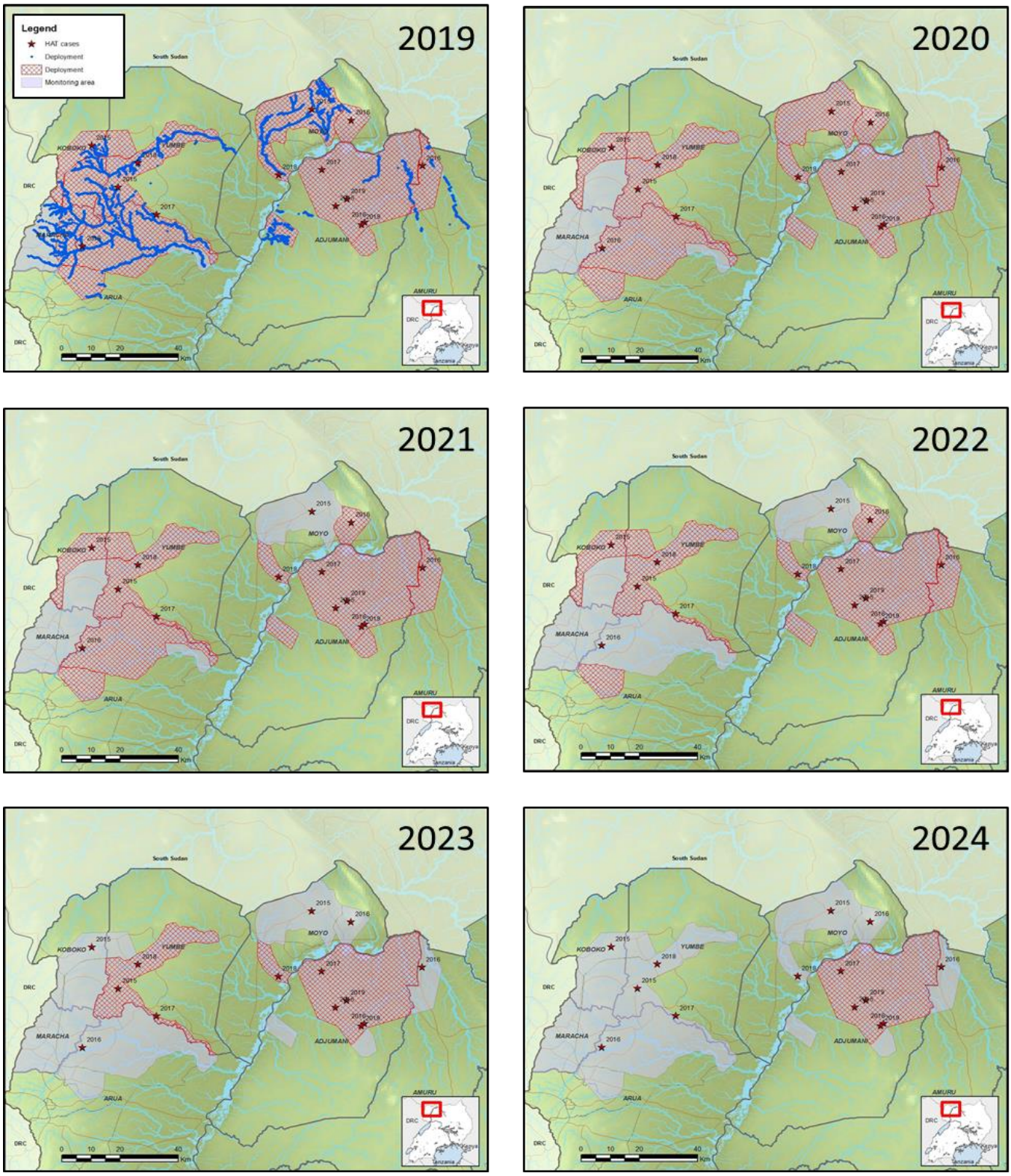


Figure 27 Map of West Nile demonstrating the stages of vector control withdrawal between 2019 and 2024. Hatched areas indicate where Tiny Targets are deployed, grey areas indicate where targets were previously deployed but have now been withdrawn. Image taken from (222)

Objectives

- The overall objective of this chapter was to assess the risk of trypanosomiasis rebound when withdrawing tsetse control in a historic HAT focus.

This was done through:

- Empirical assessment of risk by modelling rebound scenarios using the simulation model Tsetse Muse(210).
- Longitudinal surveillance of tsetse numbers using monitoring traps in areas where control has been withdrawn alongside a control area with no vector control.
- Examination of any changes in the age structure of tsetse populations once control has been withdrawn
- Evaluation of trypanosome transmission dynamics during and post-vector control.

5.3 Methods

5.3.1 Tsetse muse

The potential impact of removing tiny targets on the tsetse populations was modelled using Tsetse Muse, a deterministic simulation model of tsetse population abundance and distribution(210). Tsetse muse models a vector population made up of (i) pupae, (ii) wild adult males, (iii) sterile adult males, (iv) virgin females, (v) breeding females (vi) sterile-mated females, and (vii) females that were fertile-mated, but too late to breed in the present larval cycle. Initial parameters for the model were set out as in Table 20

Table 20 Features of the simulated population at stable carrying capacity taken from (201)

Feature			Males	Females	Source
Adults per km^2 of habitat			2500	5000	(211)
Mean adult age			24	44	(212)
Pupal period			28	26	(212)
Age at sexual maturity			5	3	(145)
Age at production of first larva			-	16	(212)
Interlarval period			-	9	(212)
Maximum adult lifespan			89	178	(213)
Death rates	Pupae per pupal period		25	25	(212)
	Eggs/larvae per larval period		5	5	(212)
	Adults per day	Average	6.14	3.07	(212)
		First day	14.21	13.62	(212)
		Young adult	2.84	1.36	(212)
		Last day	8.52	4.09	(212)
Average daily displacement, metres			124.7	106.8	(214)

To model a population with the potential for reinvasion from surrounding areas the 'non-isolated population' setting was used. The model simulates a riverine habitat split into 60 contiguous cells with each cell representing 1 kilometre (Figure 28). The 'front' indicates the point at which reinvasion can occur. Within the Ugandan equivalent, the 'front' would be represented by the areas at which tsetse control was discontinued. The simulations themselves were split into three distinct phases, first the baseline prior to any control, second the deployment of tsetse targets across 20 kilometres of habitat (from band 20 to band 40), phase three where tsetse control was removed entirely and the populations monitored for 360 days year and phase four where the vector population was monitored

for a further 360 days. As removal of all control in Tsetse Muse is not possible the simulation was set to meet minimum requirements with tsetse targets deployed over 1km at the front with a mortality rate of 0.01% on the tsetse populations in order to simulate no control as closely as possible. Over each phase, the total number of tsetse adults and the mean age of males and females were recorded as outputs. The starting population was 7500 flies (5000 females, 2500 males). Tsetse control was set to impose a 4% mortality rate on flies based on observational data collected on the impact of tiny targets on *G. fuscipes fuscipes* in Uganda (215)

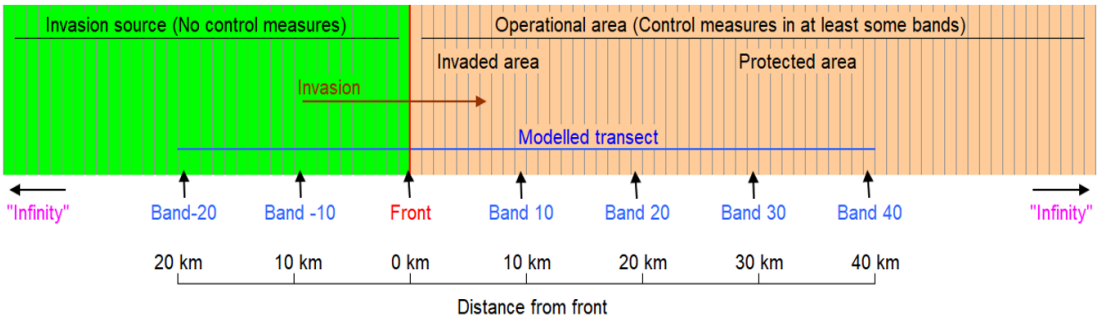


Figure 28 Layout of 60 contiguous cells in Tsetse muse demonstrating the 'front', invasion source and controlled area. Image taken from (206)

Two different simulations were run each with specific habitat mortality rates to reflect different types of habitats with regards to tsetse suitability and survival. One scenario, hereafter referred to as the 'even' scenario, modelled a transect over which the habitat was equally suitable for tsetse and so imposed an even mortality rate across all cells. The 'uneven' scenario reflected a transect over which the habitat became increasingly less favorable for tsetse. The uneven scenario simulated the general decline in habitat suitability moving upstream from a large perennial river with substantial riverine vegetation to small seasonal streams with scant vegetation near the river's source(216). Here, the mortality rate was increased by 1% per kilometer from bands 20 to 40 (Table

21). Simulation outputs were also evaluated at a specific user-defined area level. The 20km riverine intervention zone was split into: five kilometers downstream which bordered the non-intervention zone and therefore had higher likelihood of reinvasion from the neighbouring uncontrolled area, ten kilometers at the center of the intervention zone and the five kilometers located at the upstream edge (Table 22)

Table 21 Mortality rates in two scenarios run in Tsetse muse along with their respective starting mortalities

Scenario	**Baseline mortality range (%)**	**Year**	**Stage**	**N days of control**	**N days of monitoring**	**Km² controlled**	**% mortality of vector control**
Even	100	1	Control	360	0	20	4
		2	Monitoring	1	359	1	0.01
		3	Monitoring	1	359	1	0.01
Uneven	100-126	1	Control	360	0	20	4
		2	Monitoring	1	359	1	0.01
		3	Monitoring	1	359	1	0.01

Table 22 Layout of kilometer bands in Tsetse Muse output into user defined areas

Downstream	Centre	Upstream
Kilometers		
20 to 25	25 to 35	35 to 40

5.3.2 Empirical study

Field site

The study site was located in Maracha district in West Nile, Uganda. Maracha's economy is primarily based on agriculture with cassava, beans, maize and coffee being major crops of the region(217). The main species of tsetse fly found in this area is the HAT vector *G. fuscipes fuscipes*. Cattle, pigs, humans and Nile monitor lizards account for the majority of bloodmeal sources (218).

Tiny Targets

Tsetse control was carried out through the deployment of Tiny Targets, these 25 x 50cm rectangles are comprised of a 25x25cm panel of blue-coloured polypropylene cloth flanked by black polyethylene netting of the same size which is impregnated with pyrethroid insecticide. When flies are visually attracted to the targets, the black colour induces a landing response resulting in the fly picking up a lethal dose of insecticide. Tiny Targets were deployed along rivers at 100m intervals along both riverbanks at a density of 20 targets per kilometer. Targets are deployed on a six-monthly basis, due to the observation that their performance declines after six months of use(207).

Analyses of the impact of withdrawing tsetse control intentionally were carried out for two occasions. On the first occasion, deployment of targets was interrupted for six months ('Interrupted control') only and for the second ('Scale-back'), deployment of Tiny Targets ceased for 24 months.

Interrupted deployment.

As part of an initial large-scale field trial (207), Tiny Targets were deployed biennially across an area of 500 km^2 covering parts of Maracha and Arua Districts. In 2014 we compared, for one year only, catches from monitoring traps in areas where targets were

deployed once (Arua, Ayi, Olluffe), twice (Inve, Aiivu and Kubala) a year nor not at all (Koboko) (Figure 29). Targets were deployed biannually (February and July) in the original (Phase 1) Inve, Aiivu and Kubala blocks (7 x 7 km) but annually (February only) elsewhere (Arua, Ayi, Olluffe) (219). The abundance of tsetse was assessed using Pyramidal traps, each operated for ~20 days/month with catches collected and counted at 24 h intervals. Traps were deployed at 34 sites in areas with a single deployment, 31 sites for areas with two deployments and 15 sites where no targets were deployed. For further details of trap locations and intervention areas, see Tirados *et al.* 2015(207).

