ECOLOGY OF A VECTOR-BORNE ZOONOSIS IN A COMPLEX ECOSYSTEM:

TRYPANOSOMIASIS IN SERENGETI, TANZANIA

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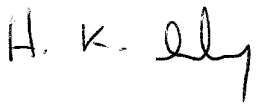
2009

Submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

DECLARATION

I declare that the research described within this thesis is my own work and that this thesis is my own composition.



Harriet K. Auty

Edinburgh, 2009

For my parents, Tessa and David

Acknowledgements

Without the kind assistance of many people who have provided samples, support and discussion, this study would not have been possible. I would like to thank my supervisors Sarah Cleaveland, Sue Welburn, Kim Picozzi and Eric Fevre. Particular thanks go to Sarah Cleaveland, whose enthusiasm continues to be an inspiration.

Funding for this PhD was provided by the Medical Research Council, Department for International Development and the Grieg Foundation.

I am grateful to Tanzania National Parks (TANAPA) and Tanzania Wildlife Research Institute (TAWIRI) for allowing me to conduct research within Serengeti National Park. It is a very special place and an honour to work there. I would like to thank everyone in the Frankfurt Zoological Society, but particularly Markus Borner, Christine Schelton and Festo Msoffe, for their invaluable support.

Particular thanks must go to Dr Richard Hoare and Dr Robert Fyumagwa and everyone else at the Tawiri-Messerli Wildlife Veterinary Programme. I am enormously grateful for their help; as well as carrying out all warthog immobilisations, they kindly collected many samples for me. In addition Sian Brown provided photographs and cake, both equally fantastic. The Tanapa Veterinary Unit, particularly Dr Titus Mlengeya and Dr Morris Kilewo, provided kind assistance and discussion. Dr Imna Malele, Hamisi Nyingilili and Ali Fussa from the Tsetse and Trypanosomiasis Research Institute in Tanga helped me dissect many many tsetse flies. Thanks must also go to Ed Sayer and Claire Lewis at Grumeti Reserves and to William Ngowo at Maswa Game Reserve.

I would not have got very far in Tanzania without the support of everyone at Carnivore Disease Project, particularly Christine Mentzel, Tiziana Lembo, Katie Hampson, Ernest Eblate and Machunde Biggambo. Joseph Masoy provided much appreciated assistance and cheerfulness. Magai Kaare, whose MSc thesis provided the back ground for this study, is much missed.

The fantastic community of researchers in Serengeti provided support, encouragement, boxes of wine and most importantly, radio calls whenever dead animals were spotted. There are too many people to list here, but in particular, I would never have found so many dead things without Ingela Janson, Patrik Jigsved, Anne Hilborn, Laura Simpson, Stephanie Eby, Felix Borner and Anna Estes, and I would never have caught so many warthogs without Dennis Rentsch and Eli Knapp.

Many people in Edinburgh provided help and support. In particular, Darren Shaw kindly provided statistical advice. Pauline McManus helped in more ways than I can remember. Bea Wissmann, Jenna Fyfe, Tim Connelly, Jo Halliday, Darryn Knobel, Paul Bessell, Neil Anderson and Lucas Matemba are the best colleagues and friends anyone could have wished for. Finally, I would particularly like to thank Andrew, without whose love, support and mojitos writing up this thesis would have been a lot harder.

Contents

List of figures

List of tables and photographs

Abbreviations

BCT	Buffy coat technique
BIIT	Blood incubation infectivity test
bp	Base pairs
CATT	Card agglutination trypanosomiasis test
CI	Confidence interval
dNTP	Deoxynucleotide-triphosphate
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
ELISA	Enzyme-linked immunosorbent assay
GR	Game Reserve
GIS	Geographic information system
GPS	Global positioning system
FMDV	Foot and mouth disease virus
HAT	Human African trypanosomiasis
HCT	Haematocrit centrifugation technique
IDE	Infectious disease ecology
ITS	Internal transcribed spacer
LRT	Likelihood ratio test
NCAA	Ngorongoro Conservation Area Authority
NCA	Ngorongoro Conservation Area
NCBI	National Centre for Biotechnology Information
NIMR	National Institute for Medical Research (Tanzania)
OR	Odds ratio
PCR	Polymerase chain reaction
SARS	Severe acute respiratory syndrome

SNP	Serengeti National Park
SME	Serengeti Mara ecosystem
SRA	Serum resistance associated
TANAPA	Tanzania National Parks
TAWIRI	Tanzania Wildlife Research Institute
TBR	*Trypanosoma brucei* PCR primers
TTRI	Tsetse and Trypanosomiasis Research Institute
VSG	Variable surface glycoprotein
WD	Wildlife Division
WNV	West Nile virus

Abstract

Unravelling the complexities of a disease with multiple wildlife host and multiple tsetse vector species is no easy task. After over a century of field observations, experimental studies, anecdotal evidence and conjecture, the role of wildlife in the transmission of trypanosomes is still unclear. Recently, however, frameworks used in the studies of other vector-borne diseases with wildlife reservoirs showed that not only is it possible to understand transmission, but that spatio-temporal predictions of human disease risk and targeted control are realistic aims, even in such complex systems. This thesis explores the epidemiology of human African trypanosomiasis (HAT) in the Serengeti-Mara ecosystem in Northern Tanzania, where recent cases in tourists have highlighted the disease as a public health and economic concern.

Assessment of the prevalence of trypanosome infections in different wildlife species is the first step in investigating the relative importance of different species in disease transmission. Identification of trypanosomes relies on sensitive and specific diagnostic tests. Polymerase chain reaction (PCR) protocols based on interspecies differences in the length of the ribosomal internal transcribed spacer (ITS) regions have been widely used in livestock to identify multiple trypanosome species in one PCR reaction. This study represents the first assessment of these protocols on blood samples collected from wildlife. Clonal sequence analysis of PCR products revealed a large range of trypanosomes circulating in wildlife, including *Trypanosoma congolense, Trypanosoma brucei, Trypanosoma simiae* Tsavo*, Trypanosoma godfreyi* and *Trypanosoma vivax.* In addition sequences similar to known sequences, termed *Trypanosoma simiae*-like and *T. vivax*-like trypanosomes, may reflect further diversity. However, further characterisation is needed before ITS protocols can be used widely for epidemiological studies in wildlife.

The prevalence of *T. brucei* s.l. and *T. congolense* varied widely between species. This variation was predominantly explained by taxonomic classification, suggesting intrinsic differences in response to trypanosomes. *Trypanosoma brucei rhodesiense*, the subspecies responsible for HAT, was identified in lion, hyaena and reedbuck. Age significantly affected the prevalence of *T. congolense* in lion and hyaena, with the highest prevalence in sub-adults. The lack of statistically significant differences in prevalence between animals sampled live or after death confirmed that post-mortem sampling provides a method for increasing sample sizes in wildlife studies. The complex relationship between tsetse density and prevalence of trypanosome infections illustrated the difficulties of assessing data from diverse ecosystems with many potential confounding factors.

A cross-sectional study of *Glossina swynnertoni* and *Glossina pallidipes*, the main tsetse species in Serengeti, highlighted the difficulties of integrating the results of microscopy and PCR to generate meaningful measures of the prevalence of transmissible *T. brucei* infections for epidemiological studies. However, PCR results suggested that *G. pallidipes* may be more important as a vector of *T. brucei* s.l. than has been previously recognised. Spatial variation in both tsetse density and the prevalence of trypanosome infections suggests human disease risk is heterogeneous.

The results of this study, along with relevant literature, are considered within the context of frameworks used for other vector-borne diseases and the implications for disease management discussed.

Chapter 1: Introduction

Vector-borne pathogens with wildlife reservoirs present perhaps one of the biggest challenges for both understanding transmission and developing control strategies. Pathogens not only interact with multiple host and vector species, but exist within complex ecosystems.

Human African trypanosomiasis (HAT) in East Africa is an example of a zoonotic, vector-borne disease where, despite over a century of research, many fundamental questions remain. The causative agent *Trypanosoma brucei rhodesiense*, transmitted by tsetse (*Glossina* spp.), can infect a wide range of wildlife species, but the role of these species in maintenance and transmission of the pathogen, or the relative importance of difference wildlife species is unknown.

The aim of this thesis is to investigate the ecology of HAT in the Serengeti Mara ecosystem (SME) in Tanzania. HAT epidemics have occurred in this area in the past, and sporadic cases indicate a continuing public health concern. In addition, recent cases in tourists to Serengeti National Park drew international attention and threatened to affect the tourist industry, therefore also presenting an economic concern. The SME contains one of the highest densities and diversities of wildlife in the world. How much this wildlife is contributing to transmission of HAT remains to be seen.

This chapter will introduce general approaches and frameworks used for diseases with wildlife reservoirs, and for other vector-borne diseases. The current knowledge of the role of animal hosts and tsetse vectors in the transmission of trypanosome infections will be reviewed. As trypanosomiasis has been the subject of many thorough reviews (for example Mulligan, 1970; Maudlin *et al.*, 2004), this chapter will review only aspects of the literature relevant to the questions addressed in this thesis.

1.1 Wildlife reservoirs of disease

Forty four percent of human pathogens and 54% of livestock pathogens also infect wildlife hosts (Cleaveland *et al.*, 2001). Wildlife have been implicated both in the maintenance of endemic diseases (e.g. the transmission of malignant catarrhal fever from wildebeest to cattle in the SME (Rossiter *et al.*, 1983)) and in the emergence of new diseases (e.g. severe acute

respiratory syndrome (SARS) in Asia (Guan *et al.*, 2003)). Pathogens that infect wildlife in addition to other species are by nature generalist pathogens, which present particular challenges both in understanding transmission dynamics and in management and control.

The ability of a pathogen to infect multiple hosts is also a risk factor for human disease emergence; 75% of emerging human pathogens are zoonotic (Taylor *et al.*, 2001). In particular, emerging diseases (infectious diseases that are newly recognised, newly evolved, or have recently increased in incidence, or expanded into a new geographic, host or vector range (Bengis *et al.*, 2004)) are often associated with wildlife (Daszak *et al.*, 2000). The most common drivers of emergence of human pathogens are changes in land use or agricultural practices, and changes in human demographics (Woolhouse & Gowtage-Sequeria, 2005), which are often associated with increased contact between people and wildlife. For example, the establishment of piggeries close to tropical forest in Malaysia allowed Nipah virus from fruit bats to infect pigs, and then pig farmers (Chua *et al.*, 1999; Field *et al.*, 2001).

1.2 Identifying disease reservoirs

Understanding the potential role of wildlife as reservoirs has important implications for disease control. Sometimes it is possible to control disease by directing efforts at the target population without consideration of the reservoir. For example, vaccinating people against yellow fever is effective for disease control, without understanding the complex non-human primate reservoir system (Robertson *et al.*, 1996). However, in many disease systems this is not possible and effective disease control is dependent on understanding the reservoir system. For example, control of Ebola haemorrhagic fever is hindered by uncertainty over the wildlife reservoirs of Ebola virus (Groseth *et al.*, 2007).

Using the definition of Haydon *et al.* (2002), a reservoir is ‘one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population’. The critical community size is the minimum size of a closed population within which the pathogen can persist. Populations that exceed this size can be maintenance populations. In smaller populations, termed non-maintenance populations, the number of hosts is insufficient for the pathogen to persist. These populations may however still be important. In complex

systems, several smaller populations which could not maintain the pathogen alone may combine to form a reservoir. Those which are essential for maintenance form a maintenance community. Others may not be essential for maintenance but can still be part of the reservoir. This is illustrated in Figure 1-1, which shows potential reservoir systems for rabies in Zimbabwe.

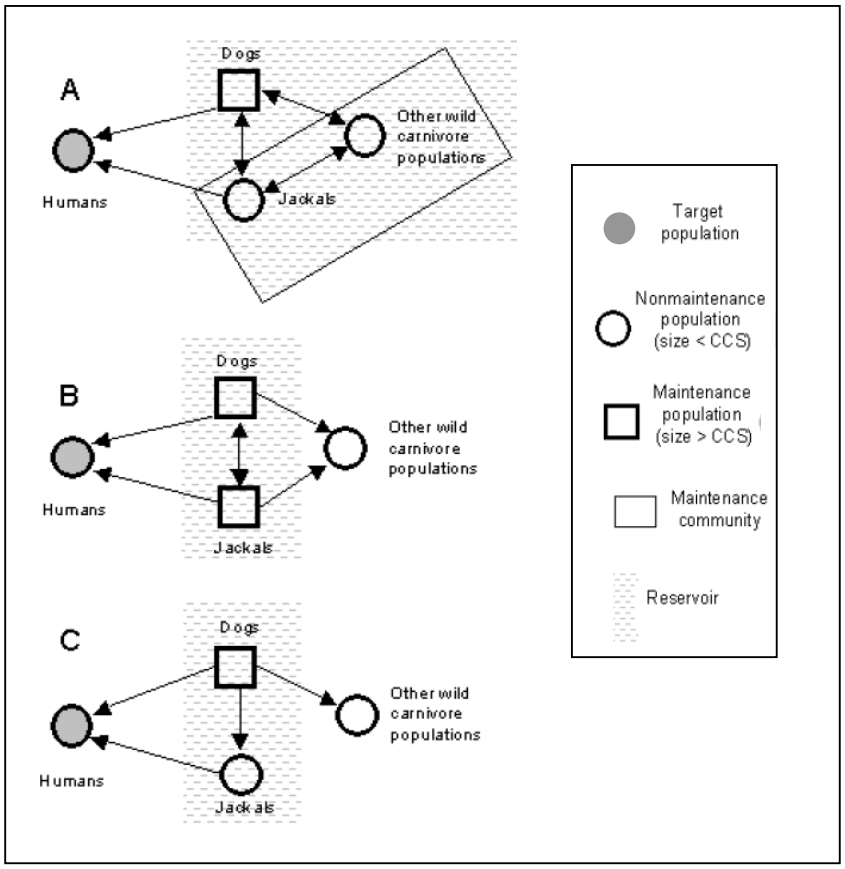


Figure 1-1: Potential reservoir systems for rabies in Zimbabwe

(reproduced from Haydon *et al.*, 2002)

Rabies is able to persist in domestic dog populations, but the role of jackals and other carnivore species is unclear.

A: Neither jackals or other carnivores can maintain infection, but together they form a maintenance population. The reservoir comprises dogs, jackals and other carnivores.

B: Jackals are able to maintain infection independently. Rabies cannot persist in other carnivore populations.

C: Domestic dogs are the only maintenance population. Control of rabies in domestic dogs should lead to rabies elimination.

In addition to the target population and the maintenance population, some reservoir systems also have source populations, which provide transmission links between the maintenance and target populations. For example, foot and mouth disease virus (FMDV) is able to persist in African buffalo. Although impala are unable to maintain FMDV infection independently, they remain an important route of infection for cattle, so would be termed a source population (Bastos *et al.*, 2000). In this example, the reservoir would comprise both buffalo and impala.

Definitive identification of a reservoir is difficult. The ultimate proof of a reservoir requires isolation of the target population from transmission from the potential reservoir, followed by declining disease incidence and eventually elimination. For example, culling to reduce mountain hare densities on grouse moors in Scotland resulted in a decline in both the overall tick population and the prevalence of louping-ill virus in red grouse. This provided evidence that mountain hares were acting as reservoirs (Laurenson *et al.*, 2003).

There are few examples, however, of studies that definitely identify reservoirs, particularly because of the randomisation and replication necessary to obtain meaningful results when studying ecosystems that have inherently high variability. The controversy that continues over the role of badgers in transmitting *Mycobacterium bovis* to cattle in the UK illustrates that even large scale, carefully designed, expensive studies do not always produce conclusive results (Woodroffe *et al.*, 2006). Consequently, intervention studies, often in the form of control programs, can act as quasi experiments and allow inferences to be made about reservoir status. An example of this is the vaccination of cattle against rinderpest, which eventually confirmed that cattle act as the reservoir for rinderpest, and the virus is unable to persist in wildlife alone. After cattle vaccination began in the 1950s, the wildebeest population in the SME increased nearly ten-fold as mortality of yearlings fell dramatically (Sinclair, 1979a; Dobson, 1995). The continued detection of rinderpest virus antibodies in buffalo in the Serengeti Mara ecosystem raised the possibility that wildlife could maintain infection independently. However the lack of antibody response in buffalo born since 1985 confirmed the disease had not continued to circulate in buffalo, and it is suspected that previous sporadic exposure originated from undetected outbreaks in cattle (Kock *et al.*,

2006; Rossiter *et al.*, 2006). Rinderpest eradication is now close to being declared with over 130 countries officially declared rinderpest free (World Organisation for Animal Health).

In the absence of definitive evidence, accumulation of other information can support the existence of a reservoir. Presence of the pathogen (or genetic material or antibodies) provides evidence of infection. Although this does not provide evidence of transmission to the target population, it is an important step in identifying potential reservoirs. Consistency in the genetic or antigenic characteristics of the pathogen in the reservoir and target population supports the potential for transmission, but does not provide information about direction of transmission.

1.3 Vector-borne diseases

Zoonoses with a wildlife reservoir that are also vector-borne present a particular challenge. Transmission is dependent on infectious vectors biting susceptible hosts. In addition to host and pathogen factors, the biology of the (potentially multiple) vector species must be considered, with vector population dynamics, ecology, and competence all important factors in disease epidemiology. The relative importance of these factors varies between disease systems. In some systems the main source of heterogeneity in transmission from host species is selective feeding by vectors (for example in transmission of West Nile Virus (WNV)), whereas in others (for example Lyme disease in North America) variation in reservoir competence between host species has more influence (Kilpatrick *et al.*, 2006).

Terms used to describe vector-borne disease systems are not always consistent. For clarity, terms will be used in this thesis according to the definitions below.

1.3.1 Vector factors affecting disease transmission

Vector competence

Vector competence is the inherent permissiveness of a vector for the infection, replication and transmission of a pathogen (Woodring *et al.*, 1996). Vector competence is usually measured by experimental infections, using for example the fraction of WNV-infected mosquitoes that will transmit virus in a subsequent bite (Kilpatrick *et al.*, 2005). Vector competence and vectorial capacity are often used interchangeably. However, vector

competence refers to the intrinsic factors that influence the ability of a vector to transmit a pathogen, where as vectorial capacity refers to all the factors that influence vector pathogen interactions, including behavioural and environmental factors as well as vector competence (Hardy *et al.*, 1983; Beerntsen *et al.*, 2000). For example, a vector species could be capable of transmitting a pathogen in the laboratory, i.e. have high vector competence, but not choose to feed on host species which carry the pathogen in the field (Beerntsen *et al.*, 2000).

Vector ecology

Vector population dynamics, ecology and behaviour differ considerably between disease systems, ranging from ticks, with life cycles which can take several years to complete and low mobility, to mobile insect vectors with complex host choice behaviour and short life cycles (Randolph, 1998). These differences in biological attributes affect disease transmission, with higher transmission rates necessary for insect-borne pathogens than tick-borne pathogens, to account for higher vector mortality in insect vectors (Hudson *et al.*, 1995). Vector feeding frequency, host preferences, survival and abundance all have important effects on transmission.

1.3.2 Host factors affecting disease transmission

Reservoir competence

Reservoir competence is the probability of an exposed host infecting a feeding vector, and depends on three criteria: (a) the susceptibility of the reservoir host when exposed to an infected vector (b) how effectively the pathogen can proliferate in the host and (c) the degree and length of infectivity to other vectors (Richter *et al.*, 2000). This is often assessed using experimental infections. For example, in assessment of the reservoir competence of different avian species in the transmission of WNV, reservoir competence was calculated as an index of (i) susceptibility (proportion of exposed birds that became positive); (ii) infectiousness (proportion of exposed vectors that became infectious per day); and (iii) duration of infectiousness (the number of days of infectious viraemia) (Komar *et al.*, 2003).