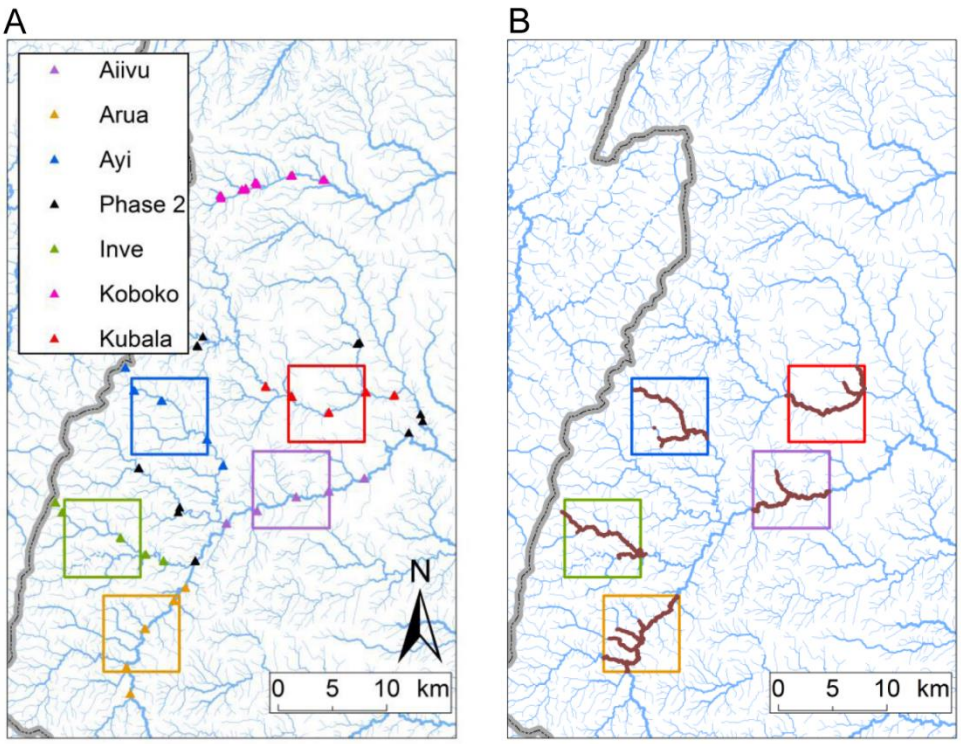


Figure 29 Locations of traps and tiny targets in NW Uganda. (A) Locations of monitoring traps for each intervention block (7 x 7 km squares) and rivers (Enyau, Oluffe and Kochi). (B) Locations of targets along rivers (indicated by brown sections) in 2013. Figure adapted from (203)

Scale-*back*

Between January 2015 and December 2019, Tiny Targets were deployed across 2500-6000 km^2 of NW Uganda (209). As part of a national strategy to scale-back tsetse control, regular deployment of Tiny Targets ceased in Maracha district from January 2020 onwards (Figure 27).

The abundance of tsetse across all intervention areas is monitored by an extensive network of ~100 pyramidal monitoring traps (20/district) (Figure 32) operated routinely by LSTM researchers based in Arua. The network includes 20 traps deployed along the Oluffe, Oru and Ayi rivers in Maracha district where Tiny Targets were deployed regularly between December 2012 and January 2020. Monitoring traps are also deployed along the Enyau River in Arua district where no tsetse control is carried out.

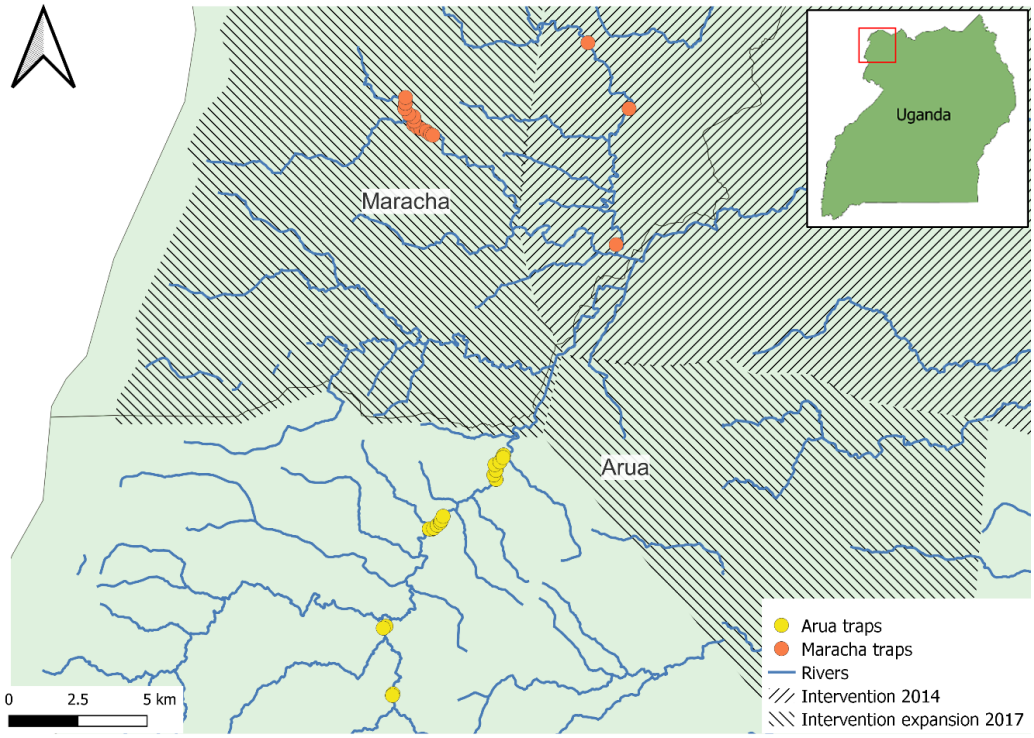


Figure 30 Location of trapping sites in Arua and Maracha

To complement these traps, a second set of traps were established specifically to monitor the rebound of tsetse in Maracha. These 12 traps, established in July 2019 were deployed solely along the Oluffe river, a large perennial river which was likely to provide a high chance of catching tsetse year-round. Catches from these traps were transported to a laboratory in Arua for analyses of age and infection status (see below).

Figure 31 Tiny Target deployed along river in West Nile, Uganda

In addition to the traps monitoring the impact of control operations in Maracha, an addition 25 traps were deployed along the Enyau river in Arua district (Figure 30). Tiny Targets are not deployed along this section of the Enyau river and hence catches provide a comparative insight into an uncontrolled tsetse population.

Analyses of age and infection status

Once flies were collected and transported from monitoring traps to the laboratory, they were refrigerated for five minutes. Following refrigeration, the sex of flies was assessed on

their external genitalia. All live female flies were then age graded by ovarian dissection using the technique described by Saunders (43). Dissections were carried out by trained laboratory technicians using a Zeiss Stemi 2000 dissecting microscope. Once the salivary glands, mouthparts and midguts were isolated they were screened for trypanosomes in a drop of 0.9% saline using a compound microscopy, either a Leica DM500 or a Zeiss Primo Star fitted with a dark-field filter. The mouthparts were separated from the head and inspected for parasites under a compound microscope at either 200x or 400x.

The salivary glands were removed through careful detachment of the head from the thorax. The head and attached salivary glands were then placed in a drop of saline and the salivary glands removed from the head.

Figure 32 Pyramidal trap deployed on the Oluffe river in Maracha district

The ovaries, spermathecae and uterus of female flies were removed by making incisions using forceps in the second abdominal segment allowing the tissues to be extracted.

Ovaries were then classified into categories one to seven depending on the developmental state of each ovary as described by Saunders (43).

Following ageing, the midguts were removed from the abdomen and macerated using dissecting forceps. Tissues were classified as positive upon visualisation of live, motile trypanosomes. All tissues and carcass from each fly were stored individually in Eppendorf tubes filled with 100% ethanol. A subset of these were transported back to LSTM for molecular analysis.

Environmental variables

The scale-back of Tiny Targets in Maracha was predicted to lead to an increase in abundance. The density of tsetse populations is also affected by environmental factors such as temperature, rainfall and normalized difference vegetation index (NDVI)(220). Accordingly, remotely-sensed data on monthly soil moisture, precipitation, NDVI and mean temperature was collected. All data was collected from a centroid located within Maracha district at 3.222606°N, 30.90008°W. Soil moisture data was collected from the European Space Agency Climate Change Initiative (https://www.esa-soilmoisture-cci.org/data) from June 2016 to December 2020, data for 2020 was not available at the time of analysis. Mean normalized difference vegetation index (NDVI) and temperature data were collected from June 2019 to December 2021 from MODIS (https://modis.gsfc.nasa.gov/data/dataprod/mod13.php). Mean monthly precipitation data was collected from the Climate Hazards Center CHIRPS (221)

General statistical methods

Statistical analyses on catch data were carried out using the open source statistical sotfware R (222). The 'glmmADMB' package was used to fit generalized lineal mixed models (glmm) to catch data using a negative binomial data distribution and a log link

function. Overall trends following the deployment of withdrawal of targets was conducted with site and day of capture specified as random effects and months a fixed effect.

For analyses where I assessed the statistical significance of differences in proportions, I fitted data to a general linear model (glm) with a binomial error distribution and a logit link function. The statistical significance of differences in catches or proportions were assessed using the 'glht' function from the 'multcomp' package. Finally, the predicted means and their 95% Confidence Intervals based on the model parameter estimates are presented.

The statistical differences in the frequency of different ovarian age classes were assessed using Chi-squared tests.

5.4 Results

5.4.1 Simulations of scale-back

Even scenario

Prior to implementing control, the starting density of flies was homogenous across all areas with mean densities of 7500 flies/km. Following one year of control, mean total fly numbers (male and female) were reduced by 95.6% downstream, 99.9% in the center and 96% in the upstream area (Figure 34). Following the removal of control for two years, tsetse numbers were predicted to rise to 88.2% of their original pre-intervention values in the downstream area, and 61.1% and 69.8% in the center and upstream areas respectively.

Uneven

The simulated starting population densities predicted that the impact of poorer habitat would result in fly densities declining from 7500 flies/km^2 to a mean of 2036.7 flies/km^2 across the riverine transect (Figure 33). One year of control reduced fly densities across all areas by 97.8% with average fly densities of 46.6 flies/km^2.

Rebound was not as rapid as in the even scenario with fly densities recovering to only 34% of pre-intervention values (Figure 33). The uptick in fly numbers was also not seen as a result of the poorest habitat existing at the upstream edge of the intervention area.

In the uneven habitat, similar levels of control were seen with upstream and downstream areas experiencing reductions in fly densities by 95.3 and 98.7% respectively. Like the even scenario the highest level of control was seen in the center of the intervention area with vector densities reduced by 99.9% of their pre-intervention levels. When the population was allowed to rebound with no control, the recovery of flies was markedly slower in the center and upstream areas of the transect with populations recovering to only 14.3% and 2.2% of their original value. The downstream area recovered fastest with fly densities reaching 71.3% of their pre-control density.

In both habitat simulations, one year of vector control was predicted to have the largest effect on mean age of flies upstream of the intervention zone. In the even simulation the smallest effects on age were seen in the downstream flies whilst in the uneven simulation flies in the center of the intervention zone experience the smallest change in age distribution. Across the three user defined areas, the mean male age increased from 16.78 days following one year of control to 19.79 days after two uncontrolled years in the even habitat simulation. In the uneven scenario a larger increase in male age was seen with an increase from 16.20 to 19.37 days (Table 23). Mean female age increased very slightly in the uneven scenario with an average increase of 0.19 days, a difference which would be undetectable in practice. A far larger increase in mean age of 8.7 days was seen in the even model simulation.