In reality, 'realised reservoir competence' is usually a more meaningful term as it also incorporates the exposure of hosts to infected vectors in the field (LoGiudice *et al.*, 2003). The term 'reservoir potential' (Mather *et al.*, 1989) indicates the average number of infected

vectors produced by an individual of a given host species, and is the product of the number of vectors fed by an individual of a given species and realized reservoir competence.

In the absence of experimental data on reservoir competence, authors have attempted to identify criteria for classifying reservoirs of vector-borne diseases. However, as with definitions of reservoirs discussed above, this is not an easy task. For example, Silva *et al.* (2005) suggest five criteria by which wildlife species could be classified as primary reservoirs for cutaneous leishmaniasis (they term primary reservoirs those responsible for maintaining the parasite enzootic cycle in nature): (a) Overlap between geographical and temporal distribution of vectors and hosts; (b) survival of the reservoir host long enough to guarantee disease transmission; (c) infection prevalence higher than 20% among hosts; (d) maintenance of the parasite in skin lesions or blood (at quantities large enough to infect the vector easily); and (e) presence of the same *Leishmania* species in the reservoir and humans. Whilst these criteria can all contribute to building evidence that a species is acting as a reservoir, this approach does not prove transmission is occurring, and several of these criteria could be met by species that are not reservoirs, leading to potential misclassification (Chaves *et al.*, 2007).

Variation in reservoir competency between host species has important consequences for disease transmission within an ecosystem. The 'dilution effect' refers to the reduction in disease risk resulting from increased biodiversity (reviewed by Keesing *et al.*, 2006). In terms of vector-borne disease dilution effect is often used more specifically to mean a reduction of disease risk due the presence of a diversity of relatively incompetent reservoir hosts (Ostfeld & Keesing, 2000). Within an ecosystem, host species with low reservoir competency may still have an important effect on transmission dynamics, by acting as dilution hosts. The most effective dilution hosts are popular feeding sources for vectors, are present at high density and have low reservoir competence (LoGiudice *et al.*, 2003). Increasing the number or density of incompetent hosts leads to a dilution effect, whilst decreasing incompetent hosts can lead to increased transmission as vectors feed more on competent hosts.

The role of dilution hosts can be illustrated in transmission of *Borrelia burgdorferi*. The causative agent of Lyme disease in the United States, this spirochaete is transmitted by *Ixodes* ticks. The principal reservoir is the white footed mouse (*Peromyscus leucopus*),

which is able to infect 40-80% of the larval ticks feeding on it. Several species, such as Eastern chipmunks (*Tamias striatus*), are moderately effective reservoirs. Most other species are incompetent reservoirs, seldom able to infect the ticks that feed on them. Changes in ecosystem composition, for example due to habitat fragmentation, have important consequences for Lyme disease transmission. Changes usually favour the white footed mouse, a generalist in habitat and dietary requirements, whilst other species are more likely to be lost. The loss of species that are incompetent reservoirs, or dilution hosts, such as red and grey squirrels (*Tamiasciurus hudsonicus, Sciurus carolinensis*) increases the spirochaete prevalence in the tick population (LoGiudice *et al.*, 2003).

The 'rescue effect' is used to refer to the maintenance of the disease agent at a relatively constant prevalence even when host populations fluctuate due to the presence of multiple competent reservoirs (Ostfeld & Keesing, 2000). In the Lyme disease system, shrews (*Blarina brevicauda* and *Sorex* spp.) can act as rescue hosts. At high densities of white footed mice, shrews contribute to the dilution effect. However at low mouse densities, the reservoir competence of shrews, although lower than that of mice, is sufficient to maintain spirochaete transmission in the ecosystem (LoGiudice *et al.*, 2003).

As can be seen in the preceding sections, in recent years the study of the interactions between hosts, vectors and pathogens, within the larger scale of an ecosystem and all its processes, has become a rapidly expanding discipline. The field of infectious disease ecology (IDE) has arisen partly as a result of the realisation that many diseases cannot be understood without considering the other aspects of the ecosystems in which they exist (Ostfeld *et al.*, 2008). This is highlighted both by the emergence of new diseases, such as SARS, and the continuing difficulties in controlling old ones, such as human African trypanosomiasis. HAT has not been considered before within the frameworks that have been widely used for other zoonotic vector-borne diseases.

1.4 Introduction to Human African Trypanosomiasis (HAT)

HAT is a debilitating disease that is fatal without treatment. More common in remote areas, it is likely that many cases are never diagnosed and die without treatment (Ekwanzala *et al.*, 1996). Epidemics can be explosive, disrupting communities and causing whole areas to be abandoned (Cattand *et al.*, 2001).

During the early Twentieth Century HAT was easily the most important public health problem in East Africa (Ekwanzala *et al.*, 1996). Efforts by the colonial authorities to control the devastating epidemics had wide ranging sociological and ecological effects. For example in Tanzania the main strategies for HAT control were resettlement and bush clearance. Between 1920 and 1934, over 130 000 people were forcibly moved into tsetse-free sleeping sickness settlements (Hoppe, 2003). Between around 1930 and 1960 approximately 2000 square miles of brush was cleared (Hoppe, 2003). Whilst not a method widely used in Tanzania, destruction of wildlife was the mainstay of control policies in Southern Africa. The numbers of animals killed was staggering; for example in Zimbabwe in one year alone (1954) 36,910 animals were shot (reviewed by Ford, 1970).

Although thought to be mainly under control by the 1960s, HAT is re-emerging in many countries as a serious public health concern (for example Angola (Stanghellini & Josenando, 2001), Democratic Republic of Congo (DRC) (Van Nieuwenhove *et al.*, 2001), Sudan (Moore & Richer, 2001) and Uganda (Fevre *et al.*, 2001)). The problem is exacerbated in countries debilitated by civil unrest, where lack of disease surveillance and control and increased mobility of people and livestock can lead to increased disease incidence (Fevre *et al.*, 2001; Moore & Richer, 2001; Lutumba *et al.*, 2005).

Two distinct forms of HAT exist, caused by subspecies of *Trypanosoma brucei* sensu lato and differing in clinical appearance and geographical location. *Trypanosoma brucei gambiense* is found in West and Central Africa and manifests as a chronic disease with a long incubation period, with death occurring several years post infection. Separated by a boundary that approximately follows the Rift Valley, *Trypanosoma brucei rhodesiense* is found in East and Southern Africa. The disease pattern in Rhodesian sleeping sickness is characterised by rapid progression of clinical signs, with death frequently occurring within six months without treatment. A third subspecies, *Trypanosoma brucei brucei*, does not infect humans but is found in livestock and wildlife across sub-Saharan Africa. The three subspecies comprise the subgenus *Trypanozoon* and are morphologically indistinguishable.

T. brucei s.l. is transmitted by tsetse flies (*Glossina* spp.). Trypanosomes undergo cyclical development in tsetse before infective forms can be injected into new hosts when the tsetse feed (Figure 1-2).

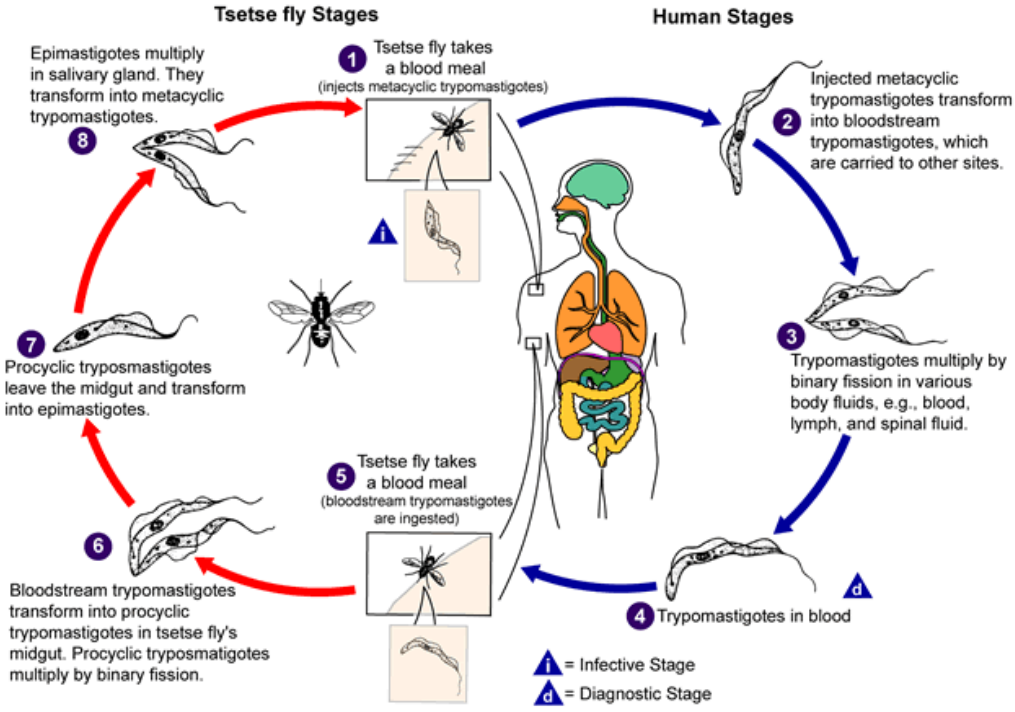


Figure 1-2: Trypanosome life cycle showing circulation between tsetse and mammalian hosts (reproduced from http://www.dpd.cdc.gov/dpdx/)

1.5 Animal reservoirs of HAT

Trypanosomes were observed in the blood of wildlife species at the same time that they were first linked to *nagana* in cattle (Bruce, 1895). It was assumed that wildlife played a role in both human and animal trypanosomiasis long before it was confirmed experimentally. Large scale wildlife extermination widely conducted in Southern Africa was predominantly based not on removal as wildlife as reservoirs of trypanosomes, but on the assumption that wildlife provided an important food source for tsetse, and removal of wildlife would lead to the disappearance of tsetse (Ford, 1970). Debate on the justification of wildlife destruction raged for many years, illustrated by Yorke (1913):

> *"It may seem an act of vandalism to slaughter the wonderful fauna of Africa, but surely when it is definitely proved that this fauna is antagonistic to civilisation, that which stands in the path of progress must be removed."*

The first experimental studies to confirm the zoonotic nature of the disease relied on parasites isolated from animals causing sleeping sickness in human 'volunteers', and showed that bushbuck (Heisch *et al.*, 1958) and domestic cattle (Onyango *et al.*, 1966) could be infected with *T. b. rhodesiense*. However, epidemiological studies to determine the role of reservoir hosts have been limited by the difficulties of differentiating *T. b. rhodesiense* from *T. b. brucei*, and the necessarily small sample sizes of these experiments were unable to shed light on the relative importance of different animal species.

The blood incubation infectivity test (BIIT) resolved the ethical concerns of human volunteers, relying on the ability of *T. b. rhodesiense* to survive the trypanocidal effects of human serum (Rickman & Robson, 1970). Whilst useful for initial studies on the role of animal reservoirs, results in field studies were often inconsistent (Geigy *et al.*, 1971), and it was discovered that the serum resistance initially demonstrated by *T. b. rhodesiense* was affected by passage through rodent hosts (Targett & Wilson, 1973).

The discovery of the serum resistance associated (SRA) gene, responsible for the resistance of *T. b. rhodesiense* to human serum (Xong *et al.*, 1998), finally allowed reliable differentiation between *T. b. rhodesiense* and *T. b. brucei* through the development of polymerase chain reaction (PCR) -based protocols, and opened the door for more extensive studies on the role of animal reservoirs in trypanosome epidemiology.

Use of the SRA technique revealed that a high proportion of cattle in Soroti District, Uganda, carry *T. b. rhodesiense*, with up to 18% of cattle infected (Welburn *et al.*, 2001). In this area, movement of *T. b. rhodesiense* infected cattle through markets or restocking programs has resulted in expansion of foci into new areas (Fevre *et al.*, 2001). At the height of an epidemic in Tororo District, Uganda, it was estimated that a fly infected with *T. b. rhodesiense* was five times more likely to have picked up the infection from domestic cattle than from an infected person (Hide *et al.*, 1996). Sheep, goats and pigs have also been shown to be capable of harbouring *T. b. rhodesiense*, with the prevalence in pigs in South Eastern Uganda reaching 13.9% for *T. brucei* s.l. and 4% for *T. b. rhodesiense* (Waiswa *et al.*, 2003). Declining wildlife populations in these parts of Uganda mean wildlife is not generally regarded as an important source of infection. Cattle are frequently referred to as the reservoir of HAT in Uganda. It is clear that they represent an important source of infection but the

exact nature of the reservoir, particularly in terms of the importance of other livestock species, is not known.

Control programs aimed at reducing the prevalence of *T. b. rhodesiense* in cattle through the use of trypanocides, or preventing tsetse contact through insecticide use, are currently in place in Uganda (Kabasa, 2007). Although the focus of this project is implementing disease control, the impact of these programs on the incidence of human disease will also provide information on the role of cattle in disease transmission.

1.5.1 Wildlife reservoirs of HAT

The importance of wildlife in trypanosome transmission remains unclear. From current knowledge, the question can be asked: what is the evidence that wildlife is acting as a reservoir for trypanosomiasis? Adapting Haydon *et al*'s (2002) definition of a reservoir this means: Can trypanosomes infections be permanently maintained in wildlife (one species or several species) and be transmitted from wildlife to man?

Whilst few rigorously conducted case control studies exist, widespread anecdotal evidence suggests that hunters, fishermen, honey gatherers and other people entering areas inhabited only by wildlife are at high risk of disease (Davey, 1924). In the face of the massive epidemics of the early 20th century, colonial administrations resettled hundreds of thousands of people out of tsetse areas, incidentally creating experiments in pathogen persistence. In Tabora District of Tanzania, Jackson (1955) describes how an area of 800 square miles adjacent to the Ugala river was depopulated in 1925 to prevent further cases of HAT, and all access for prospecting, hunting and timber cutting prohibited. Despite these measures, sleeping sickness cases continued to occur in his staff when they entered the area. This demonstrates that in the absence of man or livestock, human infective trypanosomes continued to circulate in wildlife and tsetse populations. Similar situations were seen in Kibondo district in Tanzania, and in Samia District in Uganda (Fairbairn, 1948). The recent occasional cases of HAT in tourists and staff in Serengeti National Park in Tanzania, in the absence of livestock or of other cases in man, also support this.

HAT is characterised by its ability to persist in specific geographic foci (Hide, 1999). It has often been suggested that wildlife is responsible for long term maintenance of human

infective trypanosomes within foci. Many early authors regarded wildlife as responsible for disease endemicity between epidemics, whilst livestock and man were thought to be responsible for disease amplification within epidemics (Fairbairn, 1948). Hide (1999) suggests the criteria under which a focus may exist. Firstly the human infective form must be present, arising by mutation and genetic recombination, or specifically by selection. Once a transmission cycle is established, occasional human infection may be sufficient to maintain circulation of the human infective form. Epidemic selection of this strain may occur when ecological change results in an increase in the fly population or biting rate increases. The density of infective hosts is increased by close interaction between humans, animals and tsetse. After an epidemic, endemicity returns, with human infective forms circulating at a low rate until ecological changes again trigger epidemics (Hide, 1999). However, the higher proportion of *T. b. brucei* compared to *T. b. rhodesiense* found in non-human hosts and tsetse suggests the ability to resist human serum may confer a fitness disadvantage in other species (Coleman & Welburn, 2004) and the mechanism by which *T. b. rhodesiense* is able to persist is unclear. Given that understanding endemicity is important for long term control of HAT, it is perhaps surprising that more progress has not been made in understanding its focal nature.

Various reasons have been identified in association with epidemicity or expansion of a focus. In Uganda, movement of cattle infected with *T. b. rhodesiense* from endemic HAT areas to areas where HAT had never been reported have caused foci to expand (Fevre *et al.*, 2001). Within SME, authors have hypothesized that epidemics arose from increased human-fly contact, for example working in gold mines in tsetse areas (Onyango & Woo, 1971), or increased numbers of tented camps increasing exposure of tourists to tsetse (Kaare *et al.*, 2007).

1.5.2 Relative importance of wildlife species as reservoirs of HAT

Host range and prevalence

T. brucei s.l. has been identified in a large range of species. The host range and prevalence of *T. brucei* s.l. in wildlife from studies in East and Southern Africa can be seen in Table 1-1. Studies on the prevalence of *T. brucei* s.l. in wildlife have been geographically diverse and used diagnostic tests of varying specificity and sensitivity. In addition there is clearly much variation in the number of samples analysed for each species. Despite this, some species have consistently tested positive for *T. brucei* s.l., such as bushbuck, hartebeest, lion, spotted

hyaena, warthog and reedbuck, even from different geographical areas. Whilst the majority of studies have focused on mammalian hosts, the discovery of *T. brucei* s.l. in a monitor lizard shows that other classes cannot be excluded as potential reservoirs (Njagu *et al.*, 1999).

T. b. rhodesiense has been indentified in bushbuck in Utonga Ridge, Kenya (Heisch *et al.*, 1958), lion, spotted hyaena, waterbuck, hartebeest and warthog in the Serengeti area (Geigy *et al.*, 1971; Geigy & Kauffman, 1973; Geigy *et al.*, 1973a; Kaare *et al.*, 2003), reedbuck in Lambwe Valley, Kenya (Robson *et al.*, 1972; Njiru *et al.*, 2004b) and warthog in Luangwa Valley, Zambia (Awan, 1979). The prevalence of *T. b. rhodesiense* is difficult to assess due to a generally low prevalence in host populations, and to the difficulties of identification described in section 1.5. In general, *T. b. rhodesiense* has been identified in the species with the highest prevalence of *T. brucei* s.l. and may reflect a consistent ratio between *T. b. rhodesiense* and *T. b. brucei.* In other non-human host and tsetse populations, a ratio of around one *T. b. rhodesiense* to three *T. b. brucei* infections is frequently found (Coleman & Welburn, 2004).