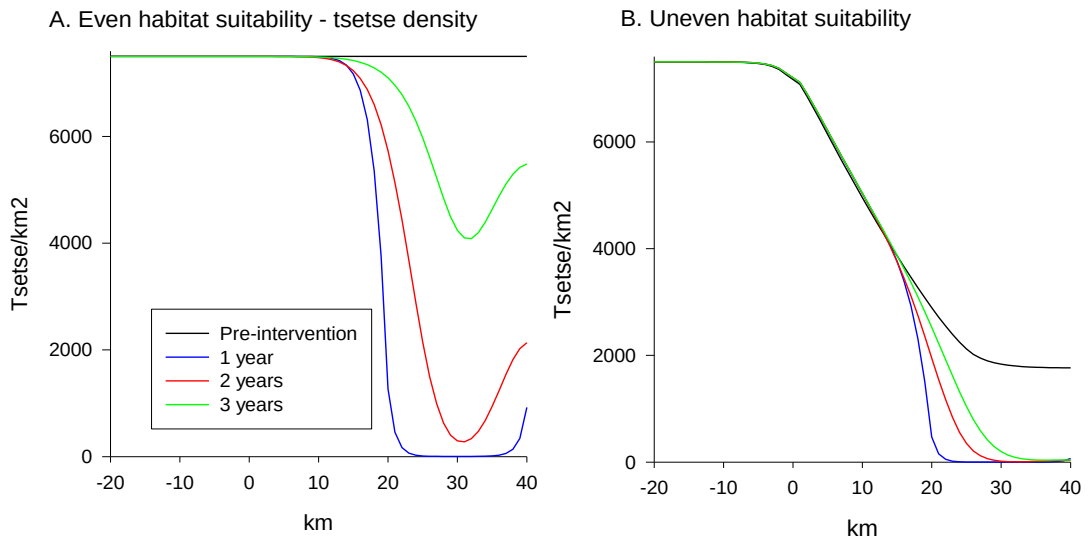


Figure 33 Total number of tsetse across the 60km transect during each simulated phase. Tsetse control was carried out in year 1.

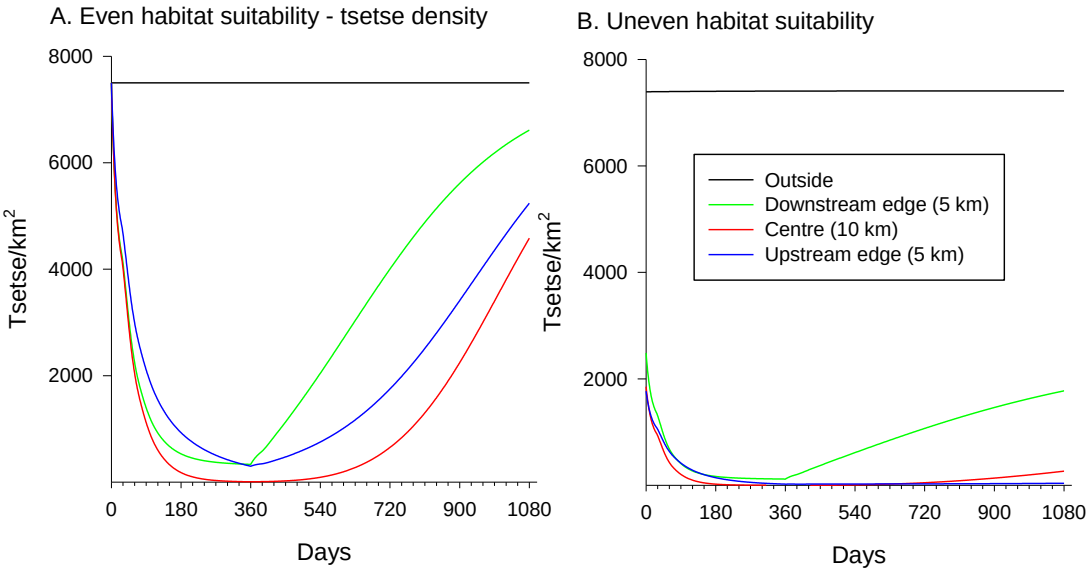


Figure 34 Total number of tsetse per kilometer² over the three year simulation represented by proximity to tsetse control

Table 23 Mean male and female age (days) throughout the simulated period aggregated by user defined zone

Scenario	Area	Pre-intervention	Control	Withdrawal 1	Withdrawal 2	Pre-intervention	Control	Withdrawal 1	Withdrawal 2
Even	Downstream	19.13	17.87	21.00	19.90	44.03	35.52	42.59	43.45
	Centre	19.13	16.54	20.54	20.02	44.03	30.32	45.36	43.73
	Upstream	19.13	15.92	20.16	19.44	44.03	38.93	43.53	43.75
Uneven	Downstream	19.24	16.67	19.63	19.53	44.24	36.81	43.37	43.98
	Center	19.20	17.05	18.66	19.20	44.32	37.99	48.45	45.51
	Upstream	19.19	14.88	19.40	19.38	44.34	42.25	44.71	43.98

5.4.2 Empirical analyses of scale-back

Interrupted deployment of Tiny Targets

The temporal pattern of catches of tsetse from areas with biannual, annual and no deployment of targets did not show any marked differences (Figure 35). In particular, while there is a small rise in catches in the period May-October in areas where targets were deployed (Figure 35 B,C), the increase was not statistically significant. Mean daily catches in areas with annual (0.8 tsetse/trap; 0.21-3.13, 95% CI) or biannual (0.4 tsetse/trap, 0.10-1.70) were lower than those where no targets were deployed (1.9 tsetse/trap, 0.80-1.88). Overall, not deploying targets for a single round of deployment did not lead to a statistically significant rebound in the abundance of tsetse in the short term (i.e., <six months).

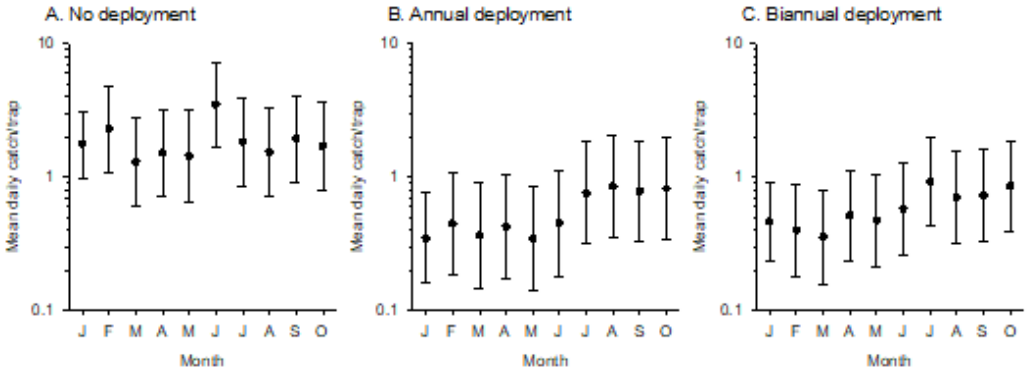


Figure 35 Mean daily catch of tsetse in January-October 2014 in areas with (A) no targets, (B) annual deployment or (C) biannual deployment of targets.

Scale-back of Tiny Targets

Between January 2019 and December 2021, a total of 5753 flies were caught in traps deployed in Maracha district. Prior to tsetse control withdrawal the mean daily trap catch in Maracha in January 2019 was 0.30 [95% CI 0.09-0.94] flies/trap/day. In December 2021 following 24 months of no control the mean catch was 0.15 [95% CI 0.04-0.60] (Figure 36). There is no evidence of catches of tsetse

increasing following the withdrawal of targets in Maracha or in Arua where no targets were deployed.

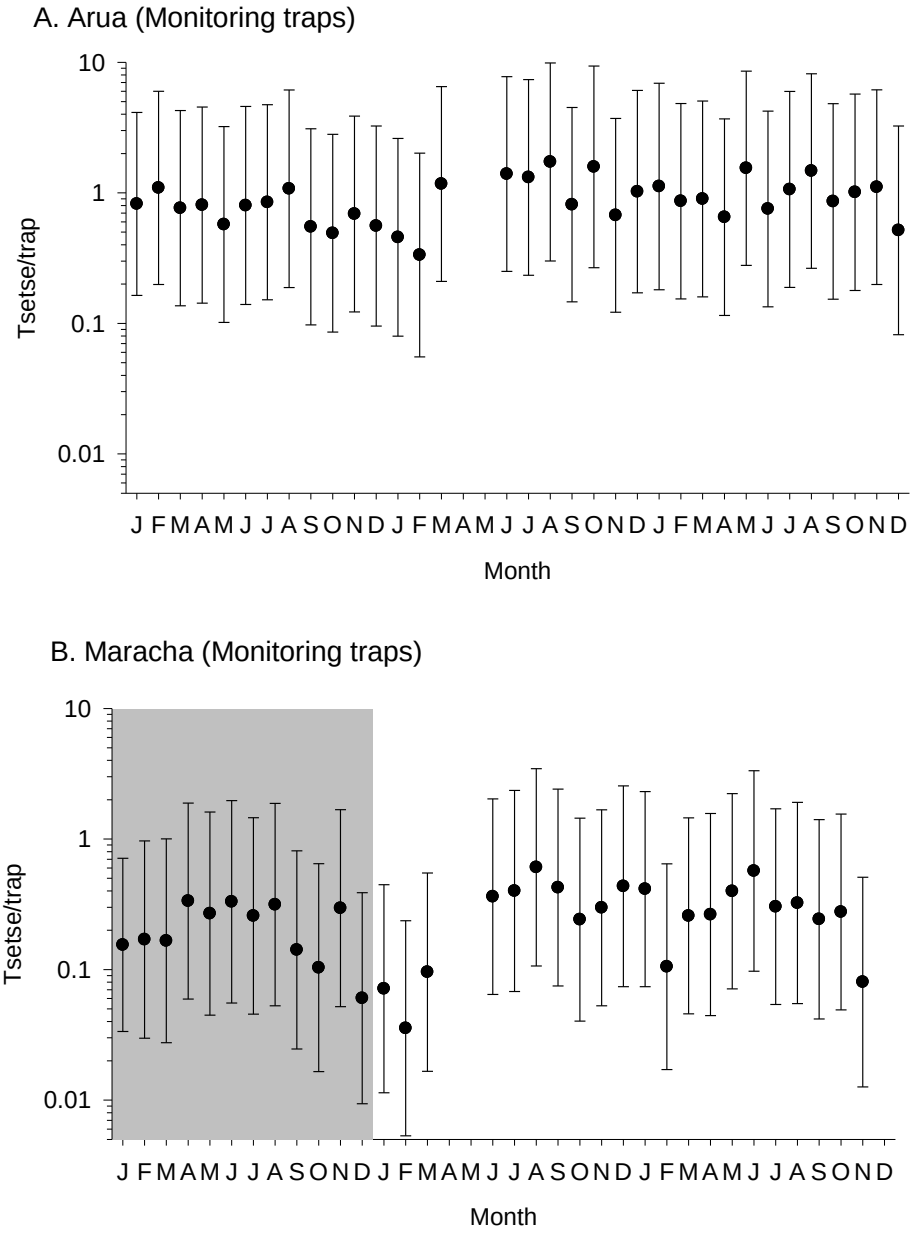


Figure 36 Mean daily catch of tsetse from monitoring traps deployed in (A) Arua and (B) Maracha between January 2019 and December 2021. Tiny Targets were not deployed in Arua and withdrawn from Maracha from January 2020 onwards

The data collected from those traps specifically established for the monitoring of flies in July 2019 onwards showed a similar trend with no clear or consistent increase in the catch of tsetse for the period January 2020-December 2021 when

targets were no longer deployed (Figure 37). Taken together, these results indicate that in contrast to the predictions of the simulation models, tsetse populations did not increase following scale back.