Table 1-1: Identification of *T. brucei s.l.* in wildlife species

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*
Mammals																					
African civet		0 (1)														0 (6)					
Bat														0 (427)		0 (2)					
Bat-eared fox						0 (2)															
Black rhinocerous																0 (5)	**7 (39)**				
Buffalo	0 (2)			0 (3)	0 (3)				0 (24)				**2 (190)**		**8 (416)**	0 (19)					0 (1)
Bushbuck	0 (2)		**2 (10)**		**2 (58)**		0 (2)	**1 (6)**	0 (7)							**2 (23)**					
Bushpig	0 (3)				0 (3)																
Cane rat																0 (1)					
Cheetah						0 (1)												0 (6)			0 (1)
Dikdik	0 (12)	0 (1)																			
Duiker	0 (9)		**1 (13)**													0 (7)					
Eland	**1 (22)**			0 (5)									0 (1)			0 (3)					0 (4)
Elephant	0 (6)				0 (1)				0 (3)							0 (20)					
Genet	0 (1)					0 (3)										0 (6)					
Giraffe	0 (62)	0 (1)		0 (1)												**1 (1)**					0 (1)
Grant's gazelle	0 (4)	0 (5)		0 (3)			0 (2)														
Greater kudu	0 (7)												**1 (16)**			0 (13)					
Grysbok																0 (5)					
Hare									0 (6)							0 (10)					
Hartebeest	0 (7)	**1 (7)**		**1 (10)**			**3 (11)**				**3 (20)**										
Hartebeest (Lichtensteins)		0 (5)																			
Hippopotamus	0 (3)				0 (2)					**4(75)**											
Impala	0 (87)	0 (10)		**1 (6)**			0 (11)					**5 (85)**	0 (23)			0 (23)					0 (15)
Jackal (black-back)				0 (2)		0 (6)										0 (1)					
Jackal (side-stripe)	0 (1)																				
Klipspringer	0 (2)	0 (1)																			

Table 1-1: Identification of *T. brucei s.l.* in wildlife species

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*
Kob									0 (3)												
Lechwe													0 (13)		**9 (50)**						
Leopard	0 (1)			0 (1)		0 (1)										0 (2)					
Lion	0 (2)			**7 (11)**		**11 (68)**	**5 (9)**		0 (1)		**24 (43)**					**3 (6)**					**2 (9)**
Mongoose (banded)						0 (3)															
Mongoose (slender)																0 (2)					
Mongoose (white tailed)						0 (3)															
Monkey (Syke's)																				0 (55)	
Monkey (Vervet)																0 (18)				0 (56)	
Olive baboon	0 (5)															0 (20)				0 (14)	
Oryx		0 (1)																			
Oribi		0 (4)																			0 (1)
Porcupine	0 (3)															0 (1)					
Puku																0 (24)					
Reedbuck	0 (2)	0 (3)					0 (10)	**3 (37)**	**1 (9)**						0 (2)						**1 (1)**
Reedbuck (Southern)		0 (4)																			
Roan antelope	0 (25)	0 (5)														0 (11)					
Sable antelope													0 (11)								
Serval	0 (2)		0 (1)													0 (2)					
Sitatunga					0 (1)																
Spotted hyaena	0 (5)			0 (3)		0 (11)	**2 (5)**		0 (1)		**13 (31)**					**2 (7)**					**1 (1)**
Steenbok	0 (6)	0 (1)																			
Thomson's gazelle	0 (5)	0 (5)		0 (7)			0 (11)														0 (24)
Topi	0 (8)	0 (5)		**2 (11)**			0 (11)		0 (7)												**1 (46)**
Tsessebe													0 (18)								
Warthog	**1 (37)**	0 (4)		**1 (14)**			**1 (13)**		0 (5)							**1 (24)**					**6 (21)**
Waterbuck	0 (1)			**3 (6)**	**1 (5)**		**1 (10)**	**1 (3)**	0 (6)		0 (1)					**2 (20)**					

Table 1-1: Identification of *T. brucei s.l.* in wildlife species

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*
Wild cat																0 (1)					
Wild dog	0 (2)	0 (1)		0 (2)		0 (4)										0 (2)					
Wildebeest	0 (9)	0 (2)		**4 (22)**			0 (10)						0 (10)			0 (5)					**1 (70)**
Zebra	0 (32)	0 (7)		**2 (22)**			0 (10)									0 (5)					0 (26)
Reptiles																					
Crocodile																0 (1)					
Monitor lizard																			**1 (19)**		
Birds																					
Ostrich		0 (1)																			

Table shows number of animals testing positive for *T. brucei* sensu lato with number of animals tested in parentheses. Diagnostic tests used were thick and thin smears, haematocrit concentration, rodent inoculation and PCR. Column headings indicate source references: 1: (Vanderplank, 1947); 2: (Ashcroft, 1959); 3:(Heisch *et al.*, 1958); 4: (Baker, 1968); 5: (Burridge *et al.*, 1970); 6: (Sachs *et al.*, 1971); 7: (Geigy *et al.*, 1971); 8: (Robson *et al.*, 1972); 9: (Mwambu & Woodford, 1972); 10: (Dillmann & Awan, 1972); 11: (Geigy & Kauffman, 1973); 12: (Irvin *et al.*, 1973); 13: (Carmichael & Hobday, 1975); 14: (Woo & Hawkins, 1975); 15: (Drager & Mehlitz, 1978); 16: (Dillmann & Townsend, 1979); 17: (Clausen, 1981); 18:(Averbeck *et al.*, 1990); 19: (Njagu *et al.*, 1999); 20: (Jeneby *et al.*, 2002); 21: (Kaare *et al.*, 2007).

Parasitaemia and pathogenicity

Parasitaemia in wildlife is typically low, reflecting the ability of native wildlife species to control trypanosome infections (Mulla & Rickman, 1988b). The mechanisms by which wildlife can control trypanosome infections are thought to be similar to the trypanotolerance seen in certain breeds of cattle such as the N'Dama. Trypanotolerant cattle may still become infected with trypanosomes and mount an immune response, however they are able to control parasite proliferation, and limit the pathological effects of the parasites, leading to lower parasitaemia and less severe anaemia compared to exotic breeds (Murray et al., 2004). In a similar manner, Cape buffalo exhibit both lower parasitaemia and fewer parasitaemic waves than cattle when infected with *T. brucei* s.l. , *T. congolense* and *T. vivax* (Dwinger *et al.*, 1986; Grootenhuis *et al.*, 1990; Redruth *et al.*, 1994).

This has been attributed at least in part to a trypanocidal factor identified in buffalo serum, thought to result in temporary clearance of trypanosomes in early stages of infection, and to suppress parasitaemia in chronic infections (Redruth *et al.*, 1994; Muranjan *et al.*, 1997; Black *et al.*, 2001). Trypanocidal activity against *T. brucei* s.l. has also been observed in other species including eland, kudu, waterbuck, and wildebeest (Rurangirwa *et al.*, 1986; Mulla & Rickman, 1988a; Redruth *et al.*, 1994; Black *et al.*, 1999). Whilst there is some evidence to suggest the acquired immunity may also play a role (reviewed by Mulla & Rickman, 1988b), other authors have concluded that the timing and magnitude of parasite surface coat specific antibody production is insufficient to be the main factor in controlling parasitaemia (Redruth *et al.*, 1994).

The generally held view of trypanotolerance in wildlife has been challenged by experimental infections of captive wildlife, in which infection with *T. brucei* s.l. caused morbidity and mortality in some species. Ashcroft *et al.* (1959) group Thomson's gazelle, dikdik, blue forest duiker, jackal, vervet monkey, serval, hyrax, 'fox' and antbear as species usually killed by infection, compared to common duiker, eland, bohor reedbuck, spotted hyaena, oribi, bushbuck, impala, warthog, bushpig and porcupine which were parasitaemic but did not exhibit clinical signs. In Ashcroft *et al.*'s experimental infections using *T. brucei* s.l. -infected tsetse, Thomson's gazelle (which showed 100% mortality, n=81) and dikdik (100% mortality, n=4) survived for an average of 4 months, during which they showed consistent

parasitaemia by microscopy. Trypanosomes were detected in cerebrospinal fluid post mortem. Frustratingly, details of other pathological changes were not reported.

The length of parasitaemia in other species varied. Warthogs showed only transient parasitaemia, with trypanosomes detectable by rodent inoculation for between one and three weeks. Duiker, impala and eland showed parasitaemia detectable by microscopy for only a few weeks, but continued to infect tsetse for several months, and could infect rodents by inoculation for over a year. Reedbuck showed initial high parasitaemia, and trypanosomes continued to be detected by microscopy for up to 20 months. In experimental infection of wildebeest to study immune response, trypanosomes were observed by microscopy for at least 7 months after intravenous infection with *T. brucei* s.l. (Rurangirwa *et al.*, 1986).

Susceptibility to *T. brucei* s.l. infection also varied with species. Some species have never been found naturally infected and are difficult or impossible to infect in the laboratory, such as the olive baboon. Almost 100% of duiker, impala, eland and reedbuck were infected after being fed on with one infected tsetse, whilst warthog were more difficult to infect (Ashcroft *et al.*, 1959).

The differences between these studies on captive animals and the situation in free ranging animals, for example in terms of force of infection, previous exposure, and concurrent stress, make it difficult to extrapolate the results. However, it is clear that in some species trypanosomes can persist for long periods at low levels of parasitaemia and still infect tsetse. This is not surprising - during the chronic stage of *T. brucei* s.l. infection in cattle, when parasites were not consistently detected by microscopy on buffy coat and thin smears, high infection rates are still seen in tsetse (Van den Bossche et al., 2005). The low levels of parasitaemia in chronic infections are often below the threshold of detection of current diagnostic techniques, whilst still sufficient to infect tsetse.

Although these experimental infections suggest that trypanosome infections can lead to mortality in some species, there is little evidence for pathogenicity in the field. Pathological changes such as mild meningoencephalitis and interstitial myocarditis have been found in small numbers of lion, hartebeest and impala infected with *T. brucei* s.l. although the clinical significance of these changes is unknown (Losos & Gwamaka, 1973). Parasites of the *T. brucei* s.l. group were found in both blood and brain tissue of two wild zebra on post

mortem examination, which were found showing weight loss and neurological signs (McCulloch, 1967). Trypanosomiasis does present a considerable threat in capture and translocation programs for black rhino, causing morbidity and mortality (McCulloch & Achard, 1985; Mihok *et al.*, 1992). However, the effect of factors such as stress associated with capture and release, and the exposure of a naïve animal to trypanosomes, are likely to explain the high susceptibility of these rhinos, which is not typical of free ranging animals.

It is usually assumed that trypanosomiasis does not present a disease risk to free ranging wildlife. Given the difficulties of assessing health in the field, survival may be altered in the absence of obvious mortality or clinical signs. This has been observed in other systems: giant gerbils, the reservoir of *Yersinia pestis* in Kazakhstan, do not show obvious clinical signs with infection. However, mark recapture studies of infected and non infected individuals detected reduced survival in infected individuals (Begon *et al.*, 2006). In Ashcroft's experimental studies (1959), although 100% mortality was reported in Thomson's gazelle, all individuals remained in good condition until a few days before death. Even minor alterations in mortality may be important due to the potential effect on transmission.

1.5.3 Animal reservoirs of T. b. gambiense

T. b. gambiense, the cause of Gambian HAT, has been found in both livestock and wildlife (Gibson et al., 1978; Jamonneau et al., 2004; Mehlitz et al., 1982), but the importance of these hosts in the maintenance of disease and transmission of sleeping sickness to man remains unclear. The existence of an animal reservoir has been suggested to explain the failure of previous human-based eradication campaigns (Rogers, 1988). However animal reservoirs appear to be of less importance in the epidemiology of Gambian sleeping sickness. The long asymptomatic phase of Gambian sleeping sickness cases provides a source of infection in the human populations and control strategies focus on identification and treatment of these cases (Welburn et al., 2001a). Whether human infection is sufficient to maintain *T. b. gambiense* in longstanding foci is uncertain. However, expanding foci are associated with human-related risk factors, such as international borders and areas of conflict, where human population displacement is common and provides the potential to move infection to new areas (Courtin *et al.*, 2008).

1.6 The tsetse vector

Tsetse (*Glossina* spp.) are obligate blood feeders through which cyclical transmission of trypanosomes occurs. There are 31 species and subspecies of tsetse, found across the fly belt of sub Saharan Africa, and of varying importance in trypanosome transmission.

In addition to *T. brucei* s.l., tsetse transmit other species of trypanosome via blood feeding, known collectively as the Salivarian trypanosomes, and classified by their development site within the tsetse (reviewed by Hoare, 1970; Stevens & Brisse, 2004). These are summarised in Table 1-2.

Subgenus	Species	Description
Trypanozoon	*T. brucei*	*T. b. rhodesiense* and *T. b. gambiense* cause HAT. *T. b. brucei* causes mild disease in cattle.
Duttonella	*T. vivax*	Causes disease in cattle
Nannomonas	*T. congolense*	Most important as a pathogen of cattle but can also cause disease in other species, including sheep, goats, pigs and horses (Hoare, 1970; Stevens & Brisse, 2004) Three groups – savannah, forest and Kilifi
	T. simiae	Causes acute, fatal disease in pigs (Hoare, 1970). Subspecies *T. simiae* Tsavo only isolated from tsetse (Majiwa *et al.*, 1993)
	T. godfreyi	Only isolated from tsetse but causes chronic, occasionally fatal disease in pigs experimentally (McNamara *et al.*, 1994)

Table 1-2: Classification and description of the Salivarian trypanosomes

Trypanosome classification has traditionally been based on factors such as morphology, host range and pathogenicity (Gibson, 2007). Since the advent of molecular technology, phylogenetic analysis has lead to reclassification of some species and discoveries of potential new species or subspecies (Gibson *et al.*, 2001; Adams *et al.*, 2008). This is explored further in Chapter 3.

1.6.1 Prevalence of trypanosome infections in tsetse

The prevalence of *T. brucei* s.l. infections in tsetse is obviously an important parameter when considering transmission dynamics. It is perhaps surprising then that there are many uncertainties surrounding methods for assessing trypanosome infection rate. Current methods for measuring prevalence of trypanosomes in tsetse are summarised below.

Dissection and microscopy

The most widespread method of assessing the prevalence of trypanosomes in tsetse populations comprises dissection and microscopic examination of the mouthparts, midguts and salivary glands of the tsetse, and relies on the differing development and maturation sites of the trypanosome subgenera within the fly (Figure 1-3).

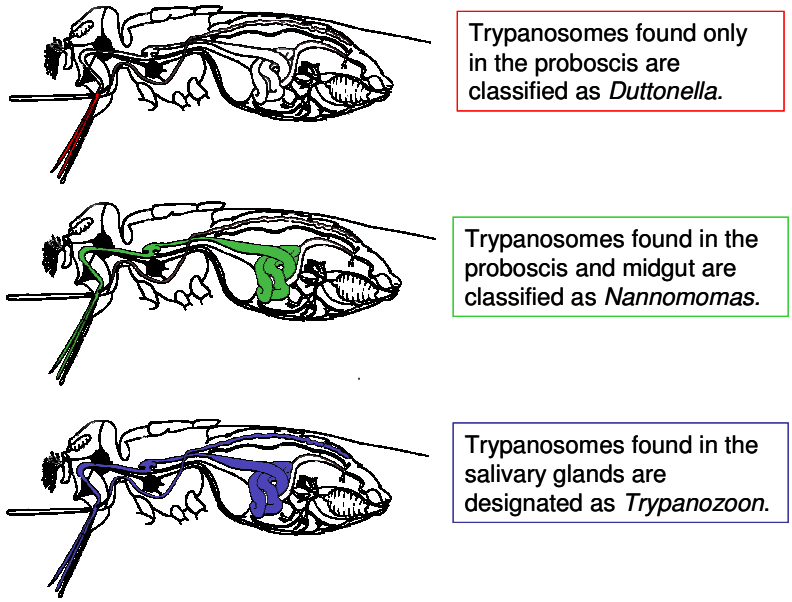


Figure 1-3: The development sites of *Duttonella, Nannomonas* and *Trypanozoon* in the tsetse fly.

Coloured areas indicate the locations of trypanosome development in each subgenus (Maudlin et al., 2004).

Although used in earlier studies (Duke 1913, reviewed by Ford & Leggate, 1961), this technique was first discussed in detail by Lloyd and Johnson in 1924 as an alternative to cumbersome rodent inoculation studies. However, Lloyd and Johnson relied principally on morphology of the developmental and infective forms, using the location within the fly only as "an additional aid", and eventually concluding that this method was too time consuming for large scale surveys. Despite this, differentiation of trypanosome species by location within the fly was adopted as the main method of assessing prevalence and has remained so for many years.

This technique has several disadvantages for use in field studies. It is not possible to differentiate below the level of subgenus, e.g. between *T. congolense* and *T. simiae*. Mature and immature infections cannot be differentiated and mixed infections cannot be identified. In addition, dissection and trypanosome identification are dependent on operator skill, and there are variations in protocols even for this widely practised technique. For example some authors only examine the midgut and salivary glands if trypanosomes are found within the mouthparts (for example Woolhouse *et al.*, 1994; Msangi *et al.*, 1998), whilst others examine all the organs (for example Moloo *et al.*, 1971; Waiswa *et al.*, 2006). Since trypanosomes are not always seen in the mouthparts in mature *T. brucei* s.l. infections, the prevalence of *T. brucei* s.l. may be underestimated by the former technique.

Molecular techniques

The development of DNA probes and subsequently PCR primers has allowed sensitive and specific identification of trypanosome species. Currently, PCR primers exist for the 11 main African trypanosome species, subspecies and subgroups. In addition, techniques have been developed to identify multiple trypanosome species in one test, based on interspecies variation in the internal transcribed spacer region of ribosomal DNA (Cox *et al.*, 2005; Njiru *et al.*, 2005; Adams *et al.*, 2006).

DNA probes and PCR primers have been used in a variety of protocols to supplement dissection and microscopy data. These include: PCR of mouthparts, midgut or salivary glands only if trypanosomes were found on microscopy in that individual organ (Morlais *et al.*, 1998; Njiru *et al.*, 2004a); PCR of mouthparts, midgut and salivary glands if

trypanosomes were found in any location in the fly (Lefrancois *et al.*, 1999; Jamonneau *et al.*, 2004); PCR of midgut if trypanosomes found at any location in the fly (Malele *et al.*, 2007); microscopy of mouthparts only, PCR of mouthparts if trypanosomes found (Lehane *et al.*, 2000; Malele *et al.*, 2003); microscopy of midgut only, PCR of midgut if trypanosomes found (McNamara *et al.*, 1995; Adams *et al.*, 2006); and PCR of whole macerated flies (Ferreira *et al.*, 2008).

These techniques have resolved some of the disadvantages of dissection and microscopy. PCR primers have high specificity and trypanosomes can be reliably identified to species or subspecies level. It has become clear that mixed infections are common, with approximately one third of PCR positive flies carrying more than one trypanosome species (Lehane *et al.*, 2000; Malele *et al.*, 2003; Njiru *et al.*, 2004a; Adams *et al.*, 2006), and up to 4 trypanosome species identified in individual flies (Lehane *et al.*, 2000; Njiru *et al.*, 2004a).

However, it is clear that the results of dissection and microscopy do not closely correlate with data generated by PCR, with only 38% (Njiru *et al.*, 2004a) to 51% (Lehane *et al.*, 2000) of *Nannomonas* and *Duttonella* infections classified as the same species by both techniques. For example, it appears that dissection overestimates the prevalence of *T. vivax*, with only 39% (Njiru *et al.*, 2004a) to 44% (Lehane *et al.*, 2000) of mouthparts only infections positive for *T. vivax* by PCR.

The majority of studies have focussed principally on technique development (Majiwa & Otieno, 1990; Adams *et al.*, 2006), confirmation of the trypanosome species circulating in a tsetse population (McNamara & Snow, 1991; Lefrancois *et al.*, 1999), identification of new trypanosome species or subspecies (Malele *et al.*, 2003; Adams *et al.*, 2008), or assessment of grouping of trypanosome species (Lehane *et al.*, 2000). Use of molecular data to assess the prevalence of infection in vector populations for epidemiological studies has been less common, and it is clear that there are difficulties reconciling this data with the results of the more traditional dissection and microscopy. The prevalence of transmissible trypanosome infections in a tsetse population is a fundamentally important parameter in disease epidemiology, but assessment of this remains a challenge, which will be explored further in Chapter 5.

1.6.2 Factors affecting trypanosome transmission by tsetse

Vector competence

For *T. brucei* s.l., vector competence is a measure of the proportion of tsetse exposed to an infected blood meal, which develop transmissible *T. brucei* s.l. infections. Vector competence is generally low, as tsetse are predominantly refractory to trypanosome infection. Trypanosomes have several hurdles to overcome to establish mature infections in tsetse. Firstly, only a small proportion of trypanosomes become established as a midgut population. This refractoriness is due to the inhibitory effects of lectins in the tsetse midgut, and is maternally inherited via the effects of midgut symbionts, *Sodalis glossinidius*, which have lectin-inhibitory activity (reviewed by Welburn & Maudlin, 1999). After establishment of a midgut population, even fewer trypanosomes overcome the second hurdle; maturation to form an infective metacyclic population in the salivary glands. The conditions necessary for maturation to occur are still unclear, though antioxidants appear to be important (Macleod *et al.*, 2007).

Vector competence is affected by both intrinsic and extrinsic factors, which relate to these immune effects. Differences in vector competence between tsetse species may arise from the presence of different *S. glossinidius* genotypes (Geiger *et al.*, 2007). Teneral flies (those which have not yet taken a blood meal) are most susceptible to infection with *T. congolense* and *T. brucei* s.l. (not *T. vivax* as there is no midgut stage) because the lectin level in the midgut is low, only increasing in response to a blood meal. After periods of starvation flies are also more susceptible to trypanosomes (Kubi *et al.*, 2006). Maturation of *T. brucei* s.l. infections is sex linked, with males maturing significantly more infections than females (Milligan *et al.*, 1995). The proportion of trypanosomes differentiating from blood stream to metacyclic forms is affected by environmental temperature (Macleod *et al.*, 2007).

Tsetse population dynamics

Glossina are *k*-strategists, producing a smaller number of offspring with more investment and hence better survival in each one. After a single mating shortly after emergence, female tsetse flies produce live young which develop through first and second stage larvae inside the uterus, and can deposit a third stage larva every 9 or 10 days. This feature, combined with the tsetse's adaptation to dry environments, means there is much less seasonal variation in density than with other insect vectors such as mosquitoes. The large amount of research that

has been conducted on dynamics of tsetse populations means they can now be modelled with some confidence (Hargrove, 2004). Whilst midgut infections have no effect on tsetse survival, salivary gland infections confer a fitness disadvantage (Maudlin *et al.*, 1998). The effect this has on transmission dynamics is unclear, since parasite-induced mortality is not seen until flies are aged over approximately 50 days, which is unusual in wild populations (Maudlin *et al.*, 1998).

Feeding preferences

Both male and female tsetse feed on blood, taking meals approximately every 3 days. Tsetse are highly mobile and show strong host seeking behaviour. Tsetse species occupy different ecological niches, including different host preferences. For example *Glossina swynnertoni*, one of the predominant tsetse species in Serengeti, Tanzania, feed predominantly on warthog, buffalo and giraffe, even when other species are more prevalent (Moloo *et al.*, 1971; Rogers & Boreham, 1973). However, some tsetse species are adaptable. In Zimbabwe selective elimination of warthogs caused the proportion of suid blood meals taken by *Glossina morsitans* to drop from 77% to 10%. Tsetse switched to feeding on bovids and elephant, and after elimination of elephants, 90% of blood meal identifications were from bovids, mainly kudu (Vale & Cumming, 1976).

1.7 Other routes of transmission

In areas outside the tsetse range, *T. vivax* has adapted to mechanical transmission via biting flies. The relative importance of mechanical transmission within tsetse areas remains uncertain (Hall & Wall, 2004). Experimentally, mechanical transmission of *T. brucei* s.l. has been reported, but is not thought to be important epidemiologically (Mihok *et al.*, 1995).

The prevalence of *T. brucei* s.l. in lions and hyaenas is consistently high. Since these species are not popular hosts for tsetse it is possible that this reflects oral transmission of trypanosomes from infected prey. *T. brucei* s.l. infections have been reported in cats and dogs fed infected goats (Moloo *et al.*, 1973), and bush babies fed infected rats (Heisch, 1952). Oral transmission is reported with other trypanosome species such as *Trypanosoma microti* in rodents, both experimentally (Maraghi *et al.*, 1995) and in the field (Smith *et al.*, 2006).

The research described in this chapter illustrates the substantial amount of research that has been conducted on HAT in the last century. It is now an appropriate time to look at the ecology of trypanosomes in SME for several reasons. The development of new molecular diagnostic tests, such as the SRA/PLC multiplex PCR for differentiating *T. b. brucei* and *T. b. rhodesiense*, provide the potential for more sensitive and specific identification of trypanosomes, something which has often limited studies in the past. Sophisticated statistical techniques now allow the influence and interactions of multiple factors to be considered. This development has been particularly important for studies on complex ecosystems, where the confounding effects of multiple factors can make relationships difficult to unravel. The frameworks reviewed in this chapter for infectious disease ecology provide new ways of looking at trypanosome ecology. Perhaps their biggest value is in encouraging critical examination of the information that we already know about trypanosome transmission within an ecosystem, and in highlighting what information is still needed. Finally, the public health and economic threats of HAT have made it an important concern in Serengeti and research on HAT is encouraged and supported by the park and research authorities.

The second chapter of this thesis describes the study site of the Serengeti Mara Ecosystem and summarises research specific to this area. Chapter 3 assesses the use of ITS PCR primers for identifying trypanosome infections in samples collected from wildlife. Chapter 4 uses this data to consider risk factors associated with trypanosome infections in wildlife species. Chapter 5 assesses the prevalence of trypanosome infections in tsetse populations in SNP. Chapter 6 is a final discussion.

Chapter 2: Overview of the Serengeti- Mara ecosystem

This chapter introduces the Serengeti-Mara ecosystem (SME) where this study was conducted. To explain the background to this study, the history of human African trypanosomiasis (HAT) in Tanzania, and specifically in SME, are summarised, and an account of the previous research specific to trypanosomiasis in SME is presented.

2.1 Human African trypanosomiasis in Tanzania

A small number of cases of HAT in tourists to Serengeti and Tarangire National Parks recently attracted international media attention (Jelinek *et al.*, 2002), but HAT is endemic in Tanzania with on average 300 cases reported each year. Reported cases between 1996 and 2004 can be seen in Table 2-1. It is likely that the cases reported are only a small proportion of the true number. In Uganda it has been estimated that for each reported HAT death, 12 deaths go unrecorded, predominantly due to people not seeking health care, or through misdiagnosis (Odiit *et al.*, 2005). The problem of under-reporting in Tanzania may be even more severe; health care infrastructure is less developed than Tororo District of Uganda, where Odiit *et al*'s study was conducted, and there are difficulties assimilating data at a national level (Matemba, 2008).

The location of districts listed in Table 2-1 are shown in Figure 2-1. At least 26 cases are known to have occurred in Serengeti during this period (Section 2.4). Although some of these cases were diagnosed outside Tanzania, several cases diagnosed at district hospitals in Northern Tanzania do not appear in these records. Historically, several HAT foci are described in Tanzania. Hide *et al* (1999) described five foci, based on previous epidemics, shown in Figure 2-2. However, variations in disease reporting and administrative boundaries make it difficult to look at the distribution of foci over time. For example, all the cases in Table 2-1 in 2004 were diagnosed at one health care centre – the National Institute for Medical Research's Tabora Research Centre, which is the national centre for HAT research (Matemba, 2008).

Region	District	1996	1997	1998	1999	2000	2001	2002	2003	2004
Kigoma	Kibondo	212	115	98	112	134	123	99	10	2
	Kasulu	155	198	172	156	191	79	68	16	7
	Kigoma	-	-	-	-	-	-	6	-	-
Manyara	Babati	12	19	15	12	-	-	-	-	-
	Hanang	5	3	8	-	2	-	-	-	-
Arusha	Monduli	-	19	6	-	-	-	-	-	-
Tabora	Urambo	1	-	7	12	27	38	59	98	64
Rukwa	Nkasi	4	-	-	-	-	-	-	-	-
	Mpanda	5	-	-	-	-	-	-	4	79
Mbeya	Chunya	6	-	-	-	-	-	-	-	-
	Total	**400**	**354**	**326**	**292**	**336**	**240**	**232**	**128**	**152**

Table 2-1 : Cases of human African trypanosomiasis diagnosed at district hospitals in Tanzania between January 1996 and June 2004 (NIMR/Ministry of Health)

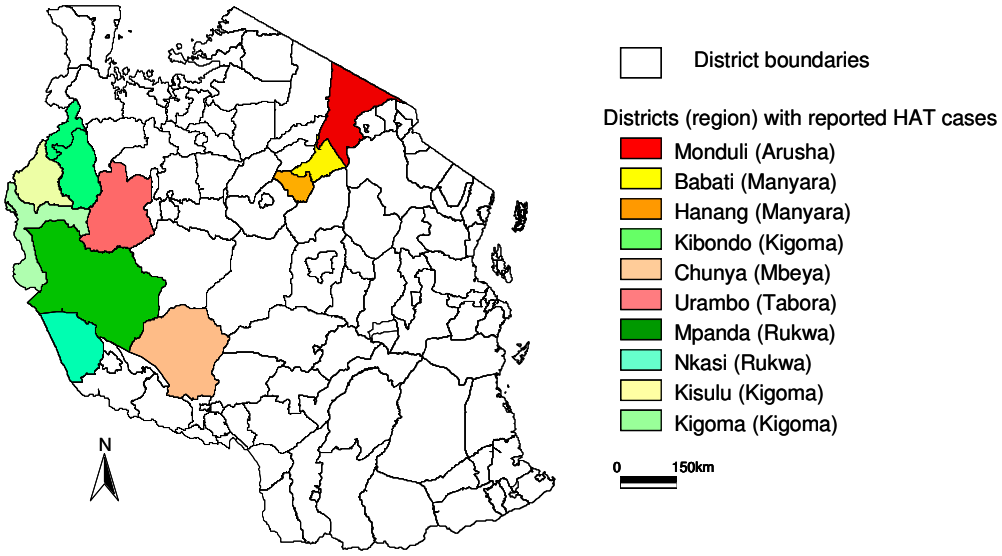


Figure 2-1: Districts in Tanzania reporting cases of human African trypanosomiasis between January 1996 and June 2004

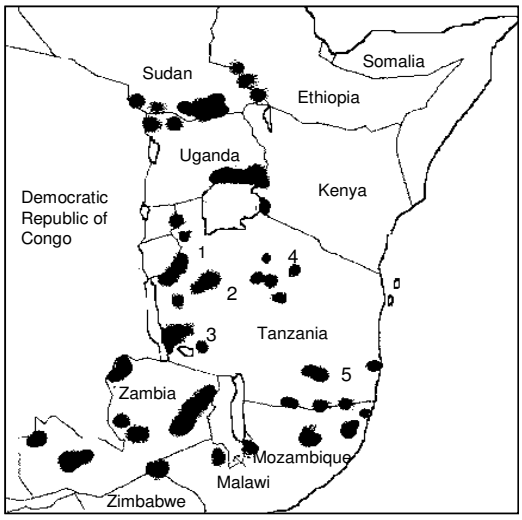


Figure 2-2: Map of historical HAT foci in East Africa, adapted from Hide (1999)

Tanzanian foci are labelled: 1 Kasulu focus; 2 Tabora focus; 3 Rungwa River focus; 4 Maswa focus; 5 Matandu River focus

Although well known for its success in eradicating tsetse from the island of Zanzibar through the release of gamma-irradiated male flies (Vreysen *et al.*, 2000), mainland Tanzania has not achieved any sustainable tsetse control in recent years. Since 2003, the focus has been on community based tsetse control programmes, including training workshops for district tsetse field officers, distribution of educational material to schools, farmers and extension officers, sensitisation campaigns and deployment of insecticide impregnated targets (Daffa *et al.*, 2005).

All HAT cases reported in Tanzania at present are thought to be caused by *Trypanosoma brucei rhodesiense*. There are no known *Trypanosoma brucei gambiense* foci in Tanzania, with the last suspected case occurring in 1958 (Kilonzo & Komba, 1993). However, concerns remain that the immigration of refugees from Rwanda and the Democratic Republic of Congo (DRC) into Western Tanzania could introduce *T. b. gambiense*, which is responsible for the epidemics in DRC (Van Nieuwenhove *et al.*, 2001).

2.2 Animal trypanosomiasis in Tanzania

Trypanosomiasis is a major constraint to livestock keeping in Tanzania with approximately 50% of rangeland infested with tsetse. Losses due to mortality and reduced milk yield are estimated at US$7.98 million annually (Daffa *et al.*, 2005).

2.3 The history of HAT in Tanzania

The first documented case of HAT in Tanzania occurred in 1902 (Fairbairn, 1948). Authors have suggested that this followed introduction of the disease from Uganda (Fairbairn, 1948), DRC (Fairbairn, 1948) or Mozambique (Kilonzo & Komba, 1993). It would be surprising if this really represented the first introduction of HAT. The theory that trypanosomes persist in ancient foci, with epidemics triggered by any disruption of the fine ecological balance, is thought more likely (Ford, 1971; Hide, 1999; Maudlin, 2006). This is supported by evidence of the genetic stability of *T. b. rhodesiense* isolates within foci (Hide *et al.*, 1996), and the identification of distinct strains of *T. b. rhodesiense* in Uganda compared to Zambia (Hide *et al.*, 1991).

HAT in Tanzania in the early Twentieth Century was characterised by substantial epidemics. The largest of these, around Lake Victoria, is thought to have killed over 50 000 people (Kilonzo & Komba, 1993). Epidemics also occurred in Southern and Western Tanzania. Establishment of sleeping sickness camps and extensive bush clearing by the German colonial administration meant the outbreaks around Lake Victoria were largely under control by 1914. However, over the next three decades, epidemics continued to occur in many areas of Tanzania, with 23 955 cases diagnosed between 1922 and 1946 (Fairbairn, 1948). The control strategies of the British colonial authorities focused predominantly on bush clearance and forced resettlement of people into tsetse-free areas (Hoppe, 2003). The disease was eventually brought under control, although further epidemics occurred in 1957 in Tabora and Kasulu.

The causal agent of HAT in Tanzania is somewhat unclear. The outbreaks around Lake Tanganyika and Lake Victoria are historically attributed to *T. b. gambiense*, whilst *T. b. rhodesiense* is thought to have spread north after introduction from Mozambique. Concurrent *T. b. gambiense* epidemics in DRC suggest that the cases arising around Lake Tanganyika may indeed have been caused by *T. b. gambiense*. However, retrospective analysis of the

epidemic in Uganda in 1900 have implicated *T. b. rhodesiense* (which was then yet to be described) rather than *T. b. gambiense* as was thought at the time (Fevre *et al.*, 2004). Cases arising around Lake Victoria, reportedly spreading gradually south from the epidemic in Uganda, may have been caused by *T. b. rhodesiense.* Attempts to establish the retrospective distribution of *T. b. rhodesiense* and *T. b. gambiense* in Tanzania have been inconclusive; detailed medical records have not been retained as was the case in Uganda (Matemba, 2008).

2.4 Trypanosomiasis and Serengeti National Park

Trypanosomiasis has helped to shape the history of Serengeti National Park (SNP). However its influence follows on from the effects of another disease – rinderpest. The rinderpest epidemic that swept Africa in the late nineteenth century arrived in East Africa in 1890 and killed over 95% of cattle. Rinderpest also devastated populations of wild ungulates, particularly wildebeest and buffalo. In the area that would become SNP, famine, followed by disease, killed a large proportion of the human population (reviewed by Ford, 1971).

Tsetse had previously been widespread but with the disappearance of their wildlife and livestock hosts, tsetse populations declined. However, with the disappearance of wildlife, livestock and people, areas of thick vegetation increased. Once the wild ungulate populations began to recover, tsetse expanded rapidly into the new woodland areas. In areas depopulated by war, disease and famine, bush spread fast and tsetse followed. A battle began between people and tsetse; whilst people retracted from the encroaching bush, colonial administrators attempted to clear bush and prevent the tsetse advancing, illustrated by Swynnerton's description of the neighbouring area of Shinyanga (1925):

> *The natives, til we turned them, were definitely 'on the run'. Everywhere on the edges of the cattle-areas there was the same advance of the young bush, and the tsetse, and everywhere else inside them are still the live roots of the suppressed bush. The natives themselves were highly alarmed, and some said to me 'Where will the end be?' I replied, 'Unless you stand firm and yourselves attack, the end will be in little more than twenty years with the death of your last beast'.*

Against this backdrop of rapidly expanding tsetse populations, an outbreak of HAT began. The first cases were diagnosed in Ikoma in 1925, reportedly spreading from an outbreak in neighbouring Maswa which began in 1922. Between 1925 and 1946, 2119 cases were diagnosed around Ikoma (Fairbairn, 1948). The epidemic only came to an end in the late

1940s, when an increasing human population and expanding agriculture had reclaimed large areas from the tsetse fly (Ford, 1971). The gazetting of SNP in 1951 may have been timely, given the land use pressure from a burgeoning human population.

In 1964, after a long period with no reported HAT cases, sporadic cases were seen, with between one and 14 cases diagnosed per year between 1964 and 1969 (Onyango & Woo, 1971), including the first case in a tourist. Within the next few years, a large amount of research was carried out, providing much of the current knowledge on the role of wildlife in trypanosomiasis transmission. This research is summarised below.

Three tsetse species were identified in SNP. *Glossina swynnertoni* was found in the highest numbers and widely distributed. *G. swynnertoni* is part of the *morsitans* group, found mainly in open woodland. Small numbers of *Glossina pallidipes*, also a member of the *morsitans* group, were mainly confined to areas of riverine vegetation. *Glossina brevipalpis*, part of the *fusca* group, was localised to small areas of dense thicket (Moloo *et al.*, 1971; Moloo & Kutuza, 1974). In 1970, 6348 *G. swynnertoni* and 623 *G. pallidipes* were dissected, with no mature *T. brucei* s.l. infections found (Moloo *et al.*, 1971). In 1971, dissection of a further 3500 *G. swynnertoni* still failed to find any salivary gland infections (Rogers & Boreham, 1973). Wanting to confirm the role of *G. swynnertoni* in transmission of *T. brucei* s.l. , a pooled rodent inoculation technique was used in 1972 to analyse over 10 000 flies, giving a overall prevalence of 0.08% (Moloo & Kutuza, 1974).

Concurrent analysis of blood meals showed *G. swynnertoni* to have a wide host range, including bovids, suids, elephant, hippopotamus, primate, carnivores, aardvark, and birds, but the most popular were warthog (25.6% of feeds identified), buffalo (26.6%) and giraffe (12.2%) (Moloo *et al.*, 1971). A follow up study in the same area found 40% of feeds identified were from warthog followed by 36% from buffalo (Rogers & Boreham, 1973).

During the same period, *Trypanosoma brucei* sensu lato was found in 3.5% of cattle in the villages surrounding the park with BIIT results suggesting a *T. b. rhodesiense* prevalence of 1.4% (Mwambu & Mayende, 1971). Examination of blood from 3000 people in and around the park, using thick and thin blood smears and the haematocrit centrifugation technique, found no trypanosome infections, despite the diagnosis of four cases of HAT in park staff within the preceding four months (Onyango & Woo, 1971).

Sampling of wildlife in 1966 and 1971 identified *T. brucei* s.l. in wildebeest, topi, hartebeest, waterbuck, impala, warthog, lion and hyaena (Sachs *et al.*, 1967; Baker, 1968; Geigy *et al.*, 1971). Further sampling in 1971 confirmed *T. b. rhodesiense* in hyaena, lion, hartebeest and waterbuck by BIIT and infection of human volunteers (Onyango *et al.*, 1972; Geigy & Kauffman, 1973; Geigy *et al.*, 1973a)

By 1993 the Serengeti focus (also known as the Ikoma focus) was considered to be inactive, as no cases had been reported during the preceding 20 years (Kilonzo & Komba, 1993). However anecdotal evidence suggests that cases continued to occur in SNP during the 1970's and 1980's. For example cases are thought to have occurred in workers clearing roads to the Nyaruswiga telecommunication tower in Death Valley during the 1980s (TANAPA, 2001).

However a recent increase in the number of cases reported has raised concern. Since 1990, 30 cases have been reported, both in Tanzanians from within or around SNP, and from visitors to either SNP or nearby Tarangire National Park. Cases reported since 1990 are listed in Table 2-2. The number of cases per year peaked at 16 in 2001. Reporting of cases through international disease reporting forums such as ProMedmail (www.promedmail.org) drew media attention and international interest to the situation (Jelinek *et al.*, 2002). This situation was particularly concerning. Tourism in SNP is a major source of revenue in Tanzania, and any perception of a serious public health threat to tourists was likely to affect tourism. TANAPA, in conjunction with the Tsetse and Trypanosomiasis Research Institute (TTRI), Tropical Pesticides Research Institute (TPRI), National Institute for Medical Research (NMRI) and the Ministry of Health conducted some research, such as examining blood smears from workers in the park, and instigated some control measures. In addition, investigations began into the role of wildlife as reservoirs of HAT for a MSc thesis (Kaare, 2003).