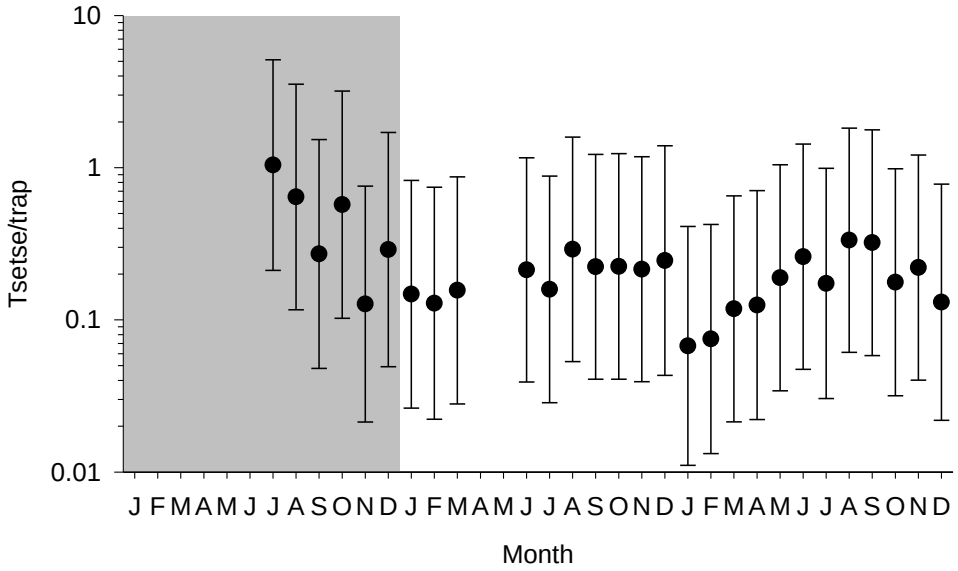


Figure 37 Mean number flies caught per trap per month in Maracha

To investigate the potential impact of environmental changes on tsetse catch, mean trap catches were plotted against environmental variables (Figure 38). Environmental variables were not found to have a significant effect on mean trap catch.

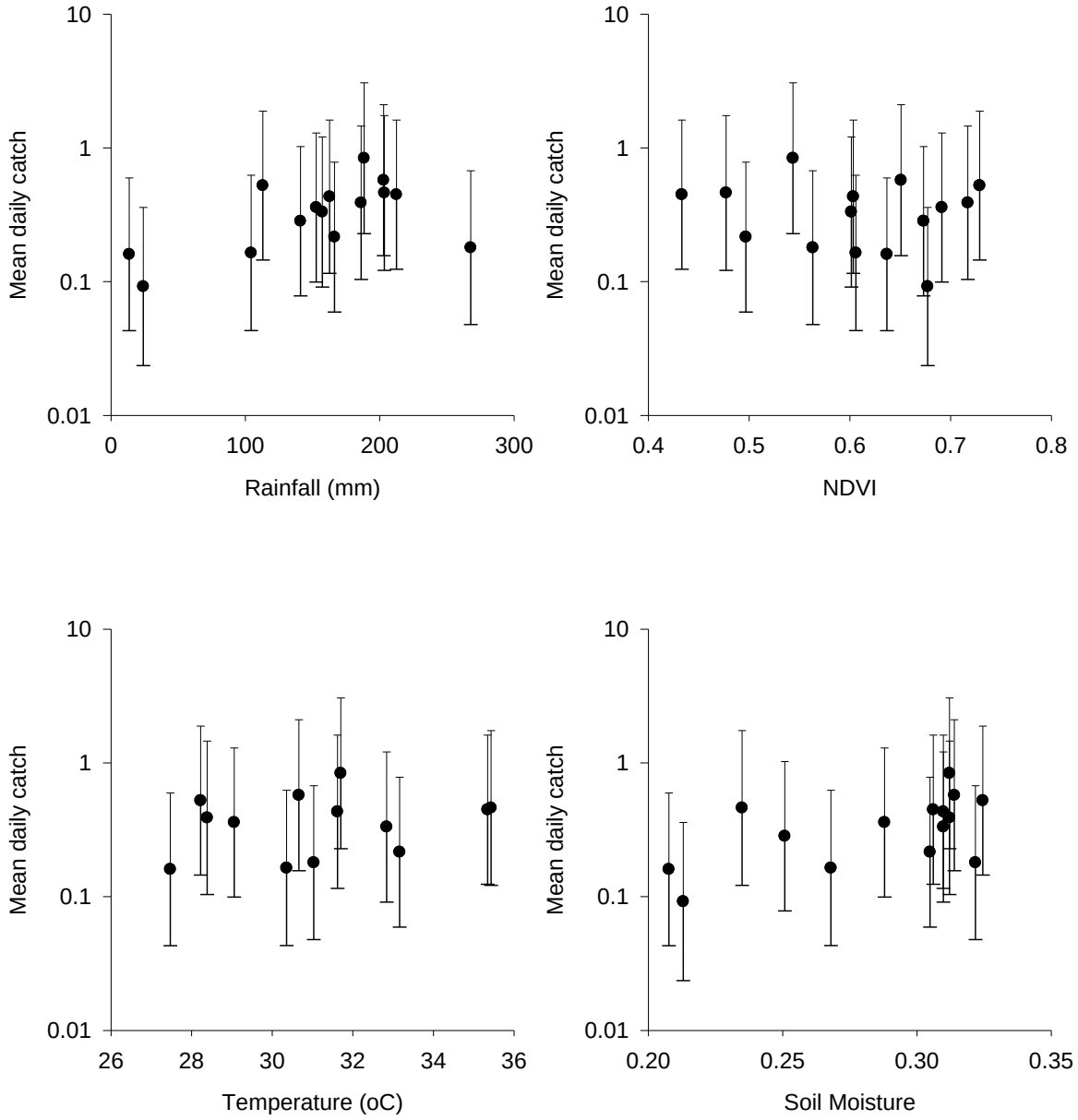


Figure 38 Mean rainfall, NDVI, temperature and soil moisture plotted against mean daily catch

5.4.3 Infection data

Of the 3842 flies caught between July 2019 and December 2021, 62.57% (2404/3842) were dissected and screened for trypanosome infection using light microscopy. The number of flies dissected per month ranged from 10 to 201 in November 2019 and August 2020 respectively. Over the 30 months of dissections 2.82% (45/2404) of flies were found to be carrying trypanosomes, consisting of 23 mouthpart infections, 15 midgut infections and 1 infected

salivary gland, five midgut and mouthpart positive and one fly with all three tissues found to be infected (Table 24).

Table 24 Percentage of flies from Maracha dissected found to have positive tissues.

MP-mouthpart, SG-salivary gland MG-midgut

Year	Total dissected (n)	MP		SG		MG		MP+MG		SG+MG		SG+MP		MP+SG+MP	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%
2019	377	5	1.33	0	0.000	0	0	0	0	0	0	0	0	0	0
2020	1103	13	1.18	0	0.000	2	0.18	0	0	0	0	0	0	0	0
2021	923	10	1.08	2	0.22	14	1.51	5	0.54	0	0	0	0	1	0.11

5.4.4 PCR data

A total of 618 tsetse flies were analysed using species-specific PCR at LSTM and screened for *T. brucei s.l.*, *T. congolense* Forest and *T. vivax* using species-specific PCRs (as described in chapter three). Of those screened, 25 flies (4.05%) were found to positive for trypanosome DNA (Table 25). *T. vivax* accounted for the highest number of trypanosomes positive flies (3.56%) with *T. brucei s.l. and T. congolense* Forest accounting for 0.32% each. Of the 25 flies positive by PCR, only two *T. vivax* positive flies had also been found to be positive by dissection. Due to the COVID-19 pandemic, no flies from January 2020 onwards were transported to LSTM for screening.

Table 25 Results of screening tsetse flies from Maracha using species-specific PCR.

TB-T. brucei s.l., TC-T.congolense Forest and TV-T.vivax

Year	**Total screened (n)**	**TB**		**TC**		**TV**		**TB+TC**		**TB+TV**		**TV+TC**	
		n	%	n	%	n	%	n	%	n	%	n	%
2019	552	2	0.36	1	0.18	21	3.8	0	0	0	0	1	0.18
2020	66	0	0	0	0	0	0	0	0	0	0	0	0

5.4.5 Ovarian dissection data

A total of 1964 flies were dissected and aged by ovarian category. Sample sizes per month ranged from a low of 77 in quarter 1 of 2021 and a maximum of 254 flies in quarter 3 of 2021. Ovarian dissection data was grouped by year quarter with quarter one consisting of January to March, April-June quarter two, July-September quarter three and quarter four October-December and the mean ovarian category per quarter calculated.

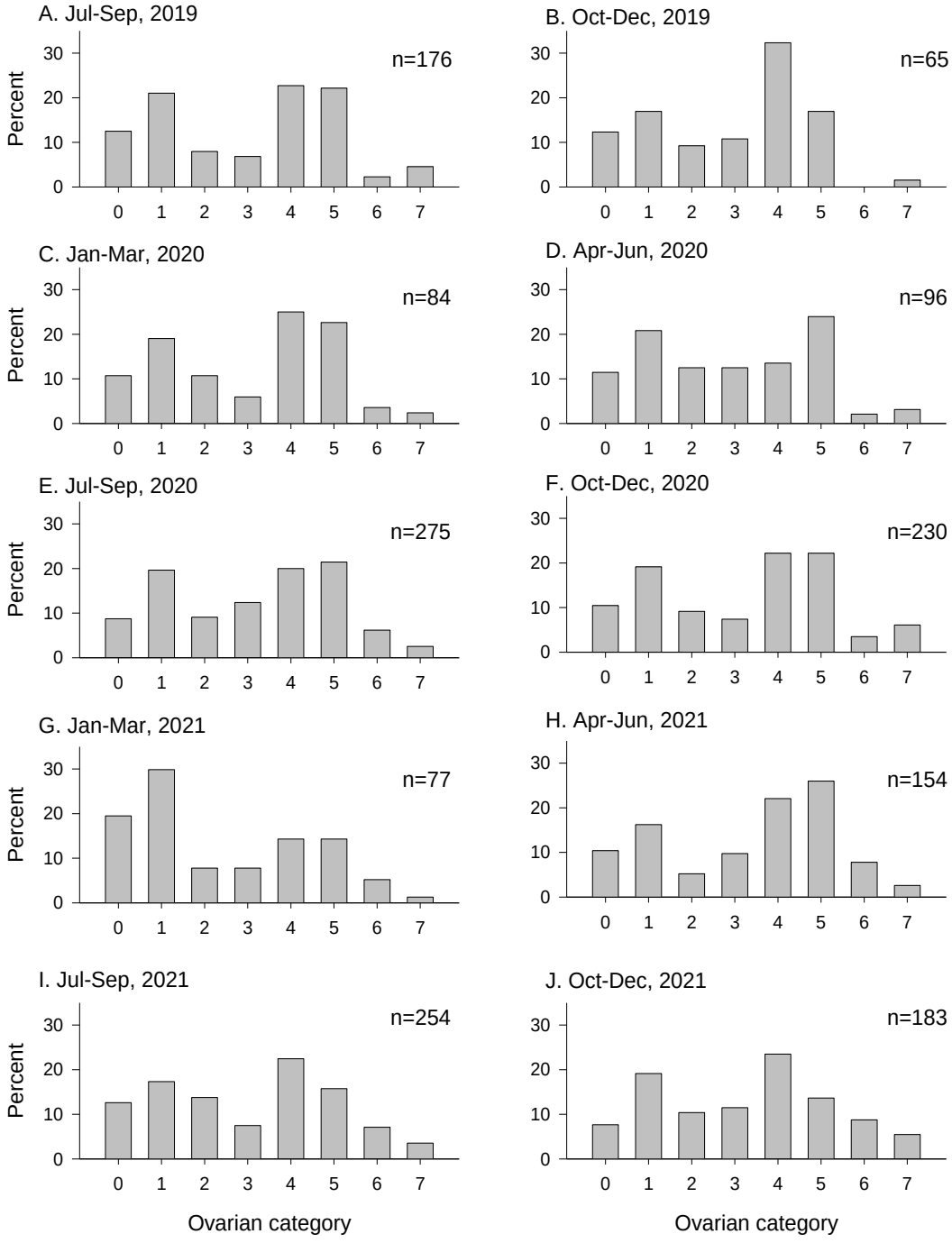


Figure 39 Distribution of flies according to ovarian category

The results show that the frequency of tsetse in different ovarian age classes were generally bimodal, with peaks in Ovarian Categories 2 and 4 (Figure 39).This pattern is typical of tsetse caught from stationary baits, reflecting the paucity of young tsetse (Category 0) attracted to stationary baits (223)and the large numbers Category 4 because this comprises flies from categories 4, 8 and 12 etc. Statistical Analyses of the frequency distributions for the 14 quarters between July 2019 and December 2021 showed that there was a significant difference between the distributions (χ^2=84.13, df=63, P<0.05). The standout contrast is between the low mean age category in Jan-Mar 2021 (2.38) and the high mean age in the following quarter (3.39) (Figure 40).