Date of Diagnosis (year or m/yr)	Sex	Age (years)	Nationality	Background	Reference
1990	M	49	USA	Tourist - Tanzania, Rwanda and Kenya	(Panosian *et al.*, 1991)
1993	M	67	USA	Tourist - Tanzania, Rwanda and Kenya	(McGovern *et al.*, 1995)
1995	-	-	Tanzania	Serengeti Serena Lodge staff	(TANAPA, 2001)
1997	-	-	Tanzania	Diagnosed at Endulen Hospital, from Makao, Meatu District	(TANAPA, 2001)
01/98	F	30	Australia	Tourist - Serengeti National Park only	(ProMED-Mail, 2000a)
1999	F	54	USA	Tourist - National Parks in Northern Tanzania	(Sinha *et al.*, 1999)
1999	M	49	USA	Tourist - National Parks in Northern Tanzania	(Sinha *et al.*, 1999)
05/00	M	37	USA	Tourist - Serengeti National Park only	(ProMED-Mail, 2000b)
09/00	F	-	Tanzania	Diagnosed at Musoma Hospital, from Serengeti District	(TANAPA, 2001)
09/00	M	2	Tanzania	As above	(TANAPA, 2001)
10/00	M	30	UK	Tourist - Ngorongoro Conservation Area, Lake Manyara and Serengeti National Parks, Mombasa	(ProMED-Mail, 2000b)
02/01	M	33	Italy	Tourist - Lake Manyara and Serengeti National Parks, Ngorongoro Conservation Area	(Ripamonti *et al.*, 2002)
02/01	M	30	Italy	Tourist - Tsavo National Park in Kenya, Ngorongoro Conservation Area, Serengeti National Park	(Ripamonti *et al.*, 2002)
02/01	F	44	UK	Tourist – Nairobi, Amboseli National Park in Kenya, Lake Manyara and Serengeti National Parks, Ngorongoro Conservation Area	(Jelinek *et al.*, 2002)
02/01	M	48	USA	Tourist – Zambia then Tanzania including Serengeti National Park	(ProMED-Mail, 2001a)
03/01	M	41	Sweden	Tourist - Ngorongoro Conservation Area and Lake Manyara, Tarangire and Serengeti National Parks	(Jelinek *et al.*, 2002)
03/01	M	68	South Africa	Tourist - Serengeti National Park only	(Jelinek *et al.*, 2002)
03/01	F	27	Norway	Researcher - Serengeti National Park and Ngorongoro Conservation Area	(Jelinek *et al.*, 2002)
03/01	M	-	Tanzania	Serengeti Balloon Safaris Staff	(Kaare, 2003)
03/01	M	60	Holland	Tourist – Tarangire National Park only	(Jelinek *et al.*, 2002)
03/01	F	53	Holland	Tourist - Ngorongoro Conservation Area and Lake Manyara and Serengeti National Parks	(Jelinek *et al.*, 2002)
04/01	F	55	Holland	Tourist – Tarangire National Park only	(Jelinek *et al.*, 2002)
06/01	M	29	USA	Reseacher - Serengeti National Park	(Kaare, 2003)
08/01	M	14	Tanzania	Unknown	(Kaare, 2003)
08/01	-	-	Tanzania	Kirawira Serena Lodge staff	Pers. comm..
10/01	M	32	Belgian	Tourist – Serengeti National Park only	(ProMED-Mail, 2001b)
06/04	M	45	South Africa	Researcher - Serengeti National Park	Pers. comm.
07/05	-	-	USA	Tourist – Serengeti National Park	(ProMED-Mail, 2005)
11/07	F	-	Germany	Tourist – Serengeti National Park	Pers. comm.
12/07	M	38	Tanzania	Worked in Serengeti National Park	(Sindato *et al.*, 2008)

Table 2-2: Reported cases of HAT around Serengeti between 1990 and 2007

Blood slides were taken from 1197 National Park staff in 2001, and were all found negative by microscopy (Magoma, 2001). This was repeated in SNP and surrounding villages in 2005 with the same results (L. Matemba, pers. comm.). In Gambian HAT, the long asymptomatic period makes active disease surveillance such as the examination of blood slides from healthy individuals described above, an effective method of case detection and control (Cattand *et al.*, 2001). However, in Rhodesian HAT, the short disease duration and severity of clinical signs mean that efforts are usually better focused on improving diagnosis of sick people, either through active case seeking or at the health centre level. District hospitals around SNP are not confident at diagnosing HAT, through low of awareness of the disease or lack of reagents for diagnosis (pers. obs.). It is therefore very difficult to estimate the incidence of HAT in people living around SNP. Two recent suspected cases in Serengeti district occurring in men thought to be exposed to tsetse when poaching wildlife in SNP (K. Hampson, pers. comm.) suggest that cases are occurring.

Kaare *et al*'s study (2007) was the first to use molecular tools to identify trypanosomes in animals in SNP. Analysis of samples from cattle in villages surrounding the park found a prevalence of 5.6% for *T. brucei* s.l. and 1.1% for *T. b. rhodesiense,* using species specific PCR primers for *T. brucei* s.l. , and SRA PCR primers to differentiate *T. b. brucei* and *T. b. rhodesiense*. *T. brucei* s.l. was found in lion, spotted hyaena, reedbuck, topi, warthog and wildebeest. However, *T. b. rhodesiense* was identified only in warthogs. The prevalence of *T. b. rhodesiense* in warthogs was 9.5%, significantly higher than the prevalence in cattle (Kaare *et al.*, 2007). This unusually high prevalence suggested that warthogs may be particularly important in HAT transmission, and raised the possibility that HAT control strategies could be developed to target warthogs, such as insecticide treatment of burrows.

Measures put in place to reduce the tsetse population included clearing vegetation along main roads, installation of insecticide-impregnated targets along main roads, around ranger posts and camp sites, and establishment of strategic de-flying areas to spray vehicles. The impacts of these measures on the tsetse population or human exposure to tsetse have not been assessed and they have proved difficult to maintain.

What is clear from Table 2-2 is that even in years without a large number of cases (with the associated increase in awareness and potentially disease reporting) a case of HAT is usually reported every one to two years. Of the last 4 cases in Table 2-2, each was reported through a

different source, and each report claimed this to be the only case reported since 2001 or 2002. Assimilation of this information is obviously important to gain a true picture of the pattern of cases over time. The factors which could trigger an increased number of cases remain uncertain. It has been hypothesized that increased incidence may be associated with increased exposure of people to tsetse. During the 1960s increased man-fly contact was associated with increasing tourism and the building of the necessary infrastructure (Onyango & Woo, 1971) and it has been suggested that recent cases may be linked to an increase in tented camps within SNP bringing people into contact with pristine wildlife areas where the density of tsetse is high (Kaare *et al.*, 2007).

2.5 Introduction to the Serengeti-Mara Ecosystem

The ecology and management of SME has been the subject of much research, the conclusions of which are summarised in three Serengeti books (Sinclair & Norton-Griffiths, 1979; Sinclair & Arcese, 1995; Sinclair *et al.*, 2008). Extensive background data, including information on wildlife density and diversity, vegetation, climate, human interactions and management, provides an unusual opportunity for studying the interactions of pathogens in an ecosystem which is already well-characterised. The opportunities this allows in terms of integrating ecological and disease data have already been demonstrated. For example, detailed information on population dynamics and social structure of lions, hyaenas and jackals allowed modelling of canine distemper virus transmission, and showed the importance of social structure in disease transmission (Craft *et al.*, 2008).

2.5.1 History and management

The Serengeti-Mara ecosystem (SME) covers more than 30 000km^2 and extends from Northern Tanzania into South Western Kenya. Defined by the extent of the annual wildebeest migration, it comprises the Serengeti National Park (SNP), Maasai Mara National Reserve, Ngorongoro Conservation Area (NCA), and Grumeti, Ikorongo and Maswa Game Reserves, shown in Figure 2-3. The Serengeti National Park is the centre of this ecosystem and covers 14 763km^2.

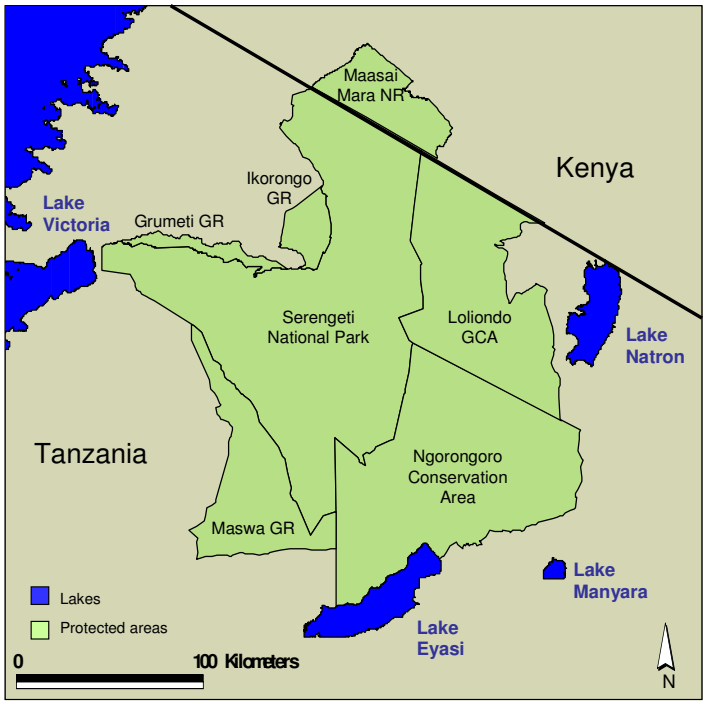


Figure 2-3: Protected areas comprising the Serengeti-Mara ecosystem

Serengeti National Park is just one part of the Serengeti-Mara ecosystem, which also comprises Ngorongoro Conservation Area, Loliondo Game Controlled Area (GCA), Maswa, Grumeti and Ikorongo Game Reserves (GR) and the Maasai Mara National Reserve (NR).

An area of southern and eastern Serengeti was first designated as a game reserve in 1929, becoming a protected area in 1940, and gaining national park status in 1951. Boundaries were adjusted in 1959 to cover more of the areas used by migrating wildebeest, and only minor changes have occurred since then. Serengeti National Park was one of the first areas to be proposed as a UNESCO World Heritage Site, and was gazetted as such in 1981.

The SME covers six districts in two regions - Mara region: Tarime, Bunda and Serengeti districts; and Shinyanga region: Bariadi, Meatu and Maswa districts. Serengeti National Park is managed by Tanzania National Parks (TANAPA). The Game Reserves are managed by the Wildlife Division (WD). The Ngorongoro Conservation Area Authority (NCAA) is responsible for management of the Ngorongoro Crater and surrounding areas. Wildlife research in Tanzania is overseen by Tanzania Wildlife Research Institute (TAWIRI).

2.5.2 Ecology

The climate is tropical with mean temperatures of around 27^0C. The typical rainfall pattern shows peak rainfall between March and May ("long rains"), and a smaller peak between November and December ("short rains") (Sinclair, 1979b). There is a rainfall gradient across the ecosystem, with more than 1000mm per year falling in the northwest, compared to 450 mm per year in the south east (Boone *et al.*, 2006). This gradient is also seen in the vegetation, changing from short grass plains in the south east, to taller grass species and increasing woodland in the central, north and west, dominated by Acacia spp. Along the main rivers, gallery forest can be found.

The SME is well known for having the largest herds of migrating ungulates in the world. Almost the entire population of 1.1 million wildebeest undergoes an annual migration (Mduma & Hopcraft, 2008), calving on the short grass plains in February and March then moving back towards northern Serengeti and the Maasai Mara National Reserve for the dry season (Figure 2-4). In addition to the wildebeest, there are around 180 000 zebra and 330 000 Thomson's gazelle, which also undergo seasonal migration (Mduma & Hopcraft, 2008).

This abundance of herbivores supports a high concentration of large predators, with around 7500 hyaenas and 2800 lions (Hofer & East, 1995). The diversity of other species is also high. A list of mammalian species can be seen in Appendix 1. Studies of other classes have been less extensive and the continuing discovery of new species (for example Drewes, 1997) suggests there are many yet to be described.

Figure 2-4: Approximate routes of annual wildebeest migration

The majority of the Serengeti wildebeest population undergoes an annual seasonal migration, concentrating in the southern grasslands in the wet season (December to May) and moving north west to the woodlands of Serengeti National Park and the Masai Mara Game reserve during the drier months (Frankfurt Zoological Society).

2.5.3 Interactions between people and the park

The park is bordered to the west by people of a variety of ethnic origins, including Ikoma, Isenye, Kurya, Sukuma, Zanaki, Jita, Ikizu, Ngoreme, Taturu, and Luo. Agropastoralism predominates, with people subsisting on small scale livestock and crop production. The density of people in these areas continues to increase (Campbell & Hofer, 1995), particularly adjacent to park boundaries. The "hard borders" this produces are mitigated by the buffer zones provided by Grumeti, Ikorongo and Maswa Game Reserves. To the east of the park the human density is lower. This area is dominated by the Maasai, pastoralists who rely heavily on livestock and live in communities that are more widely dispersed. Within the national park tourism is the only land use permitted, and settlement is limited to park and tourism

personnel. Game reserves allow tourism and commercial hunting. The NCA allows tourism, settlement, livestock and cultivation. However, wildlife poaching and cattle incursions present continuing concerns for the national park and game reserve management (Campbell & Hofer, 1995; Hilborn *et al.*, 2006).

Tourists began to visit SNP during the 1960s, although closure of the border to Kenya in 1977 effectively prevented tourism which did not rebound until the 1990s. Tourism in Tanzania now contributes substantially to the economy, with earnings from tourism standing at over US$862 million per year. In additional US$12 million is generated by utilization of wildlife (predominantly tourist hunting) (Tanzania Economic Survey, 2006). SNP attracts more tourists than any other park in Tanzania, with over 20% of Tanzania's 644 124 tourists per year visiting the park (Tanzania Economic Survey, 2006).

The majority of tourist facilities such as lodges and campsites are concentrated in Seronera, the central transition zone between the plains and the woodlands, although in recent years development of other areas of the park has increased. In addition to tourists, there are approximately 1000 people living within the park, comprising park personnel, lodge staff and researchers.

2.6 Spatial analysis and geographic information systems

The increasing availability of high resolution satellite imagery and development of powerful and accessible geographical information systems (GIS) have allowed increasingly sophisticated analysis of spatial heterogeneity, and the factors which influence it. In SME spatial analysis has been used to study predictors of hunting behaviour in lions (Hopcraft *et al.*, 2005), protection of wildebeest migration routes (Thirgood *et al.*, 2004) and vulnerability of black rhinos to poaching (Metzger *et al.*, 2007). Recently a detailed vegetation map was developed to assess the influence of landscape factors such as rainfall and topography on vegetation distribution (Reed *et al.*, 2009).

GIS are particularly appropriate for vector-borne disease studies. Disease risk is usually heterogeneous due to the habitat requirements of the vector. GIS has been used both for prediction of disease risk (Guerra *et al.*, 2002; Kabatereine *et al.*, 2004), and for investigations into pathogen transmission dynamics (Tran *et al.*, 2004). GIS have been used

widely in trypanosomiasis research elsewhere, but the spatial aspect of trypanosomiasis transmission in SME has never been addressed.

Habitat requirements of different tsetse species have been well characterised and distribution can be predicted from remote sensing or vegetation data. One example of the practical use of this type of information is the software program Tsetse Plan (Vale & Torr, 2003). Tsetse Plan is an Excel-based computer programme designed to help in the planning and implementation of tsetse control operations. Whilst the main aim of this program is to provide support for those using bait technologies to control tsetse, the first stages of the program ("feasibility study" and "pre-treatment") use established knowledge on tsetse habitat preferences, in combination with details specific to the study area, to assess the distribution and abundance of tsetse.

Within the scope of this study, it was not possible to carry out a comprehensive study of the distribution and density of tsetse populations in SME. Tsetse density is not simple to measure; trap catches reflect tsetse activity, which varies with many factors, such as temperature and host availability. However, disease transmission is likely to be heavily influenced by the heterogeneous distribution of tsetse. Tsetse Plan provided the opportunity to produce predicted maps of tsetse density, which would illustrate the comparative distributions of the different tsetse species.

2.7 Maps of predicted tsetse density

Tsetse Plan uses inputs such as predominant tsetse species, distribution of vegetation and distribution of any constraints on the tsetse population (such as control programs already in place or areas with low host density) to make spatial predictions of tsetse density using a deterministic population model. The inputs are used to set down seed populations in each square of a grid. These populations are then subjected to iterative cycles of population growth and movement, using established population parameters, to produce a value of predicted density of tsetse for each grid square. Tsetse Plan produces maps of these outputs to show the predicted spatial distribution of tsetse in the study area.

The following inputs were used to produce maps of predicted tsetse density for *G. swynnertoni* and *G. pallidipes* in SME:

- The vegetation map used was a supervised physiognomic classification of Landsat 7 satellite imagery with $30m^2$ resolution, covering the whole of the SME (Reed *et al.*, 2009). It uses a hierarchical land cover classification system on four levels to describe the predominant vegetation type (Grunblatt *et al.*, 1989), with an overall accuracy of 77% (Reed *et al.*, 2009).

- A 1 km^2 grid was overlaid over the map of SNP, Maswa, Grumeti and Ikorongo Game Reserves using ArcMap 9.0 (ESRI). Vegetation categories used by Tsetse Plan are not consistent with the classification of the vegetation map. Therefore, the proportion of pixels of each of four main vegetation types (grassland, savannah, open woodland and dense woodland) was analysed per grid square, and each square assigned to a Tsetse Plan category, according to the criteria listed in Table 2-3.

- The study area was split into areas of 50km by 50km (the largest area that can be analysed in one run by Tsetse Plan, whilst using 1km grid squares).

- For each species, the approximate density was estimated as high. This estimate was based on a trap catch with odours of approximately 100 flies per day, determined by the trap catches obtained during trapping for dissection (chapter 5).

- Tsetse constraints were left as zero for all squares. Although there are some insecticide impregnated tsetse traps in the park, there is no co-ordinated control effort and it is unlikely that these traps are in sufficient numbers to affect the distribution map. Wildlife is abundant in all areas.

- The 11 maps produced were combined to form one map for the whole study site in ArcMap (2km overlaps were included on all borders) and a smoothing filter was applied.

- This was carried out for *G. pallidipes* and *G. swynnertoni*.

TsetsePlan classifications	Tsetse Plan Description	Vegetation classification
No-go areas	Places where tsetse do not go, e.g. lakes and heavily built-up settlements.	There are no no-go areas in the study site.
Unsuitable places	Some tsetse might go into such places, but the flies do not survive there to produce self-sustaining populations. Examples are marshalnd, grasslands and open fields.	Grassland (>90%).
Little vegetation	Natural areas consisting mostly of grass or low herbs but with scattered bushes over 1m high and perhaps 1-10 small trees per hectare.	Predominantly grassland with small area of savannah or woodland (grassland 50-90%, savannah <40%, open and dense woodland combined<20%).
Thicket	Dense bushes or small tress provide a virtually complete cover. If bushes predominate they are too dense to allow ready access by humans at any season.	Predominantly open and dense woodland (>50%).
Savannah woodland	Trees provide about a 50% cover, with bushes, grasses or low herbs being insufficiently dense to stop a man strolling easily, at least in the dry season.	Predominantly savannah (>50%), or mixed grassland, savannah and woodland (open and dense woodland 20-50%, or savannah >40% and grassland >40%, or savannah 20-50%, grassland 30-50% and open and dense woodland <50%)
Riverine woodland	Large evergreen or semi-evergreen trees provide much or most of the cover with varying densities of bushes.	No grid squares contained sufficient riverine vegetation to be classified as such.

Table 2-3: Criteria for reclassifying vegetation type for Tsetse Plan categories

Vegetation categories used in TsetsePlan were not consistent with the classification system used for the vegetation map. The criteria listed above were used to reclassify each grid square.

Figure 2-5 & Figure 2-6, shown overleaf, illustrate the density of tsetse predicted from vegetation map using TsetsePlan. The scale refers to the population density as a percentage of the density that could be expected under highly favourable conditions for tsetse (in ideal vegetation, well within the climatic limits, with no man made adversities).

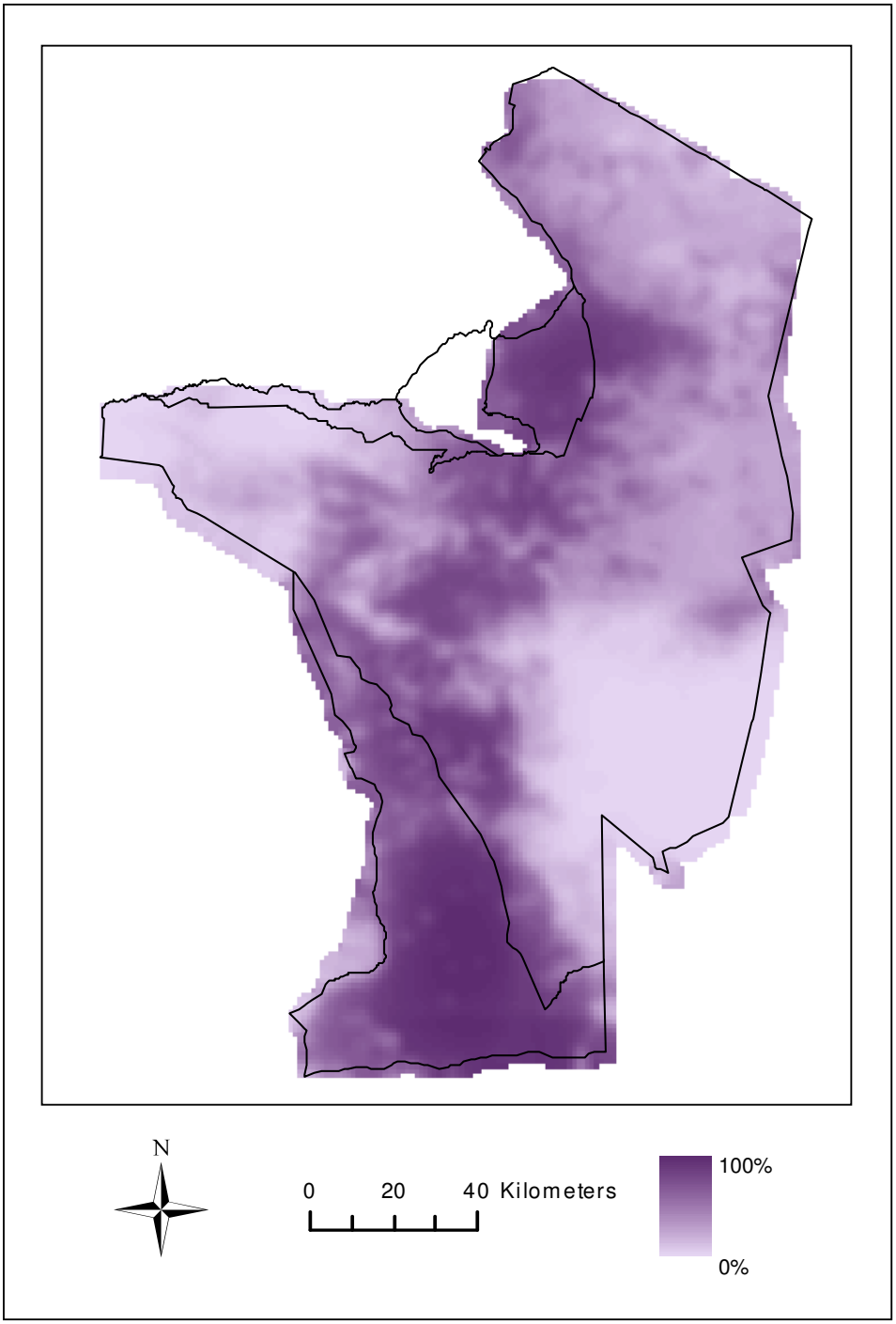


Figure 2-5: Density of *G. pallidipes* in the Serengeti National Park, Grumeti, Ikorongo and Maswa Game Reserves

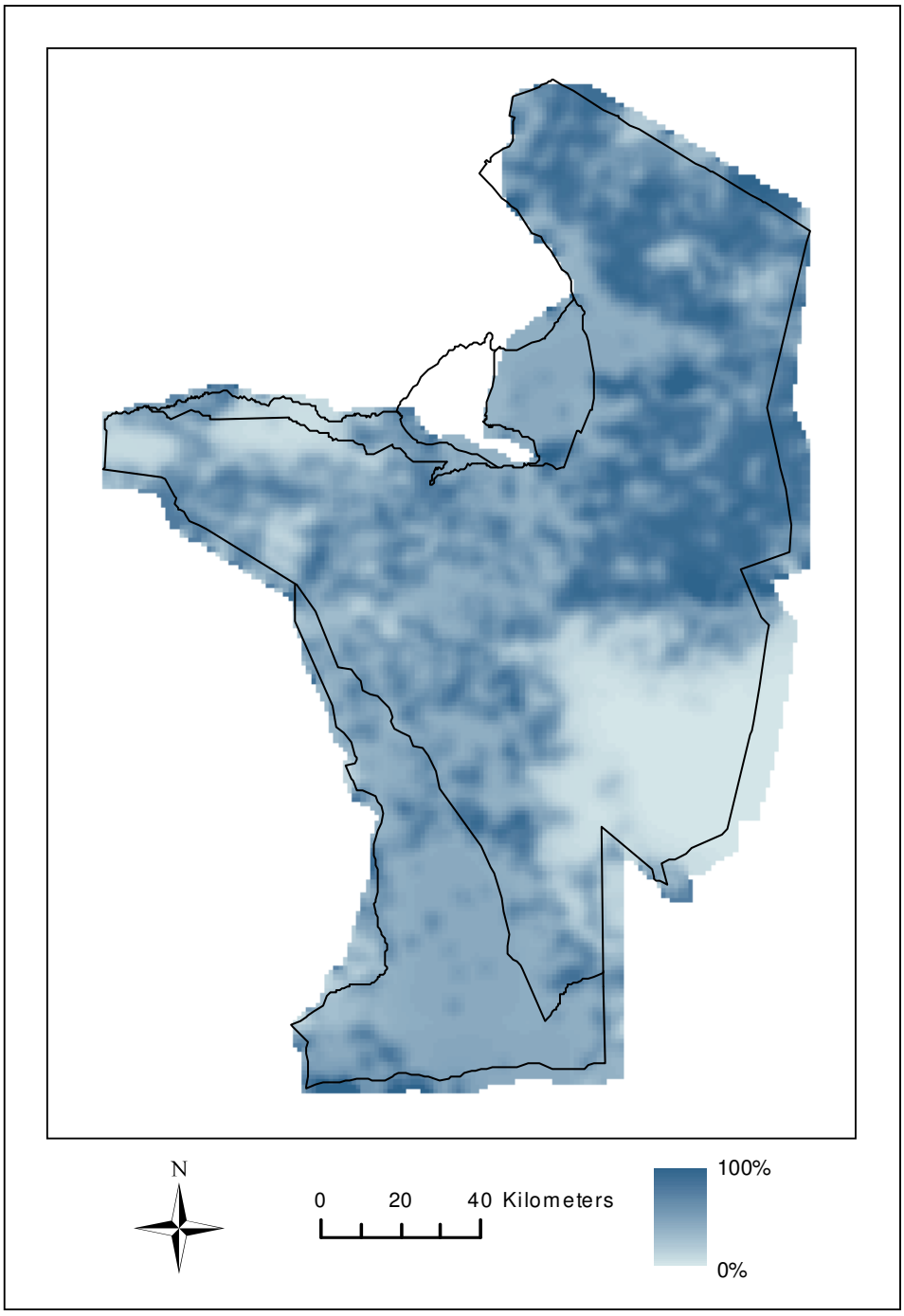


Figure 2-6: Density of *G. swynnertoni* in the Serengeti National Park, Grumeti, Ikorongo and Maswa Game Reserves.

These maps of predicted density of *G. pallidipes* and *G. swynnertoni* provide the first assessment of the spatial distribution of tsetse in SME. The different habitat requirements of the two species are demonstrated by their differing distributions. *G. pallidipes* are found in the areas of thickest vegetation. They are particularly abundant in Maswa GR and Ikorongo GR, and in a band from north to south through the centre of SNP. In contrast, *G. swynnertoni* prefer savannah areas of wooded grassland. They are widely distributed through most of SNP, but particularly in the north and east. Neither species are found on the grassland plains in the south-eastern part of the park.

Unfortunately the amount of trapping necessary to validate these maps was outwith the scope of this thesis. As with all predictive maps, these should be considered to provide an estimate of density only. However, they clearly illustrate the different distributions of the two tsetse species, and the values provide an indicator of density for comparative analyses. These values will be used in Chapter 4 to assess the effect of tsetse density on the prevalence of trypanosomes in wildlife hosts. The models and parameters used to predict population growth are potential sources of inaccuracy, since these are not specific to SME. Discrepancies may also arise from reclassification of vegetation types into the categories used by Tsetse Plan.

The distribution of tsetse in SME is not uniform and disease risk is likely to highly heterogeneous. These maps provide a starting point for further research into the spatial patterns of trypanosomiasis in SME, and may assist the park authorities in allocating limited resources for tsetse control within the national park.

Chapter 3: Assessment of an ITS PCR for identifying trypanosome infections in wildlife

3.1 Introduction

3.1.1 Importance of accurately identifying trypanosome infections

One of the first steps in reservoir studies is to identify the natural host range of the pathogen. Reliable identification of *Trypanosoma brucei* sensu lato in wildlife hosts is essential for epidemiological studies of wildlife reservoirs of human African trypanosomiasis (HAT).

Whilst the epidemiology of *T. brucei* s.l., and particularly *Trypanosoma brucei rhodesiense*, are of most interest due to the potential for human infection, the other trypanosome species found in wildlife and transmitted by tsetse flies were also of interest for several reasons. Firstly, species such as *Trypansoma congolense* and *Trypanosoma vivax* are important pathogens of livestock, and therefore important in their own right. Secondly, *T. brucei* s.l. does not exist in a vacuum but interacts with these other species, which may be important in terms of immunity and co-infections. For example the parasitaemia of chronic *T. brucei* s.l. infections increased with subsequent infection with *T. congolense* (Van den Bossche *et al.*, 2004). Thirdly, the trypanosome species share many characteristics, and knowledge of the transmission dynamics of other trypanosome species can only improve understanding of *T. brucei* s.l. .

Several studies have revealed a wide diversity of trypanosomes in tsetse (Malele *et al.*, 2003; Adams *et al.*, 2008). All new or potential new trypanosome species identified recently have been found in tsetse, including *Trypanosoma simiae* Tsavo and *Trypanosoma godfreyi*, and several trypanosomes reported to be inconsistent with any known species, but whose identity is so far uncertain, such as *T. godfreyi*-like trypanosomes, *T. vivax*-like trypanosomes and *T. brucei*-like trypanosomes. Few authors have focused on identifying trypanosomes in their wildlife hosts, presumably due to the logistical difficulties and higher costs of obtaining samples from wildlife. Indeed, there are still no identifications reported of *T. simiae* Tsavo or *T. godfreyi* in mammalian hosts, although warthogs are suspected to be the natural hosts.

3.1.2 Diagnostic techniques for identifying trypanosome infections

Since the terms sensitivity and specificity will be used frequently in this chapter, their definitions are included here for clarity. Analytic sensitivity of a test refers to the lowest concentration of a substance that can be detected by the test. The analytic specificity is the capacity of the test to react to only one specific target. In contrast, epidemiological or diagnostic sensitivity and specificity are concerned with the ability of a test to detect individuals with the condition of interest (Saah & Hoover, 1997; Dohoo *et al.*, 2003).

The main factor in the difference between analytic and diagnostic sensitivity is the ability to obtain and identify the target substance (e.g. pathogen or antibody) in the sample collected. For example collection of samples which do not contain the pathogen, or the presence of substances which inhibit a PCR reaction, will lead to low diagnostic sensitivity and false negative test results, even for a test with high analytical sensitivity. The most common reasons for differences between analytical and diagnostic specificity is contamination during the sampling procedure, or detection of for example, fragments of DNA which do not reflect current infection (Saah & Hoover, 1997).

Historically, trypanosomes were identified by examination of wet blood films, or Giemsa-stained thick and thin fixed blood smears, with species differentiated by morphological characteristics. The development of various concentration techniques improved the sensitivity of microscopy, for example by centrifuging blood in microhaematocrit tubes and examining the buffy coat/ plasma junction under the microscopy (haematocrit centrifugation techniques HCT) (Woo, 1970), or by cutting the tube to express material at the buffy coat/ plasma junction and examining this with dark ground or phase-contrast illumination (buffy coat technique BCT) (Murray *et al.*, 1977).

Sub-inoculation of blood from animals suspected to carry trypanosomes into another species, such as rodents, has been widely used. The advantages of this technique, namely the ability to isolate parasite stabilates and higher sensitivity compared to microscopy, mean there are many examples of its use in wildlife, particularly in attempts to characterise *T. brucei* s.l. (for example Geigy *et al.*, 1971; Robson *et al.*, 1972). However, trypanosome species vary in their ability to infect rodents. Laboratory rodents are particularly susceptible to *T. brucei* s.l. infection, but *T. vivax* is rarely able to establish an infection (Leeflang *et al.*, 1976). Xenodiagnosis, feeding a susceptible vector on the animal suspected to carry trypanosomes,

is not a widely used technique for identifying trypanosomes in cattle, but has been used quite extensively in wildlife studies, particularly in experimental studies, where the number of individuals is small, since it is very sensitive and often detects trypanosomes in animals negative by all other techniques (for example Ashcroft *et al.*, 1959).

Despite development of tests such as the antibody-detection enzyme-linked immunosorbent assay (ELISA), immunological techniques have not been adopted for trypanosome identification. One reason for this is that most research has focused on diagnosis of trypanosome infections in livestock, where techniques which identify current infection, rather than antibody response, are of more use. Development of an antigen-detection ELISA addressed this issue but failed to solve problems of low sensitivity and specificity (Eisler *et al.*, 2004). The only antibody detection method that is currently widely used is the card agglutination trypanosomiasis test (CATT) which is used in diagnosis of HAT caused by *T. brucei gambiense* (Magnus *et al.*, 1978).

Much research has focussed on the development of molecular tools for the identification of trypanosomes, initially using DNA probes, then polymerase chain reaction (PCR). Whilst these techniques have not yet been developed into field or pen-side tests, and remain research tools, the information they have provided has lead to much progress in the epidemiology of trypanosomiasis. Species-specific PCR primers have been developed for the main African trypanosome species, subspecies and subgroups: *T. brucei* s.l. (Moser *et al.*, 1989), *T. brucei rhodesiense* (Welburn *et al.*, 2001), *T. vivax* (Masake *et al.*, 1997), *T. congolense* savannah, *T. congolense* forest, *T. congolense* Kilifi, *T. simiae* (Masiga *et al.*, 1992), *T. simiae* Tsavo (Majiwa *et al.*, 1993) and *T. godfreyi* (Masiga *et al.*, 1996).

However, identification of all trypanosome species in a host requires up to nine different PCR reactions, which is labour intensive and costly. Recently, PCR primers have been developed that can identify multiple trypanosome species in one PCR reaction. These primers amplify the internal transcribed spacer (ITS) regions of ribosomal DNA. The target of the primers described by Cox *et al* (2005) is shown in Figure 3-1. The high rate of sequence variation in these non-coding regions results in PCR products of differing lengths, enabling trypanosomes species, and in some cases subspecies or groups, to be differentiated.

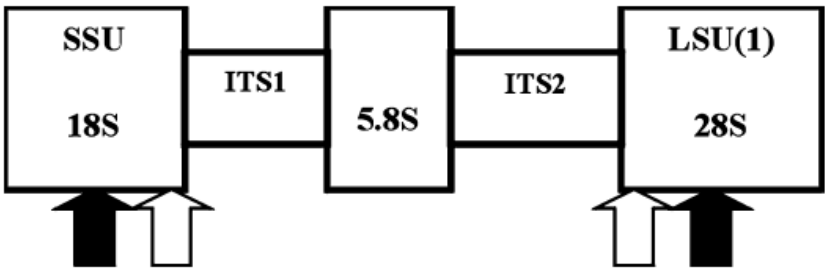


Figure 3-1: Structure of the ribosomal RNA gene locus.

The large boxes represent conserved coding regions (SSU small subunit, LSU large subunit), whilst the small boxes represent the variable spacer regions (ITS internal transcribed spacer). The nested primers developed by Cox et al are indicated by arrows, black arrows show outer primers, known as ITS1 and ITS2, white arrows show inner primers ITS3 and ITS4 (reproduced from Cox *et al.*, 2005).

Two sets of PCR primers were initially developed (Cox *et al.*, 2005 (termed "ITS" primers) ; Njiru *et al.*, 2005 ("ITS CF/ITS BR primers")). These protocols have been used to identify trypanosomes in cattle (Cox *et al.*, 2005), sheep, goats, pigs (Wissmann, 2007) and horses (Auty *et al.*, 2008). A third set of primers designed specifically for identification of trypanosomes in tsetse ("TRYP" primers) included identification of further trypanosome species but the similar lengths of some species did necessitate further tests for species differentiation (Adams *et al.*, 2006). The discovery of trypanosomes in tsetse that have ITS sequences only moderately similar to the existing trypanosome species has led to further investigations of potential new species, described at present as *T. godfreyi*-like trypanosomes (Malele *et al.*, 2003; Adams *et al.*, 2006), and *T. brucei*-like trypanosomes (Hamilton *et al.*, 2008).

The analytical sensitivity of ITS primers is lower than for the species specific primers. The analytic sensitivity of ITS is 55 pg/ml (Cox *et al.*, 2005), whereas Moser *et al.* (1989) report that TBR primers could detect 0.01pg of trypanosomal DNA in a 50µl reaction volume, equivalent to 0.2pg/ml. However, the diagnostic sensitivity of ITS primers and TBR is reported to be comparable in cattle (Cox *et al.*, 2005) and in tsetse (Adams *et al.*, 2006). Njiru *et al* (2005) found that whilst the diagnostic sensitivity was comparable for cattle samples which tested positive by HCT and BCT, for samples where no trypanosomes were observed by microscopy, the diagnostic sensitivity of ITS was much lower compared to species-specific primers.

Although species-specific primers have been used to identify trypanosomes in samples collected from wildlife, the use of ITS primers has not been assessed. The technique potentially provides a method of assessing the prevalence of the main African trypanosome species in one nested PCR. The diversity of trypanosome species found in tsetse in Tanzania (Adams *et al.*, 2006) suggests that a diverse range of trypanosomes are also likely to be found in wildlife, including potential identifications of trypanosome species which so far have only been found in tsetse. This makes the use of ITS particularly interesting. However, the parasitaemia found in trypanosome infections of wildlife is usually low (Mulla & Rickman, 1988b). The experiences of Njiru *et al* (2005) suggest that the sensitivity of ITS may be compromised in low parasitaemia populations. Consequently, since *T. brucei* s.l. is of particular interest due to its potential for human infection, *T. brucei*-specific primers will be used in addition to the ITS primers.

3.2 Objectives

To assess the use of ITS to identify trypanosome infections in wildlife, specifically:

- To assess bands produced by ITS primers using clonal sequence analysis

- To compare the diagnostic sensitivity of ITS to species-specific primers for identification of *T. brucei* s.l.

- To compare two sample preparation methods (a) using a washed filter paper disc to seed the PCR reactions and (b) eluting DNA off the filter paper and using the eluate to seed the PCR reactions

3.3 Materials and methods

3.3.1 Sample collection

Sample collection is described fully in Chapter 4. Briefly, all samples are blood samples preserved on Flinders Technology Associates (FTA®) classic cards (Whatman Biosciences, Cambridge, UK), collected from opportunistic sampling of wildlife in the Serengeti Mara ecosystem (SME) between 2002 and 2007.