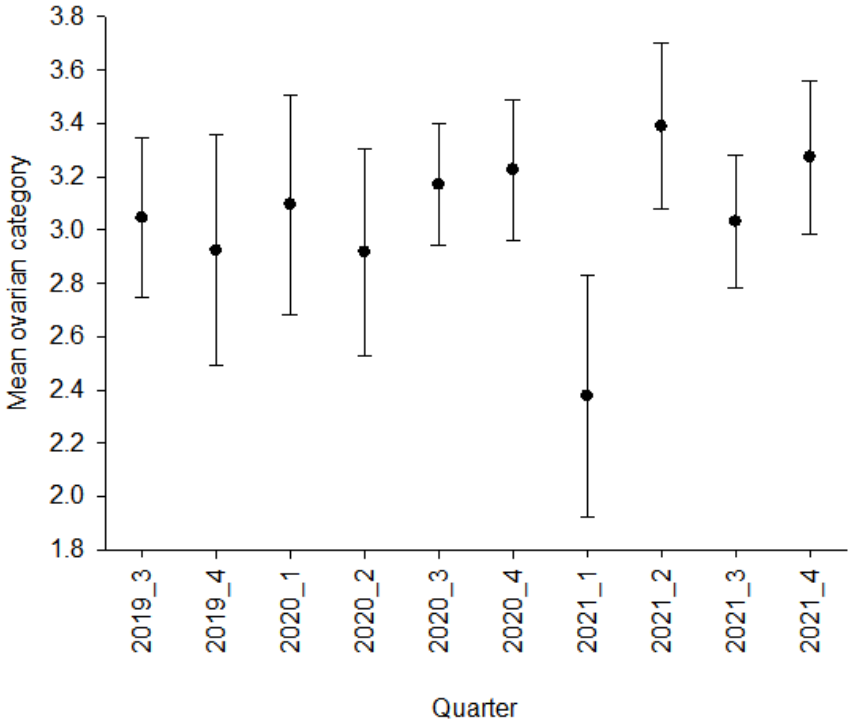


Figure 40 Mean ovarian category by year quarter

5.5 Discussion

Understanding the risks of vector control withdrawal are fundamental to the resilience of disease elimination campaigns. As the COVID-19 pandemic has demonstrated, situations may arise in which disease control programs may be halted. Knowing the risks of this withdrawal are key, both to the scheduled reduction of vector control but also provides further evidence to the potential risk of sudden removal.

Previous simulations of population recovery have predicted that the tsetse population will recover within ~3 years(207). The simulations outlined in this chapter produced more conservative estimates with a rebound up to 73% in an even habitat distribution and up to 29.3% in an uneven habitat. Similar to the Tirados *et al* findings, the greatest control was achieved at the center of the intervention area with the downstream population being bolstered by reinvasion of flies from neighboring areas.

When the rebound rates were examined, rates of recovery were found to be highly location specific. In both scenarios the downstream area had the largest rate of population recovery due to bordering a reinvasion front. In the uneven scenario this was in addition to the habitat having the lowest mortality rates in the downstream region, reflecting the impact of poorer habitat on the ability of a population to recover.

In contrast to the rebound predicted by Tsetse Muse, empirical data from Uganda showed no significant increase in catches following the interruptions of Tiny Target deployment or its withdrawal for two years. Similarly, there was no changes in the age structure

In 2011, prior to tsetse control being deployed in Maracha, monitoring traps were catching 12.4 tsetse per day (207). Previous predictions estimated that tsetse densities could rebound to ~50% of pre-intervention numbers within 2 years and 100% within

three years. The data collated in this study suggest that tsetse recovery is substantially slower than predicted with mean trap catches in 2021 found to be on average 0.41 flies/trap/day compared to 0.31 flies/trap/day in 2019 when vector control was in place.

Recovery of flies can be affected by factors such as the presence of control in downstream areas (224), degradation of habitat and environmental factors. The mean catch per trap did not rise above 1 fly per trap per day over the course of the 24-month surveillance period. It has been shown that the presence of control in downstream areas potentially prevents rapid rebound of vector populations upstream (224). Riverine tsetse largely travel along river networks and so the rate of reinvasion is likely to be slower. Furthermore, the habitat is becoming increasingly poor in terms of suitability for tsetse with agriculture and livestock grazing occurring along river networks. The slow rate of rebound recorded in Maracha may in part be due to the poorer habitat in the upper parts of the river where a large proportion of the traps were deployed.

Distribution of flies across the seven ovarian categories demonstrated the expected patterns of highest numbers of flies found to be aged between categories four and five. This is due to the age classification system being based on four ovulation cycles and so flies that are recorded as age categories four and five could in fact belong to categories eight or older. In the field it would be expected that a higher number of young flies will be caught in traps than older flies due to increased risk of mortality with increasing age. However tsetse trapping is biased with higher probability of catching individuals as they get older (223), resulting in under representation of flies in earliest age categories. One suggested reason for this is the under development of the flight muscles and lower activity rates of young flies, meaning they are less likely to be captured in traps. As a

result of this bias, it has been suggested that category zero flies must be excluded from tsetse mortality analysis based on ovarian categories (225). Due to populations of tsetse in Maracha being subject to long term control it was expected that subsequent removal of control would increase the overall mean age of the vector population. Following two years of no control, the mean age as well as the age structure of the tsetse population of Maracha had not changed. A study by Hope *et al* (226) found similar results when examining the impact of removing vector control for 12 months in West Nile. Previous studies of the impact of target deployment in Zimbabwe on savannah flies found that vector control had a clear impact on mean age with the population found to be younger following deployment of control (227).

Like age structure, the infection rates of flies also did not change significantly with the number of flies positive by dissection remaining extremely low. Again, Hope *et al* (226) had the same findings with no difference in infection rates recorded by dissection. Only two flies positive by dissection were positive by PCR (both *T. vivax*). This rate was lower than expected, particularly due to the higher sensitivity of PCR compared to microscopy. It is possible that the remaining infections identified using microscopy were non-T. *brucei*, *T. congolense* or *T. vivax* trypanosomes (i.e *T. grayi*). The low correlation between the two diagnostic methods may also be a reflection of errors during the dissection or DNA extraction of samples. Infected tissues may have not been transferred either into storage or sample tubes prior to extraction. *T. vivax* positives may have been missed due to high genetic variability of the species; the specificity of the species-specific primers may result in specific isolates not amplifying successfully.

Similarly, only two of the flies positive by PCR were also positive by dissection, this is likely due to the high sensitivity of molecular tools such as PCR. Infections may have

been missed by dissectors or the flies had not established infections and were simply carrying parasites perhaps as a result of recently feeding on an infected host.

The low catches recorded in Maracha even after 24 months of no vector control across the entire district indicate that the implementation of a xenomonitoring surveillance tool may continue to be challenged as a result of very low numbers of flies. With *T. b. gambiense* infection rates predicted to be as low as one in 10,000 tsetse flies (190) and mean catches found to be less than one fly per day the effort and cost needed to catch sufficient numbers of flies may make this an unfeasible option.

The findings of this chapter suggest that riverine flies may not rebound at the rate that was previously predicted. These results may be specific *G. fuscipes fuscipes* in West Nile and further studies are required to investigate if a similar pattern is observed in other tsetse species in other environments.

This study is limited by recording catches in mainly in Maracha district. Building upon this, collecting data in districts where targets have never been deployed would provide further information on tsetse population dynamics, in particular in relation to external variables that may have not been considered in this study.

Finally, the rebound rates found in this study have to be taken in the context of the ongoing control that continues in neighbouring districts and rivers. Although there was no significant rebound in Maracha this might not be the case when scale back occurs in neighbouring districts and larger parts of the river system are uncontrolled. The planned scale back in 2022 involves the extension of target withdrawal to include Arua and Koboko districts as well as Maracha. The findings of this chapter suggest that withdrawing control may not result in a rapid recovery of the tsetse population. Furthermore, the data indicate that the impact on mean age of flies and rate of infection

will also remain stable at least for the initial period following cessation. Despite this, the risks of a resurgence of gHAT are not to be taken lightly and so any withdrawal must be followed up with surveillance of both the tsetse and trypanosome population to maintain the historic gains made so far.

6 Discussion

With WHO disease specific targets for the elimination of gambiense and rhodesiense HAT set for 2030 (147), HAT control strategies will have to adapt to reflect the ongoing and forecast decrease in disease prevalence. With case numbers so low (<<1 case/10,000 people) current screening methods experience drops in their positive predictive value and alternative methods such as active screening are no longer justifiable due to their cost. Both forms of sleeping sickness have epidemic potential, and this has been demonstrated by a number of large historical disease outbreaks, including most recently the outbreak of rHAT in northern Malawi in 2019-2020 (147). Furthermore, gHAT can persist at extremely low levels making the development of a surveillance system that is not only cost-effective but highly sensitive essential. These requirements will have to be met to detect any potential resurgence of transmission but also to validate elimination of transmission.

The need for robust and resilient strategies that are sustainable both in terms of cost and effort required was emphasized by the COVID-19 pandemic. Active screening campaigns for HAT were postponed due to a combination of risk of SARS-CoV-2 transmission and reduction of resources allocated to trypanosomiasis control(147). Strategic deployment of low-cost surveillance in sentinel sites could play an important role in the identification of high-risk areas and the enabling of reactive disease control. There have been recent development of diagnostic tools specifically designed to confirm elimination of transmission in very low prevalence areas with a focus on detecting infection or antibodies in human hosts (228). Although these tools may improve the reliability of results obtained in screening campaigns, the testing of human populations remains logistically challenging and highly labour intensive.

The molecular detection of parasite DNA in vectors of disease (xenomonitoring) as a proxy for human infection rates and transmission may provide a low-cost alternative for HAT elimination campaigns(229). This method has previously been utilised to quantify lymphatic filariasis transmission across Africa, the Americas, Southeast Asia, the Eastern Mediterranean and the Western pacific (230–239). When HAT surveillance resources are stretched, xenomonitoring could provide real-time transmission data in a low-cost and time efficient manner.