3.3.2 Laboratory analysis - FTA card preparation

Discs were cut from the FTA card using a Harris Micro Punch™ tool of diameter 2mm or 3mm. Between sample punches, 10 punches were taken from clean FTA paper, to prevent contamination between samples. Discs were prepared for analysis using one of two protocols:

Washed disc protocol

Two discs of diameter 2mm were cut out from each sample. These were washed according to the following protocol: two washes of 15 minutes each with FTA purification reagent (Whatman Biosciences, Cambridge, UK), followed by two washes of 15 minutes each with TE buffer (Sigma Aldrich, Dorset, UK). Each disc was dried at room temperature for 90 minutes, and then used to seed a PCR reaction.

Eluted DNA protocol

Five discs of diameter 3mm were cut out from each sample. Discs were washed as described for protocol 1, then dried for 30 minutes at 37^{o}C. To each eppendorf tube (containing all 5 discs) 100µl 5% chelex suspension was added, and the tubes incubated at 90^{o}C for 30 minutes. The eluted DNA was then used for PCR reactions. Chelex is a chelating resin which is thought to prevent degradation of DNA by binding to potentially damaging metal ions (Walsh *et al.*, 1991).

All samples were analysed using ITS primers using both protocols. In addition, all samples were analysed using TBR primers with the eluted DNA protocol. A subset of 200 samples

(the first 200 samples collected) was also analysed using TBR primers with the washed disc protocol.

3.3.3 Laboratory analysis - PCR protocols

Two sets of PCR primers were used: a) ITS PCR to differentiate the main species of African trypanosomes and b) TBR protocol specific to *T. brucei* s.l..

ITS PCR

Samples were analysed using the primers described by Cox *et al* (2005). This is a nested PCR which detects differences in length of the internal transcribed spacer (ITS) regions of the trypanosome ribosomal genes, enabling differentiation of the main African trypanosome species. Expected product sizes are shown in Table 3-1.

The PCR protocol was as described by Cox *et al* (2005). PCR was carried out in 25µl reaction volumes, containing 10mM Tris-HCl pH 9.0, 1.5mM $MgCl_2$, 50mM KCl, 0.1% TritonX-100 and 0.01% (w/v) stabiliser (all combined in SuperTaq PCR buffer, HT Biotechnologies, Cambridge, UK), 2µM of each outside primer ITS1 and ITS2, 1mM total dNTP's, 1.25 Units of Biotaq (Bioline Ltd, London, UK), and one 2mm washed FTA disc. The second round reaction contained 1µl of first round product, and used inside primers ITS3 and ITS4. The ITS PCR was repeated using 5µl of eluted DNA instead of the washed disc. Each batch included one genomic DNA positive control for *T. brucei* s.l., one negative disc and one water negative control.

Species	Expected band size from NCBI database (bp)	Band sizes obtained by Cox *et al.* 2005(bp)
T. congolense	1413	1408
T. simiae (Tsavo)	954	951
T. brucei	1207-1224	1215
T. simiae	850	847
T. vivax	611	620
T. theileri	988	998

Table 3-1: Band sizes expected from NCBI reference sequences, and sizes obtained using nested ITS primers amplifying genomic DNA from trypanosome stocks

T. brucei s.l. PCR

The species-specific primers described by Moser *et al* (1989) were used for identification of *T. brucei* s.l.. PCR was carried out in 25µl reaction volumes containing 16.0mM $(NH_4)_2SO_4$, 67mM Tris-HCl, 0.01% Tween 20 (NH_4 buffer, Bioline Ltd, London, UK) 1.5mM $MgCl_2$, 800µM total dNTP's, 0.4µM of each primer TBR1 and TBR2, 0.7 Units of BioTaq Red DNA polymerase (Bioline Ltd, London, UK) and either 5µl of eluted DNA or one washed disc. The expected product is 177bp in length.

For all PCRs, thermal cycling was carried out in a DNA Engine DYAD™ Peltier thermal cycler. Cycling conditions and primer sequences can be seen in Table 3-2. All primers were sequenced by MWG Biotech. PCR products were run on a 1.5% (w/v) agarose gel at 100V, stained with ethidium bromide and visualised under an ultraviolet transilluminator (Gel-Doc 2000, Bio-Rad).

PCR	Primer Sequence
ITS	ITS 1: 5' - GAT TAC GTC CCT GCC ATT TG - 3'
(Cox *et al.*, 2005)	ITS 2: 5' - TTG TTC GCT ATC GGT CTT CC - 3'
	ITS 3: 5' - GGA AGC AAA AGT CGT AAC AAG G - 3'
	ITS 4: 5' - TGT TTT CTT TTC CTC CGC TG - 3'
	Cycling Conditions: 95ºC for 7min, 35 cycles: 94ºC for 60sec, 55ºC for 60sec, 72ºC for 120sec
TBR	TBR1 5'- CGA ATG AAT ATT AAA CAA TGC GCA GT-3'
(Moser *et al.*, 1989)	TBR2 5'- AGA ACC ATT TAT TAG CTT TGT TGC-3'
	Cycling Conditions: 94ºC for 3min, 30 cycles: 94ºC for 60sec, 55ºC for 60sec, 72ºC for 30s, final extension 72ºC for 5min

Table 3-2: PCR primer sequences and cycling conditions for TBR and ITS primer sets

3.3.4 Laboratory analysis - clonal sequence analysis

The required sequence was cloned to produce sufficient DNA for accurate sequence identification. In the cloning process DNA is incorporated into a plasmid vector which is maintained and propagated by a host organism such as *Escherichia coli*. The DNA which makes up the PCR band is isolated by extraction and then inserted into the cloning vector ("ligation"). Heat shocking causes the *E. coli* cells to take up the plasmid ("transformation"). Once the bacteria have multiplied to give many copies of the plasmid and therefore required sequence, the plasmid is purified and the PCR sequence can be identified.

DNA extraction

DNA was extracted from bands in agarose gels using a Qiagen MinElute DNA extraction kit (Qiagen). The manufacturer's protocol was followed. Briefly, the band was excised and weighed, and three volumes of buffer QG added per one volume of gel (300µl QG per 100mg gel). After incubation at 50^0C for 10 minutes to dissolve the gel, one gel volume of isopropanol was added. The sample was applied to a Qiagen Minelute column and centrifuged for 1 minute. The flow through was discarded, 500µl of QG buffer was added and the sample centrifuged again for 1 minute. Then 750µl of buffer PE was added. The sample was centrifuged for 1 minute, flow through discarded and centrifugation repeated to remove traces of buffer. 10µl of buffer EB were added to elute the DNA from the column. After 1 minute of incubation, the column was centrifuged for 1 minute to produce the eluted DNA. All buffers were provided in the kit. All centrifugation steps were carried out at 13000rpm.

Ligation and transformation

Cloning was carried out using a Qiagen PCR cloning kit. A ligation reaction mixture, containing the Qiagen pDrive cloning vector, PCR product and ligation mastermix, was incubated for 2 hours at 4^0C. Two microlitres of reaction mixture was added to a thawed tube of Qiagen EZ competent cells, mixed gently and incubated on ice for five minutes. The tube was heated to 42^0C in a heating block for 30 seconds then incubated on ice for two minutes. Two hundred and fifty microlitres of room temperature SOC medium was added to each tube, and cells plated onto Luria Bertani agar plates (LB; 10g tryptone, 5g yeast extract, 10g NaCl, 15g agar in 1l water). Inclusion of ampicillin in the agar prevented growth of colonies without the pDrive cloning vector, which contains an antibiotic resistance gene. In addition,

blue white selection was used to identify colonies with vectors which included the required sequence. The addition of a compound called isopropyl β-D-1-thiogalactopyranoside (IPTG) into the agar causes the bacteria to produce an enzyme which hydrolyses 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal), also included in the agar, to produce blue coloured colonies. However, if the vector has incorporated the DNA insert, the gene controlling production of the enzyme is disrupted and X-gal is not metabolised. Therefore white-coloured colonies indicate successful uptake of the insertion. Once the transformation mixture had absorbed into the agar, plates were incubated at 37^0C for 15-18 hours.

After a further incubation of 2 hours at 4^0C to improve colour differentiation, eight white colonies per sample were inoculated into 100µl of LB broth (10g tryptone, 5g yeast extract, 10g NaCl in 1l water), also containing ampicillin. After two hours incubation at 37^0C, 50µl of each sample was inoculated into 500µl LB broth. The remaining 50µl was incubated at 99^0C for five minutes, then PCR was performed to confirm the presence of the correct sized sequence. From two tubes with confirmed inserts, 50µl was inoculated into 2ml LB broth and incubated at 37^0C.

Plasmid purification

After incubation for 12-16 hours, cells were harvested by centrifugation for three minutes, followed by removal of the supernatant. Plasmids were then purified using the Qiagen Qiaprep Miniprep kit. Bacterial cells were resuspended in 250µl buffer P1, and transferred to a microcentrifuge tube. Two hundred and fifty microlitres of buffer P2 was added and mixed, followed by 350µl of buffer N3 then further mixing. Tubes were then centrifuged for 10 minutes. The supernatant was applied to a Qiaprep spin column and centrifuged for 1 minute. After the flow through was discarded, 0.5ml of buffer PB was added, centrifugation repeated for 1 minute and flow through discarded. The spin column was washed by adding 0.75ml buffer PE, and centrifuging for 1 minute. The flow through was discarded and centrifugation repeated to remove residual buffer. The Qiaprep column was placed in a clean tube and DNA eluted using 50µl buffer EB, which was incubated for 1 minute followed by centrifugation for 1 minute. All buffers were supplied in the kit. All centrifugation steps were carried out at 13000rpm.

PCR was repeated on the eluted DNA to confirm the presence of a sequence of the correct length. The eluate was submitted for sequencing to GATC (GATC Biotech, Germany). Each clone was sequenced with M13 forward and reverse primers.

Sequence analysis

Initial sequence inspection was carried out in Bioedit (Hall, 1999). Sequences were then compared to available reference sequences using BLAST (basic local alignment search tool). BLAST is a sequence alignment algorithm which is used to search databases for sequences with optimal alignment to the query sequence. Blast uses regions of local alignment to assess sequence similarity. Initial searching uses small sequence fragments, or words, to check for similarity then extends them to create alignments. Matching DNA pairs are scored using an identity matrix and gaps are penalised. Searches were performed using blastn program.

Three parameters were used to assess sequence similarity. The raw alignment score (S) is derived from the identity matrix and gap scores. This can be normalised to give a bit score (S') by incorporating statistical parameters which are specific to the scoring system used. The bit score can therefore be used to compare alignments across different searches. The significance of an alignment with score S is given by the expected value (E). This is the expected number of alignments that could arise by chance, with a score the same or better. A lower E value indicates a more significant hit. The E value reflects the size of database and the scoring system in use. Percent identity is calculated from the proportion of bases that are identical in the aligned sequences.

3.3.5 Statistical analysis of sensitivity

Prevalence was calculated with 95% binomial confidence intervals. Cohen's kappa statistic, usually used to assess agreement of diagnostic tests, could not be used for this dataset because it is not appropriate if the prevalence is less than 20%, or the test results differ significantly (Dohoo *et al.*, 2003). Although the analytical sensitivity of each PCR is known, there is no gold standard test and no data is available on diagnostic sensitivity. For this study, TBR was assumed to be the gold standard and the sensitivity of ITS measured in comparison, using the following formula:

$$\text{Sensitivity} = \frac{\text{Number of disease positive animals which test positive (i.e. } TBR_{pos}ITS_{pos})}{\text{Total number of disease positive animals } (TBR_{pos}ITS_{pos} + TBR_{pos}ITS_{neg})}$$

More sophisticated statistical techniques are available for assessing tests with no gold standard, using maximum likelihood or Bayesian approaches (for example Pouillot *et al.*, 2002; Branscum *et al.*, 2005). However this was not the main focus of this thesis.

3.4 Results

3.4.1 Summary of results

In total, 639 samples were analysed using ITS and TBR primers. A summary of results is shown in Table 3-3. In this table, TBR positive means tested positive using TBR primers on eluted DNA (the protocol that was followed for all samples). ITS positive means a band was detected on either the washed disc protocol or the eluted DNA protocol. Initially ITS bands were classified as trypanosome species if the band size was within 20bp of the expected size. For example, for *T. vivax*, expected to be 620, bands between 600 and 640 were classified as *T. vivax*.

Some of the results shown in Table 3-3 are surprising. For example, band sizes consistent with *T. simiae* were found in buffalo, hartebeest, hyaena, impala, lion, Thomson's gazelle, warthog, wildebeest and zebra. Previously *T. simiae* infections in wildlife have only been reported in warthogs (Claxton *et al.*, 1992; Kaare *et al.*, 2007), lions (Welburn *et al.*, 2008) and white rhinoceros (Mihok *et al.*, 1994). Band sizes consistent with *T. vivax* were found in a baboon, a species believed to be refractory to trypanosome infections (Ashcroft *et al.*, 1959).

Species	Number of samples	TBR	ITS						
			T.b.	*T.c.*	*T.s*	*T.s.T*	*T.t.*	*T.v.*	Mixed
Aardwolf	1								
Baboon	7							1	
Bat eared fox	4					1			
Buffalo	25	1		3	1			3	1
Bushbuck	2	1	1	2					1
Cheetah	3	1							
Civet	2								
Dikdik	6								
Duiker	1								
Eland	6	1	1	2					1
Elephant	1								
Genet	1								
Giraffe	11							1	
Grants gazelle	21	1		1				2	
Greater kudu	1			1					
Hare	1								
Hartebeest	11	2			1			2	
Hyaena	78	9	4	2	4			2	2
Hyrax	1								
Impala	17	3		2	2				
Jackal	12								
Leopard	4								
Lion	145	22	1	77	3			5	4
Mongoose	2								
Reedbuck	3	1					1		
Roan	1								
Rodents	45								
Serval	1								
Thomsons gazelle	45		1	2	1			6	2
Topi	18	1					1	3	
Vervet	3								
Warthog	37	1	1	2	6		7	1	7
Waterbuck	6			2				5	1
Wildebeest	57			2	3		3	2	
Zebra	62	2	1	4	5		2	1	3
Total	**641**	**46**	**10**	**102**	**26**	**1**	**14**	**34**	**22**

Table 3-3: Number of samples testing positive using TBR and ITS primers

The table indicates number of samples for each host species where PCR tested positive using TBR primers, or produced a band of expected size +/- 20bp for *T. brucei (T.b.), T. congolense (T.c.), T. simiae (T.s.), T. simiae Tsavo (T.s.T.), T. theileri (T.t.)* and *T. vivax (T.v.)* with ITS primers. Some individuals tested positive for more than one trypanosome species, each trypanosome species is included in the table and the number of individuals where this occurred is indicated ("mixed infections").

Comparison of ITS and TBR primers

The sensitivity of ITS was assessed by comparison with *T. brucei* s.l. specific primers. The number of samples testing positive using the ITS and TBR primers is shown in Table 3-4. For consistency, results using the eluted DNA protocol only were used. Using TBR only, the prevalence of *T. brucei* s.l. was 7.2% (CI 5.3-9.5). Using ITS only, the prevalence of *T. brucei* s.l. was 0.5% (CI 0.001-1.4). These proportions differed significantly between the two tests (McNemar's test, $p<0.001$, $\chi^2_1=41$). Estimating the sensitivity of ITS assuming TBR to be the gold standard gave a sensitivity of 6.5%. Even if the results of both the eluted DNA protocol and the washed disc protocol were included for the ITS primers, the sensitivity only rose to 14% ($TBR_{pos}ITS_{pos}/(TBR_{pos}ITS_{pos} + TBR_{pos}ITS_{neg}) = 6/(6+36)=14.3$).

	ITS positive	ITS negative
TBR positive	3	43
TBR negative	0	593

Table 3-4: Number of samples testing positive on eluted DNA with ITS and TBR primers.

The proportion of samples testing positive differed significantly between the two tests ($p<0.001$).

3.4.2 Comparison of sample preparation protocols

The sample preparation protocol detecting more positive samples varied with trypanosome species. For *T. congolense, T. brucei* s.l. and *T. theileri* using ITS primers, more samples tested positive using the washed disc protocol. However for *T. simiae* and *T. vivax*, and for the TBR primers, the eluted DNA protocol detected more positive samples (Table 3-5). These differences were significant for *T. brucei* s.l. and *T. vivax* with the ITS primers.

Primers	No. of samples analysed	Trypanosomes Species	Number of positive samples		X^2_{df}, p value
			Discs	Eluted	
ITS	639	*T. congolense*	104	91	X^2_1=3.2, p=0.074
		T. brucei s.l.	10	3	**X^2_1=5.1, p=0.023**
		T. theileri	11	7	X^2_1=0.90, p=0.34
		T. simiae	12	17	X^2_1=0.70, p=0.40
		T. vivax	19	47	**X^2_1=19.2, p<0.0001**
TBR	200	*T. brucei* s.l.	9	14	X^2_1=1.5, p=0.23

Table 3-5: Number of samples testing positive with ITS and TBR primers, using two sample preparation protocols

The table illustrates the number of samples testing positive by two different sample preparation protocols: the washed disc protocol and the elution protocol, for two different primer sets. X^2 and p values using a paired McNemar test show significant differences between the number of samples testing positive with each protocol for *T. brucei* s.l. and *T. vivax* with ITS primers (shown in bold).

3.4.3 Sequence analysis

There were three reasons to suggest that the bands observed when using ITS primers may require further investigation. Firstly, some of the results of ITS were surprising, with wildlife species testing positive for trypanosome species which had never before been found in that host species. Secondly, bands were often observed of a size which was not consistent with expected bands, and thirdly, even close to expected band sizes, the exact size often showed variation, making classification difficult. This can be see in Figures 3-2, 3-3 and 3-4. Clonal sequence analysis was conducted to investigate the identity of these bands. In total twenty bands were cloned and submitted for sequence analysis.

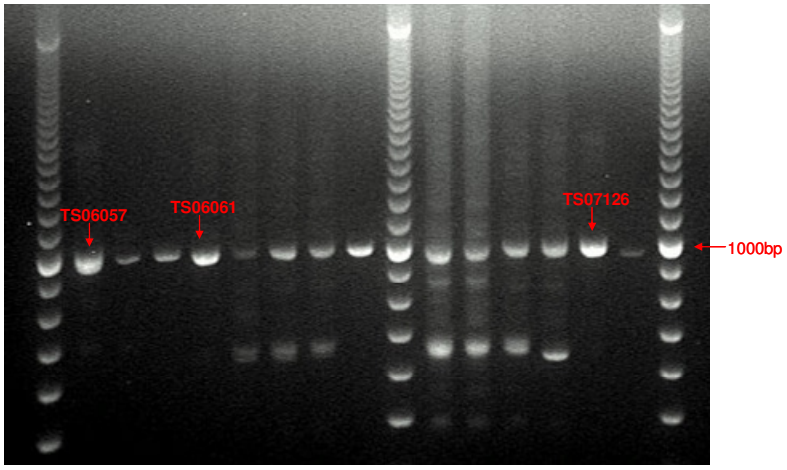


Figure 3-2: Agarose gels showing products amplified by ITS primers for band sizes 950-1000bp

Gel shows 14 bands obtained sized between 950 and 1000bp. Potential classification in this size range are *T. theileri* (998bp) and *T. simiae* Tsavo (951bp). Bands subsequently chosen for sequence analysis are labelled with sample reference numbers. A 100bp marker is included in lanes 1, 10 and 17 with double strength band at 1kbp.