The overall objective of this thesis was to develop and assess the potential for xenomonitoring to play a role in a long-term surveillance system for pathogenic trypanosomes of veterinary and medical importance. To meet the overall objective I aimed to achieve the following:

1. **Development of a qPCR-based assay for the detection of human African trypanosomes**
2. **Development of a qPCR -based assay for the detection of animal African trypanosomes**
3. **Examination of a low-cost magnetic bead method to extract trypanosome DNA from tsetse as an alternative to column-based extraction methods**
4. **Evaluate the impact of withdrawing vector control on a tsetse population in a historic HAT focus in northwest Uganda**

6.1 Development of a qPCR-based assay for the detection of human African trypanosomes

The decision of which human-infective parasite to screen for has historically been made based on location and the discrete geographic distributions of *T. b. rhodesiense* and *T. b. gambiense*. For instance, studies conducted in east and southern Africa assume that tsetse will be infected with *T. b rhodesiense* whereas those in West and Central Africa assume *T. b. gambiense*. This approach is less satisfactory for Uganda where all sub-species of *T. brucei* occur. The changing distribution of flies (189,240–242) and trypanosomes(243,244) along with the remaining need to identify to sub-species due to disparities in treatment regimens for human disease suggest that a 'catch-all' approach for screening is the logical way to move forward for diagnostic algorithms. Furthermore, in terms of molecular tools, traditional PCR remains the mainstay for large-scale screening of tsetse which is limited by the risk of contamination, difficulty interpreting gel electrophoresis results and the need for multiple PCRs to be carried out on each sample.

The assay described in chapter two is a novel multiplexed qPCR which has the additional step of post-amplification high-resolution melt analysis which aids to further improve specificity. A combination of novel and previously described primers were incorporated into an optimised assay which reduced processing time from over five hours to approximately two whilst screening for both human infective forms of *Trypanosoma brucei*. Due to the assay targets being such low copy number, there is a risk of a false negative result simply due to a lack of sufficient genomic material. The addition of a further primer set allows for differentiation between samples which have adequate DNA for single-copy gene amplification, through the detection of a pan-*T. brucei* gene also present in single-copy. Validation of the assay on a subset of

previously screened samples from a rHAT focus showed that the assay performed well, detecting all *T. b. rhodesiense* positive flies when compared to traditional PCR. The assay was highly specific, with no false positive results recorded.

In future, this assay would need further validation on a larger sample set with a higher number of positive flies to provide more robust data on its performance. One of the major constraints of this chapter was the unavailability of any *T. b. gambiense* positive flies. With only 565 cases of gHAT reported globally in 2020(147) and vector infection rates predicted to be $1x10^{-5}$(190), the low prevalence of infected flies in the field is a considerable challenge for the development of xenodiagnostic tools and will likely continue to be so as global cases continue to drop in the decade ahead.

Furthermore, in its present format, the described assay is not field-friendly. The need for a qPCR machine along with a laborious and expensive DNA extraction method are the two main barriers to this assay being applied in the field. I aim to address the DNA extraction drawback in chapter four. The development of the compact and highly-portable Magnetic Induction Cycler (MIC) also offers the prospect of a more field-friendly qPCR. This small, qPCR machine weighs only 2kg, completes runs of up to 48 samples in 40 minutes. The portability of this device lends itself to reactive screening of flies, allowing it to be transported between areas that may be at risk of resurgence whilst having the potential to produce same day data on the transmission of trypanosomes by local tsetse. The number of flies that would need to be processed to detect an infected fly remains an unaddressed but major challenge to xenomonitoring. Screening pools of flies would help improve speed of processing and efficiency. Although this is not addressed directly in this thesis, the data in chapter four on the parasitaemia of wild flies suggests that this could be viable strategy in the future along

with findings by Cunningham *et al* (81) that a single infected fly could be detected in a pool of up to 20.

An additional drawback of the assay for subspecies of *T. brucei* is the reliance on single copy genes. In order for *T. b. gambiense* or *T. b. rhodesiense* to be detected, samples must have sufficient genomic material, as was seen in the wild-caught flies, less than half met this requirement. At present, these are the only diagnostic markers available for the human pathogenic species of *T. brucei* and until further markers are available and validated this limitation will continue to exist for all sub-species-specific diagnostics. A recent study (245) found that the *T. brucei* repeat which is often used to detect members of the Trypanozoon group is not as conserved between members of the Trypanozoon group as previously thought. Examination of the single nucleotide polymorphisms that exist between *T. b. rhodesiense* and *T. b gambiense* indicates that this could be utilised as a diagnostic marker present in significantly higher copy number (245). Future comparison of the qPCR's performance against other diagnostic methods such as LAMP (81)would provide valuable further data on the potential of this method as a xenomonitoring tool. Finally, evaluation of the qPCR's performance on other types of sample such as human or animal blood spots would help uncover the applicability of the assay to other trypanosomiasis surveillance and diagnostic strategies. At present, human diagnostics are dependent on the visualisation of trypanosomes but a PCR based approach could be of use when there is ambiguity regarding the sub-species of *T. brucei* due to the difference treatment regimens for each. The method developed here might be particularly useful where there is uncertainty about the species of *T. brucei* causing HAT in a patient. For instance, the identity of the pathogen would be uncertain for Ugandans visiting northwest Uganda from say southern Uganda where rHAT occurs. The application of this assay in the

screening of cattle could also help identify reservoir hosts and distinguish *T. b. brucei* from human pathogenic species.

6.2 Development of a qPCR -based assay for the detection of animal African trypanosomes

Diagnostic and surveillance tools for animal African trypanosomiasis are predominantly reliant on the screening of the vertebrate host (mainly livestock and domestic animals) for infection. However, due to the difficulty and cost of screening animals, large scale epidemiological surveys are rarely conducted and diagnosis for treatment purposes is rarely done. The strategy for AAT control is not focused on elimination or eradication and so effective surveillance of transmission could play a critical role in quantifying and managing disease burden. This is particularly important for the transmission of *T. b. rhodesiense* because of its role in causing rapidly progressive human disease and the 2030 WHO elimination targets(153).

In chapter three, a qPCR based xenomonitoring tool was developed which screens simultaneously for the three main pathogenic species of AAT: *T. brucei s.l., T. congolense* and *T. vivax*. A combination of newly-designed and previously described species-specific primers were selected along with an internal positive control that targets a Phocid herpesvirus. The assay was found to be at least as sensitive as species-specific traditional PCR when evaluated on laboratory reared tsetse spiked with target DNA. Specificity of any xenomonitoring assay is critical to its use due to the high number of non-target trypanosomes species which have been found to circulate in tsetse across AAT foci (246–248). Therefore, the assay was challenged with a wide range of trypanosome species DNA found within tsetse with no cross-reactivity recorded. A high specificity xenomonitoring tool could prevent the potential for any unnecessary presumptive treatment of local livestock with trypanocides, a practice which at present

is threatening the long term use of these medications due to the emergence of resistance(249–253).

In contrast to the promising laboratory results, the assay's performance with wild-caught tsetse from both Tanzania and Uganda was poorer than seen in laboratory flies with sensitivities of 61.36%, 66.15% and 85.83% for *T. vivax*, *T. brucei* and *T. congolense* respectively. Running the assay in a singleplex format resulted in improved sensitivities for *T. congolense* and *T. vivax* but sensitivity remained the same for *T. brucei s.l.* It is possible that the sensitivity and efficiency of the assay is potentially being affected by the presence of bacterial endosymbionts such as *Sodalis*, *Wolbachia* or *Wigglesworthia*. For example, in wild tsetse, up to 50% of flies harbour *Sodalis* (254) and so the possible interaction between the assay's performance and the presence of endosymbionts would benefit from further investigation in future. Improvements in sensitivity could be achieved by adapting the qPCR to a nested format. Increasing the overall number of amplification cycles can increase the detection of DNA present in low quantities but has the additional risk of non-specific amplification and contamination. The specificity of the assay could be further improved through the use of hydrolysis probes. The highly-specific nature of probes help ensure any amplification seen is only due to the presence the target sequence and dramatically reduce the risk of false-positives.

Similar to the HAT qPCR, future work on this assay could involve the testing of this assay's performance on samples from animals such as blood spots on FTA cards. This assay dramatically reduces processing time for screening tsetse for the main causative trypanosomes species for AAT, from three separate assays to one. Furthermore, the risk of contamination is reduced due to decreased sample handling and the results obtained per sample can be quantified unlike with traditional PCR. Despite these

advantages, this assay would require further optimisation to be a viable xenomonitoring tool in the field sensitivity would need to be improved, particularly when screening wild tsetse in order for this assay to perform at a comparable level to currently available molecular methods such as species-specific PCR.

6.3 Examination of a low-cost magnetic bead method to extract trypanosome DNA from tsetse as an alternative to column-based extraction methods

For a xenomonitoring strategy to provide data on transmission, a large number of flies have to be processed in a time effective, field-friendly and low-cost manner. Recent studies of trypanosome prevalence in HAT foci have found infection rates of 2/5986 for *T. b. rhodesiense* (201) and 0/2184 for *T. b. gambiense* (255). One major bottleneck to this is the extraction of DNA from tsetse samples. At present, the most widely used method of DNA extraction from wild tsetse is use of a spin column-based extraction, usually provided in kit format. Although these kits produce high yields and extracts which perform well in PCR they can be complex, time consuming and expensive costing approximately £2.77 per sample (192). With the number of flies that would need extracting for effective surveillance in the tens of thousands, these drawbacks become an increasing hurdle.

Any alternative extraction method must produce high yields of high-quality DNA which can be used for subsequent molecular analysis. MagnaExtract is a novel, field-friendly and low-cost magnetic bead methodology that has previously been validated for use in detecting anti-microbial resistance genes in water samples(192). This method involves the preparation of magnetic beads prior to use which greatly reduces costs followed by a protocol which requires approximately 30 minutes to complete. In this chapter, the protocol developed by Byrne *et al* (192) was optimised for tsetse fly extraction after

which its performance was directly compared against the Qiagen DNeasy Blood and Tissue kit. Optimisation of the protocol was carried out using laboratory-reared tsetse fed bloodmeals spiked with *T. b. brucei* and performance evaluated on overall DNA yield and DNA purity. The addition of an overnight incubation step in lysis buffer and doubling the volume of beads used produced DNA extracts of comparable quality and yield when evaluated against DNeasy. Using the final optimised protocol, a series of flies spiked with a dilution series of quantified numbers of trypanosomes was extracted using both the DNeasy and MagnaExtract methods and the performance compared. The MagnaExtract method was successful in amplifying low numbers of parasites (up to 1 parasite/mL) which were subsequently detected using PCR. This method also had a 2-3 hour faster processing time compared to DNeasy indicating it could be a viable low-cost and more time efficient alternative to the Qiagen kit.

The Ct values obtained using the dilution series of trypanosomes was then applied to a sample set of trypanosome-infected tsetse from northwestern Uganda and used as an index for the number of trypanosomes present. The mean number of trypanosomes in wild flies was estimated to be between 10^3 and 10^4 depending on species. These high numbers of trypanosomes in wild flies suggests that pooling flies in groups may be a viable strategy when optimising a xenomonitoring protocol.

The extent to which flies could be pooled was not investigated in this chapter and the limit to which pools can be increased whilst still detecting infected flies would be a valuable next step. Evidence suggests that pools of up to 20 individuals should still allow identification of one infected sample. Larger pools would reduce the number of assays to be run and an overall reduction in screening costs and time. A number of pooling strategies have been described using hierarchical (256) or array-based methods (257–261) which allow for samples to be pooled whilst retaining the ability to

identify which sample within a pool is providing the positive result (Figure 41). The use of such strategies could enable flies from different traps and areas to be combined whilst maintaining the ability to determine where specific positive flies were caught from and where potential control measures may be required.

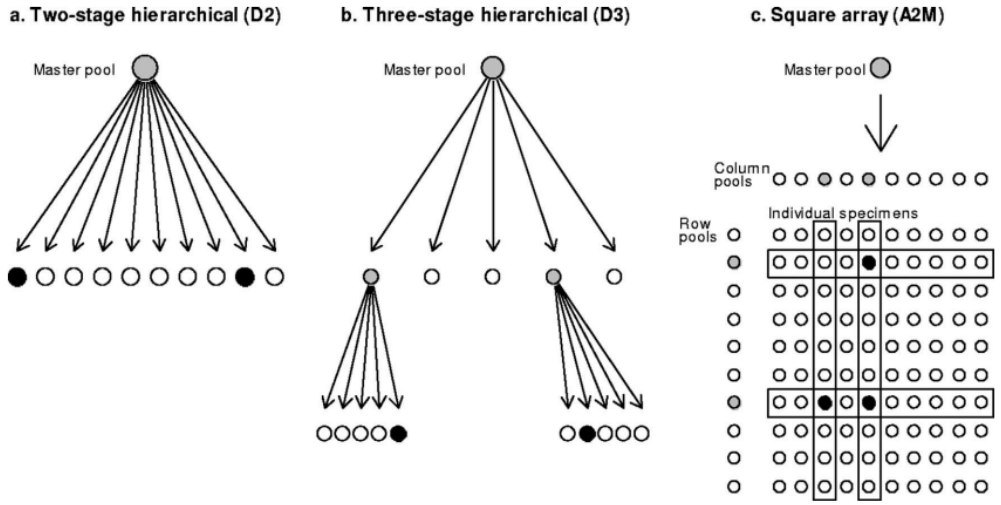


Figure 41 Schematic of three pooling algorithms, and the protocol following a positive master pool . A- a positive pool is split into individual samples. B- a positive pool is split into subpools and C- a positive master pool is broken down into rows and columns. Image taken from (257)

The MagnaExtract method was only evaluated within a well-equipped molecular laboratory setting at LSTM. Following the findings of this chapter, this method could benefit from being further validated within a low-resource field setting. The main challenge in the implementation of any tsetse DNA extraction method in the field is the requirement to homogenize a large number of flies prior to extraction. The size and structural robustness of tsetse requires homogenization with high impact to release any parasite DNA from the fly carcass. At present, this is done in the laboratory using a TissueLyser II (Qiagen) of which more compact models exist. Alternatively, the use of handheld grinders or homogenizers could be assessed. Although these alternatives are more field-friendly they are laborious and are unlikely to provide viable alternative for large-scale mechanical lysis.

6.4 Evaluate the impact of withdrawing vector control on a tsetse population in a historic HAT focus in northwest Uganda

As gHAT elimination targets edge closer, disease control strategies are changing to adapt to the combination of fewer cases and reduced risk. One consequence of this will be to ease the level of vector control gradually in areas which have had no recorded HAT cases for several years. Due to the historic epidemic nature of sleeping sickness, this easing of control must be carried out with minimal risk to disease resurgence. The increase in the number of tsetse may provide sufficient numbers of tsetse for xenomonitoring.

In this chapter the risk of tsetse population rebound following cessation of vector control was simulated using Tsetse Muse which has previously been utilised to estimate costs of sterile insect technique(210), the impact of insecticide-treated cattle(262) and the effect of tiny target deployment(207). Two simulations reflecting differing states of habitat suitability for tsetse were run and the effect on tsetse density and age examined. Following on from simulated scenarios, empirical data were collected from the field to compare empirical findings with model predictions and to examine the impact of Tiny Target withdrawal in Maracha district. I also assessed the effect of interrupted target deployment on tsetse populations. HAT transmission risk was assessed by examining tsetse infection rates and the age distribution of flies.

The simulations produced by Tsetse Muse demonstrated the impact that habitat change through general land use change or environmental degradation can have on the ability of tsetse to rebound. Higher quality habitat along with proximity to an invasion front from which tsetse from neighbouring areas could reinvade were found to improve rates of population recovery. These predictions were in broad agreement with findings

reported by Hope *et al* (226)where persistence of tsetse populations were linked with reinvasion from uncontrolled areas. These findings contrast to the predictions of Tirados *et al* (207) in which the withdrawal of targets was predicted to lead to a large and relatively rapid recovery in the tsetse population. On the one hand, present findings offer some strong assurance that scaling back vector control does not necessarily lead to a surge in transmission potential. On the other hand, numbers of tsetse caught in the post-control period were still too small (<1 tsetse/trap/day) to offer the prospect of catching sufficient numbers of tsetse to estimate the prevalence of *T. brucei* in the tsetse population. Despite this, the low numbers of tsetse and low infection rates recorded do suggest an overall low-level of transmission.

Modelling rebound for settings with highly suitable habitats ('even scenario') suggest that tsetse may rebound rapidly in such areas. The planned scale back of vector control across northwestern Uganda, which includes areas with perennial rivers and highly-suitable habitat, may lead to a large rebound in the tsetse population.

Infection rates of tsetse using dissection in West Nile were low, in keeping with a study of tsetse in nearby Koboko district north of Maracha in 2013-2014 (263). This study by Cunningham *et al* (255) found 2.4% of flies positive for trypanosomes by dissection, however unlike this study where there was a high correlation between flies positive by qPCR and those positive by PCR this was not the case in the samples from Maracha. This may be due to the flies being positive for non-target trypanosome species (i.e. *T. grayi)* or may simply be reflective of the smaller sample size with only 618 flies screened using both methods in this thesis. In terms of impact on trypanosome transmission dynamics, the withdrawal of tsetse control did not appear to have any significant impact with infection rates remaining very low throughout the withdrawal period.

The overall findings of this chapter suggest that although any future vector control withdrawal must be evidence based and carried out with caution, the risks of vector rebound to pre-control levels is lower than expected. Furthermore, any changes on tsetse age distributions appears to be minimal along with rates of trypanosomes infection.

These results are for one focus of HAT and will not necessarily reflect the rebound rates of tsetse populations in other habitats such as savannah or forest regions. Degrees of environmental degradation or land change use are also likely to have a significant impact on rebound rates. Presence of highly suitable habitat such as national parks will also provide a refuge from which tsetse can reinvade.

6.5 Final conclusions

Based on the findings of this thesis, at present, xenomonitoring has limited use as a field-based method of surveillance for either AAT or HAT in West Nile. A major challenge facing incorporation of xenomonitoring into a trypanosomiasis surveillance system is the large numbers of flies required in order to detect even one infected fly. Current estimates suggest infection rates for human infective trypanosomes in tsetse are one in every 10,000 flies (190,264). When this is considered with the finding that even after two years of vector control cessation trap catches remain at under one fly per day, the sheer number of traps and trapping days that would be required to detect to gain a true understanding of trypanosomes transmission in real time will be extremely difficult to accrue.

In summary, I developed and evaluated novel qPCR based xenomonitoring tools to provide a more time-efficient, simple and sensitive alternative to currently available traditional PCR assays. With further development, the assays I describe can be used in a

two-step screening protocol for tsetse and furthermore enable a more one-health focused approach to trypanosomiasis. With limited funding and resources for both diseases, using a more integrated surveillance approach for African trypanosomiases as a whole has the potential to be both more cost-effective and sustainable post-elimination. I also provide evidence for the potential of a more field-friendly DNA extraction which both reduces costs and sample processing with evidence to suggest the value of pooling samples.

The limited scale-back of tsetse control in Uganda has not led to a widespread rebound of tsetse and transmission. However, in the decade ahead the extent of scaleback will increase and it seems likely that in some settings tsetse populations will recover rapidly. When this happens, it will be important to determine whether *T. b. gambiense* is being transmitted. The long lag between infection and presentation of advanced symptoms in humans (~18 months), and the decline in screening capacity for human cases, means that there is a risk that a resumption of transmission will only be detected years after it has started. Xenomonitoring could play a crucial role in the early detection of a resumption in transmission and the methods developed in this thesis provide the basis for a system to meet that need.

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7 Annex

The following set of experiments were planned for a chapter investigating the potential of screening non-tsetse biting flies (specifically *Stomoxys* and Tabanids) for trypanosomes. Due to the COVID-19 pandemic, these experiments were never conducted however the initial outlines of experiments planned are outlined here.

The low numbers of tsetse caught in areas with long-term vector control poses a major challenge to xenomonitoring strategies. Other non-biting flies are often present in high numbers, particularly in areas with livestock. I aimed to evaluate the potential of using non-tsetse biting flies within a xenomonoring context. Prior to the undertaking of field-based experiments, laboratory-based proof-of concept studies were outlined and are listed below.

7.1 Experiment 1: detection of trypanosome DNA in non-vectors

Aim

Proof of concept to demonstrate that trypanosome DNA (Trypanosoma brucei brucei, Trypanosoma congolense Savannah, Trypanosoma vivax) can be detected in non-tsetse flies immediately following a spiked bloodmeal

Method

Stomoxys will be fed on bloodmeals spiked with either *Trypanosoma brucei brucei*, *Trypanosoma congolense* Savannah, *Trypanosoma vivax* or control blood. Blood will be spiked to a trypanosome DNA content of 5 ng genomic DNA/uL (equivalent of 50,000,000 trypanosomes/mL). Immediately following the bloodmeal, 10 flies from each bloodmeal group will be killed and stored. Trypanosome DNA will be extracted following the DNeasy 96 protocol for the purification of total DNA from animal tissues.

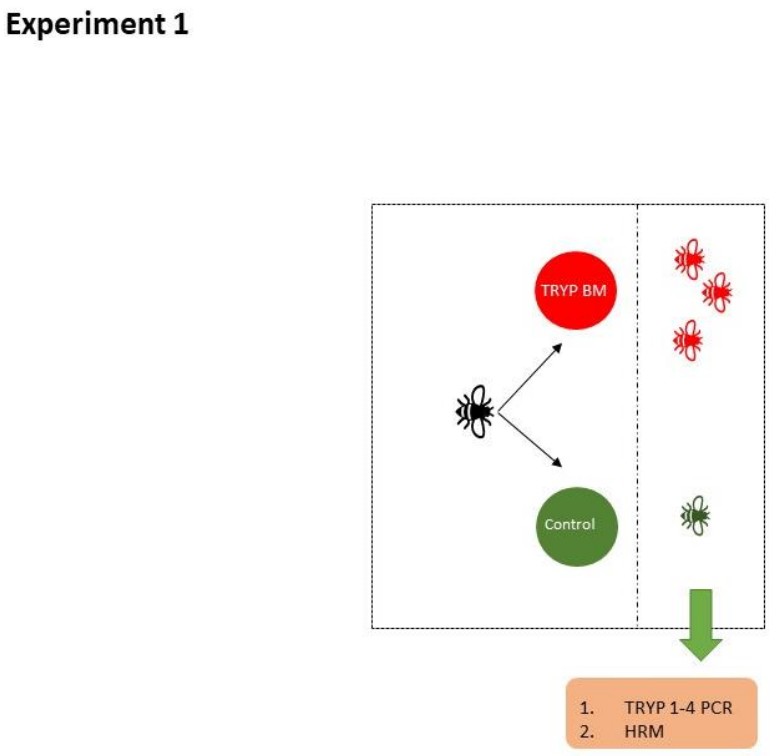

Figure 42 Stomoxys are fed either spiked or unspiked bloodmeal. Once flies have completed feeding they are killed and screened for the presence of trypanosome DNA using either qPCR (HRM) or traditional PCR.

The extracted DNA product will be screened for the presence of target trypanosome DNA using a nested generic PCR (TRYP 1-4 PCR) and qPCR method (HRM).

7.2 Experiment 2: trypanosome DNA persistence in non-vectors.

Aim

Investigate how long post-bloodmeal can trypanosome DNA be detected in a non-vector?

Method

Stomoxys will be fed on bloodmeals spiked with either *Trypanosoma brucei brucei*, *Trypanosoma congolense* Savanna, *Trypanosoma vivax* or control blood. Once fed, 10 flies from each group will be killed and stored in 100% ethanol. Twenty-four hours following blood feeding, a further ten flies (per trypanosome species group) will be killed and screened for the presence of trypanosome DNA. If DNA if present we will sample at 48 hours as well. If no DNA is detected, we will screen the flies every 12 hours following feeding (or at smaller time increments i.e., six hours). DNA will be extracted using DNeasy and the extracted product run on HRM and PCR.

7.3 Experiment 3: limit of detection of trypanosome DNA in *Stomoxys*

Aim

Dilutions of trypanosome concentration in bloodmeal on persistence and detection of trypanosome DNA

Method

A dilution series of trypanosome spiked blood (for each of the three trypanosome species) will be fed to *Stomoxys*. Flies will be killed every 24 hours (n=10 per group) following infected bloodmeals and stored in 100% ethanol. DNA will be extracted using DNeasy and the extracted product run on HRM and PCR. This process will be continued every 24 hours until 6 days (Figure 44).

Experiment 2

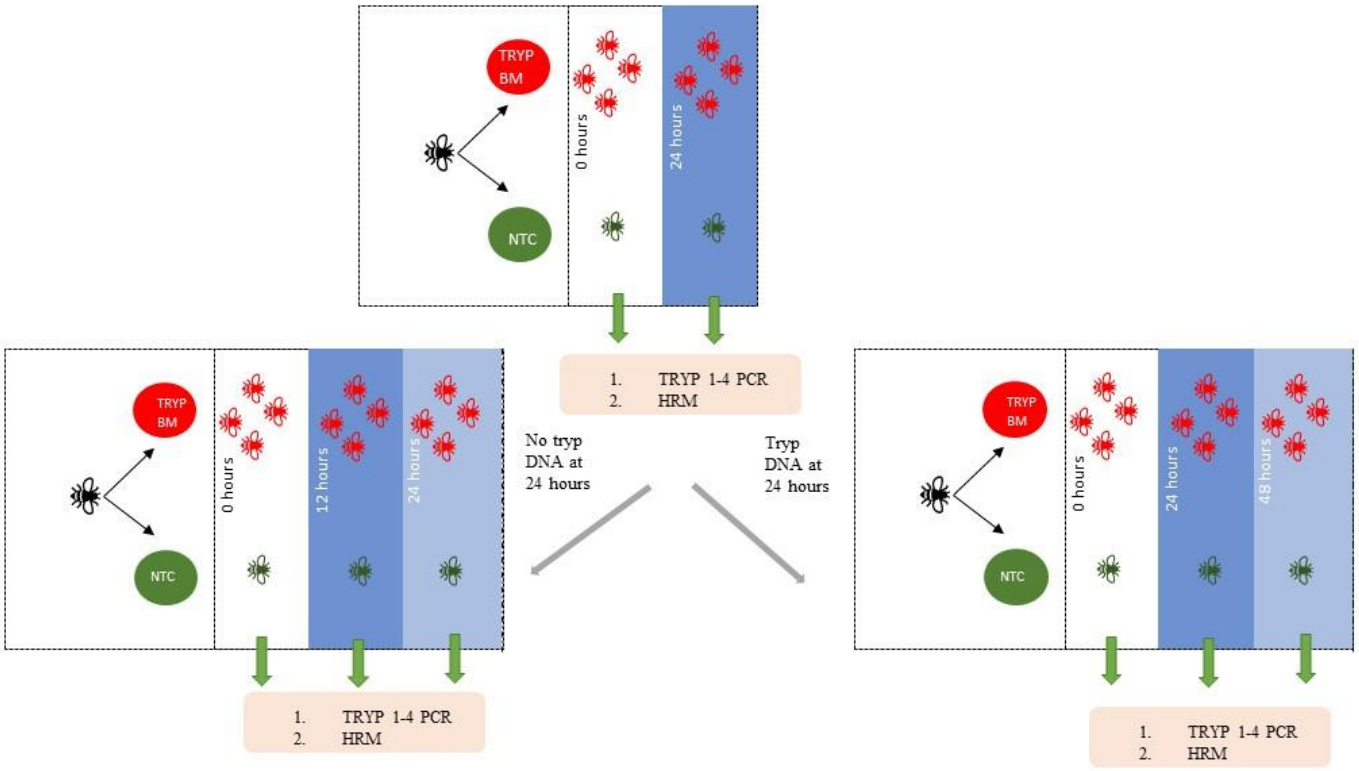

Figure 43 Schematic demonstrating the workflow of experiment 2. Flies are fed infected or uninfected bloodmeals after which they are screened at zero and 24 hours. Presence of trypanosome DNA at 24 hours will result in flies being fed and re-screened at 48 hours. No parasite DNA at 24 hours will result in flies being fed once more and re-screened at 12 hourly intervals up to 24 hours.

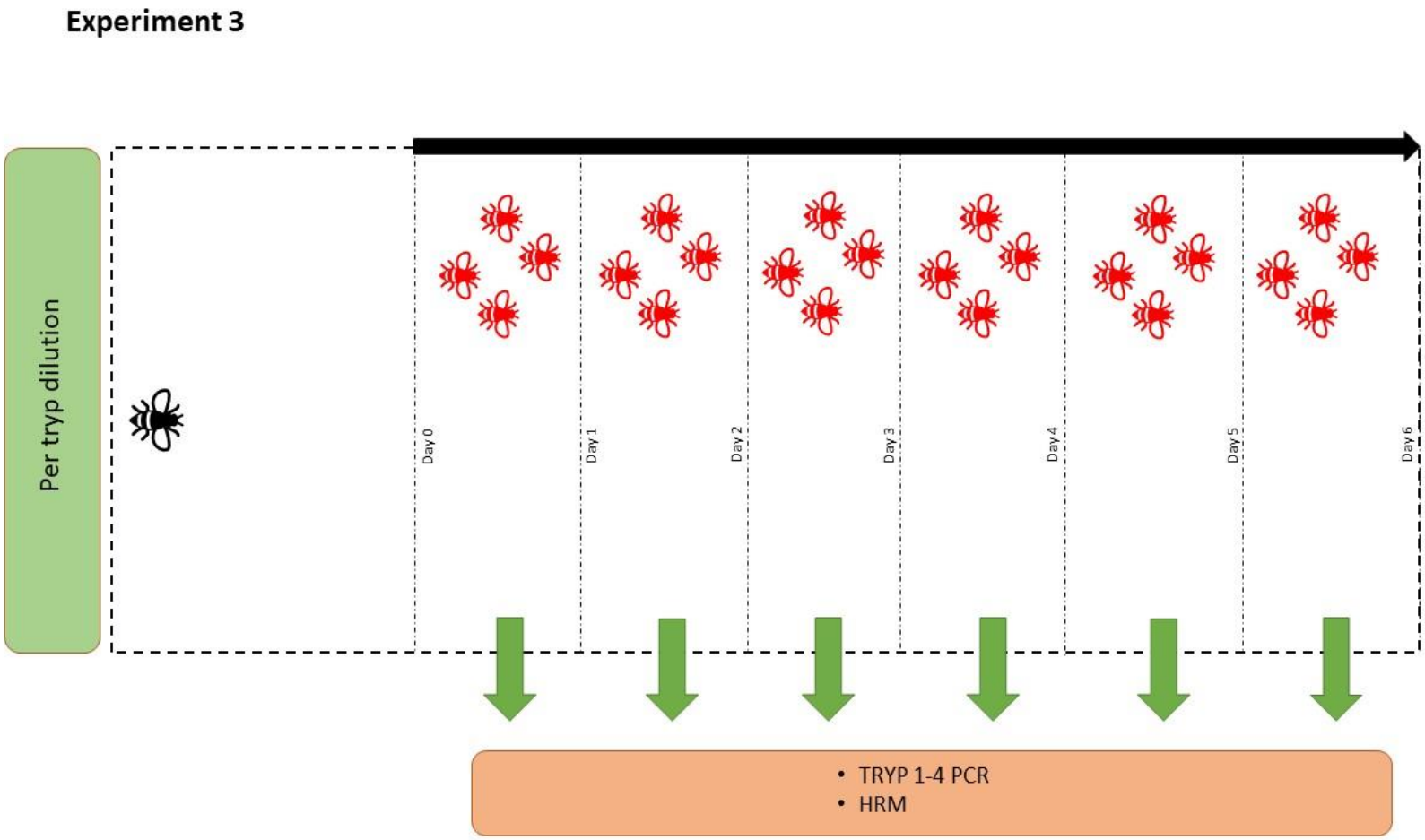

Figure 44 Flies are fed specified concentrations of trypanosomes at day zero. A subset of flies from each group are then screened for the presence of trypanosome DNA every 24 hours from day 0 to day 6.

7.4 Experiment 4: the impact of interrupted feeding on transmission potential

Aim

Flies are likely to be interrupted during blood feeds, either by other flies or the host's defensive behaviours. We aimed to investigate the impact of this interruption at a range of intervals on mechanical transmission potential.

Method

Flies will be allowed to feed on infected bloodmeals for 0,30,60, and 180 seconds before being moved off the feeding tray. Flies will then be killed and stored in 100% ethanol for further processing by HRM and PCR.

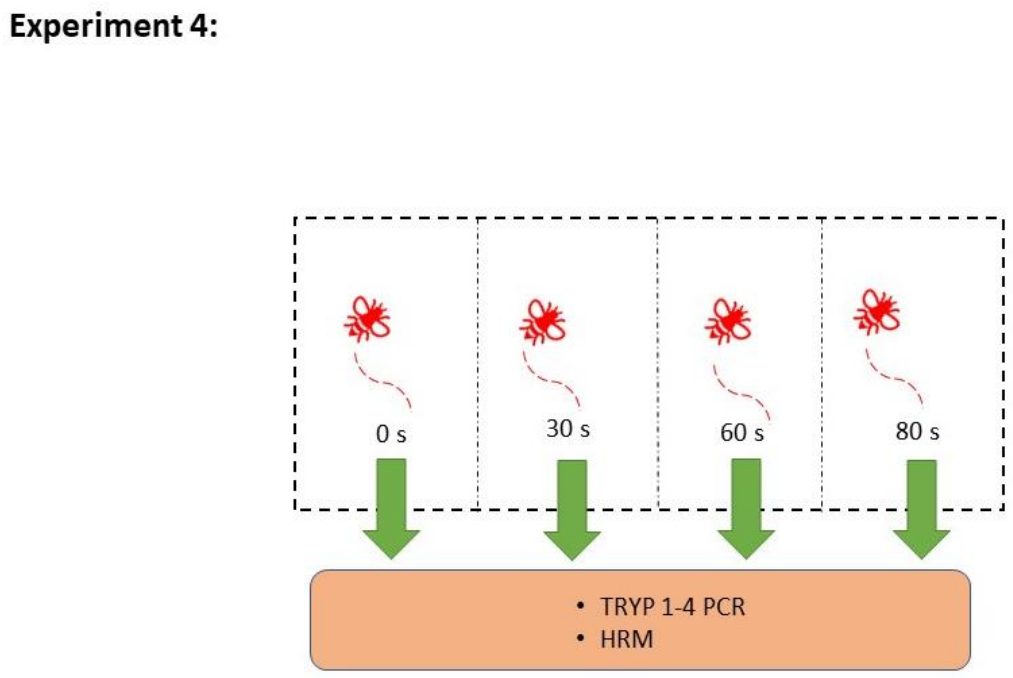

Figure 45 Flies are fed spiked bloodmeals and feeding is interrupted at either 0,30,60, or 80 seconds once feeding has commenced. Flies are then screened to investigate how quickly trypanosome uptake has occurred.