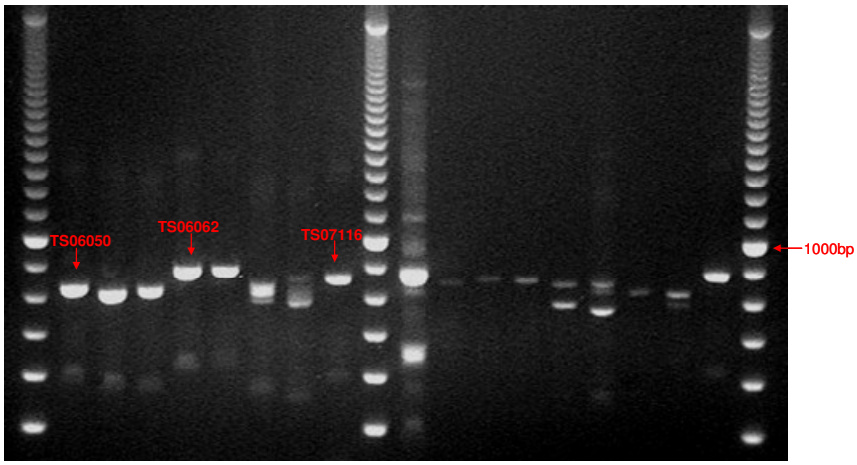


Figure 3-3: Agarose gels showing products amplified by ITS primers for band sizes 800-900bp

The gel shows 17 bands obtained between 800 and 900bp in length. *T. simiae* (847bp) is the only reference length reported within this range. Lanes 1, 10 and 20 contain a 100bp marker with double strength band at 1kbp. Three bands subsequently analysed are labelled with sample reference numbers.

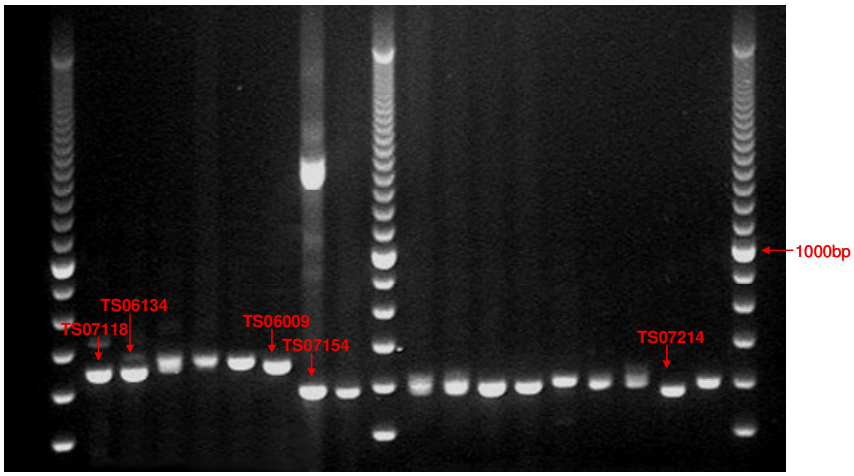


Figure 3-4: Agarose gels showing products amplified by ITS primers for band sizes 580-680bp

The gel shows 17 bands sized between 580 and 680bp. The only reference sequence expected within this range is *T.vivax* (620bp). Lanes 1, 10 and 20 contain a 100bp ladder with double strength band at 1kbp. Bands from which sequences were obtained are labelled with the sample reference number. Other bands, for example in lane 8, are likely to indicate detection of mixed infections.

Sequences were obtained for 16 bands. In addition to the bands indicated in Figure 3-2-Figure 3-4, one unknown band of approximate size 1150, which was found in eight hyaenas, two bands of approximate size 1220 assumed to be *T. brucei* s.l. , and two bands of approximate size 1410, assumed to be *T. congolense* savannah, were sequenced. For four other bands, clonal sequence analysis was unsuccessful. For two bands no transformed colonies containing the insert were observed. For the other two bands sequences were obtained but were not of sufficiently good quality to interpret.

Sequence Identification

BLAST searches were used to compare the sequences obtained to reference sequences in the NCBI database. The closest matches for each sequence are shown in Table 3-6.

Sample Number	Species	Sequence Length (bp)	Closest match on NCBI BLAST			
			Accession number	S	E	%
TS06009	Buffalo	654	DQ316043 *T. vivax*	881	0	97*
TS07154	Waterbuck	596	DQ316043 *T. vivax*	565	$7e^{-158}$	89*
TS07214	Giraffe	594	U22316 *T. vivax*	553	$4e^{-154}$	81
TS06134	Warthog	650	AY661891 *T. godfreyi* **	158	$6e^{-36}$	87
TS07118	Thomson's gazelle	646	No match			
TS06062	Warthog	878	U22320 *T. simiae*	996	0	86
TS06050	Wildebeest	824	No match			
TS07116	Wildebeest	852	No match			
TS07126	Warthog	967	U22318 *T. simiae* Tsavo	1294	0	90
TS06061	Warthog	968	U22318 *T. simiae* Tsavo	1335	0	91
TS06057	Wildebeest	988	No match			
TS07016	Hyaena	1055	No match			
64.05	Hyaena	1220	XO5682 *T. brucei*	2143	0	99
TS07112	Zebra	1207	AC159414 *T. brucei*	2024	0	97
TS07210	Lion	1407	U22315 *T. congolense*	2320	0	97
83.05	Hyaena	1420	U22315 *T. congolense*	2118	0	92

Table 3-6: Bands sequenced and closest matches to sequences in NCBI database

Table shows the closest matches obtained between bands sequenced and available reference sequences using BLAST. Score (S), expected value (E) and percent identity (%) are shown. (* percent identity for length of reference sequence, which is 534bp; ** BLAST search for ITS1 region only)

Five bands were sequenced of a length close to that expected for *T. vivax*. One sequence, from a buffalo, closely resembled a reference sequence (DQ416043) isolated from a cow in Kenya (Cortez *et al.*, 2006). However two sequences, from a waterbuck and giraffe, although most closely matching to *T. vivax*, only shared 80-90% identity with any *T. vivax* reference sequences. Until further research establishes the relationship between these sequences, and following the convention of other authors, these sequences will be referred to as *T. vivax*-like. These sequences are shown in Figure 3-5 and Figure 3-6.

One sequence from a warthog shared 87% identity with *T. godfreyi*. The reference sequence available for *T. godfreyi* includes the ITS1 only (130bp). The sequence obtained from warthog shared 87% identity for this available region, and was the most similar sequence on BLAST search if only the ITS1 region was used. All these sequences are shown in Figure 3-7.

Warthog samples at 967 and 968bp shared 90-91% identity to *T. simiae* Tsavo. Unfortunately only one reference sequence is available for *T. simiae* Tsavo so it is not possible to confirm the degree of variability usually seen. However these samples were 97% identical to each other (Figure 3-9). Similarly, a sequence from a warthog most closely matched to *T. simiae*, shared only 86% identity with the one available reference sequence (Figure 3-8). Similar findings with the TRYP primers found *T. simiae* sequences from tsetse in Tanzania to be only 93% identity to the reference sequence but 99.5% consistent with each other (Adams *et al.*, 2006).

The two sequences expected to be *T. brucei* s.l. were consistent with reference sequences (>97% identity). Other *T. brucei* s.l. sequences in the database shared 97-98% identity with each other. The sequence from a hyaena was most similar to sequence accession number X05682 and the sequence from a zebra most similar to AC159414. These sequences, together with two reference sequences, are shown in Figure 3-10.

Sequences expected to be *T. congolense* savannah were also consistent with *T. congolense* reference sequences. The sequence from a hyaena, shared only 92% identity overall. However, this was due to a section of poor quality sequence; alignment of the ITS 2 region gave 98% identity. These sequences are shown in Figure 3-11.

Unidentified sequences

Five sequences could not be identified, as they did not closely match any available reference sequences. On BLAST search all had closest matches with *Trypanosoma* spp. or the very closely related genera, *Leptomonas* or *Bodo*.

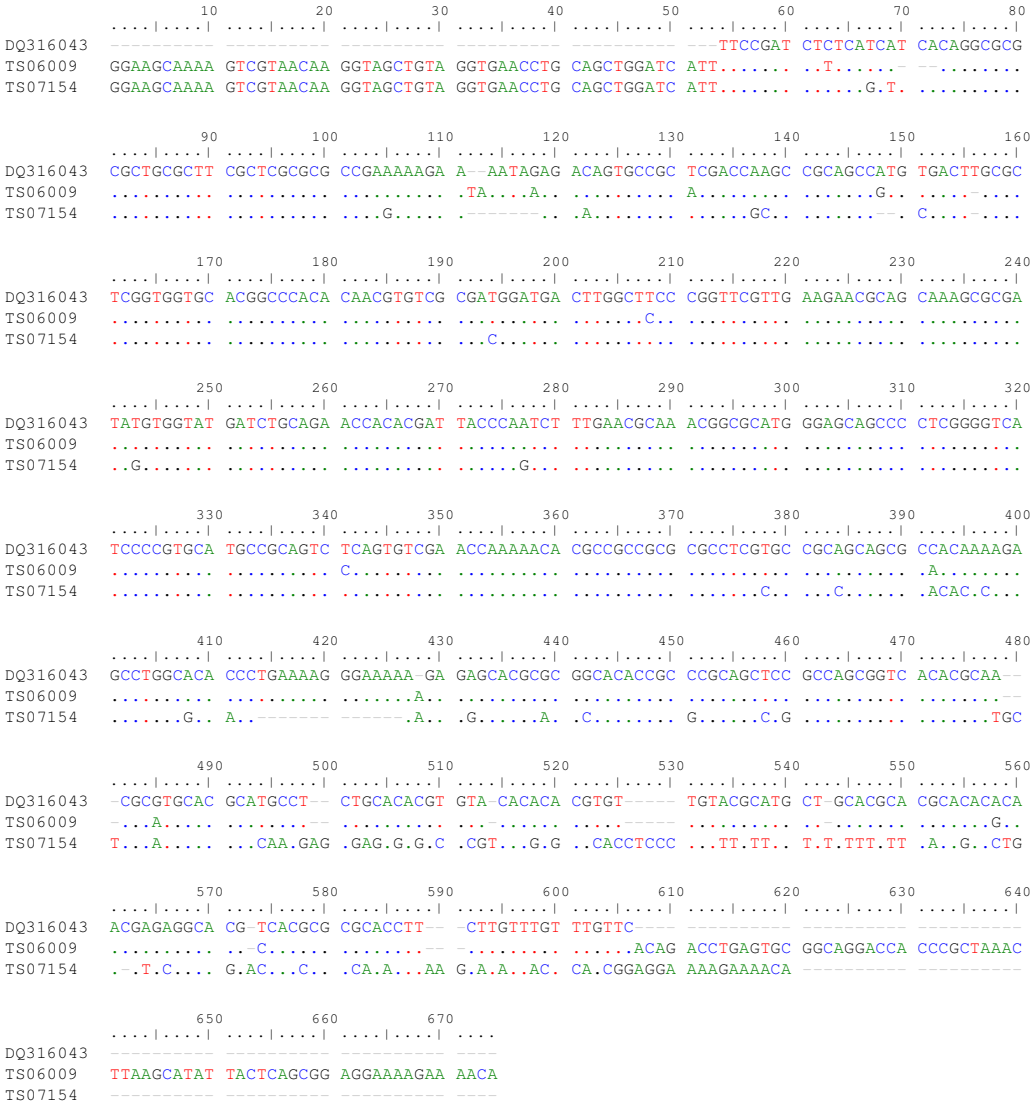


Figure 3-5: Sequence alignment for sequences TS06009 (97% identity) and TS07154 (89% identity) with *T. vivax* reference sequence DQ316043

Figure 3-6: Sequence alignment for sequences TS07214 with *T. vivax* reference sequence U22316 (81% identity)

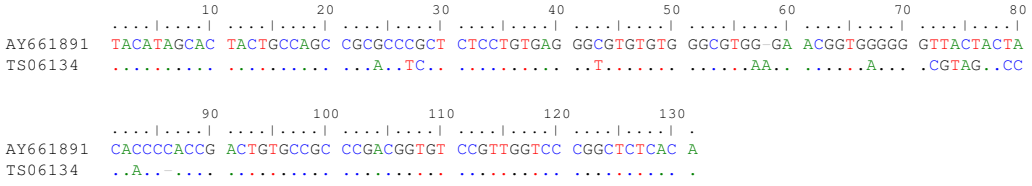


Figure 3-7: Sequence alignment for sequence TS06134 with *T. godfreyi* reference sequence AY661891 (87% identity)

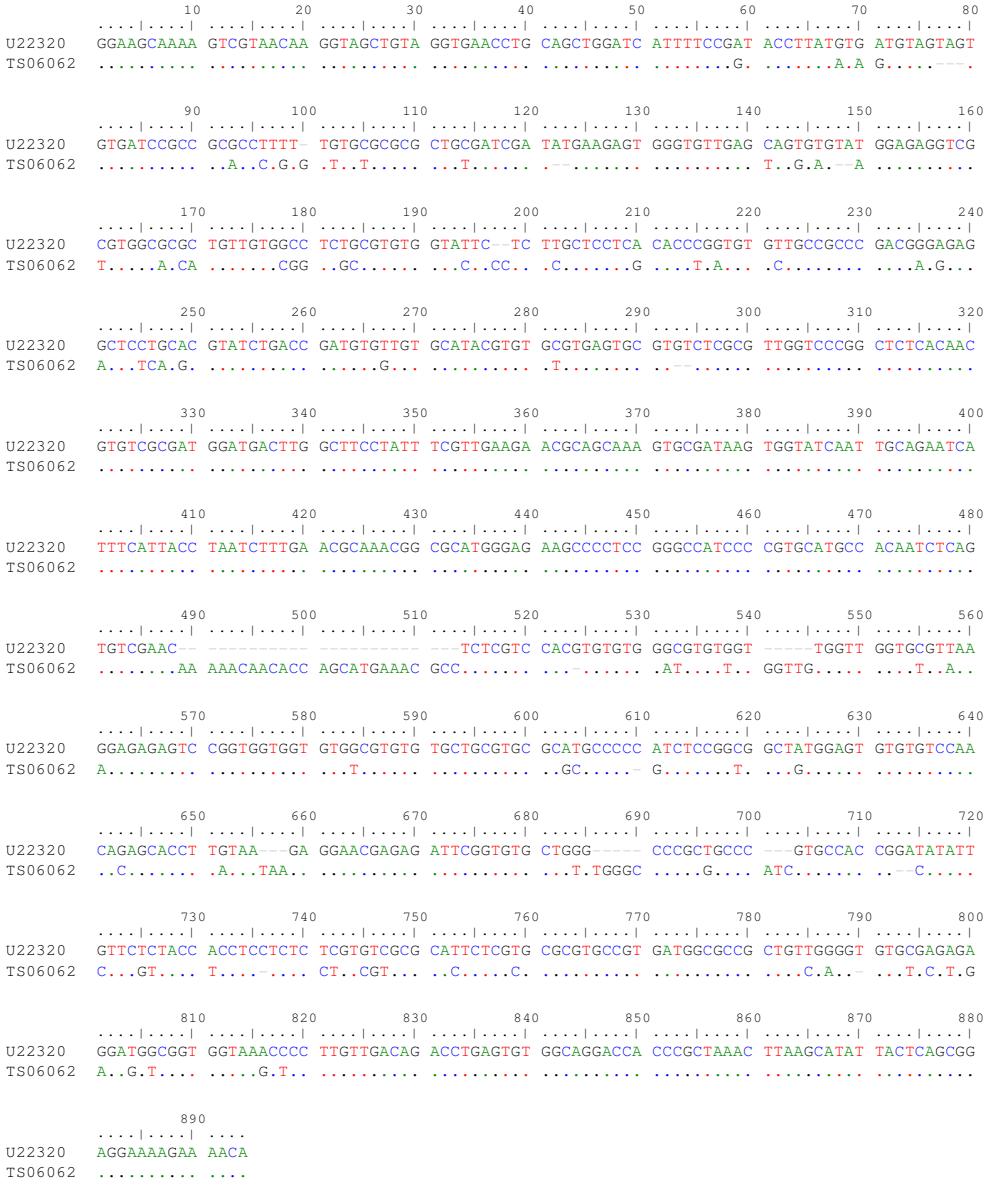


Figure 3-8: Sequence alignments for sequences TS06062 with *T. simiae* reference sequence U22320 (86% identity)

Figure 3-9: Sequence alignments for sequences TS06061 (91% identity) and TS07126 (90% identity) with *T. simiae* Tsavo reference sequence U22318

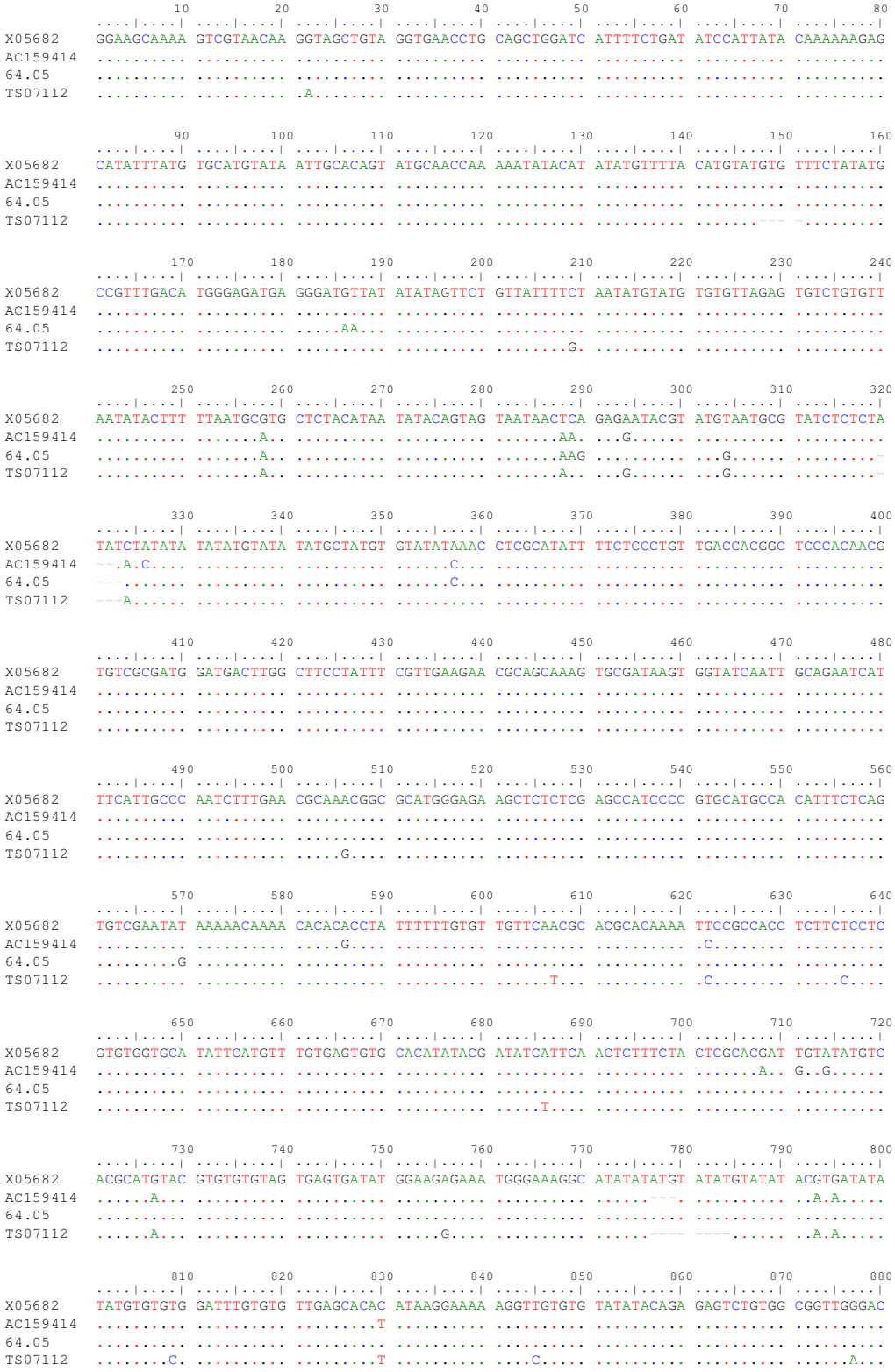
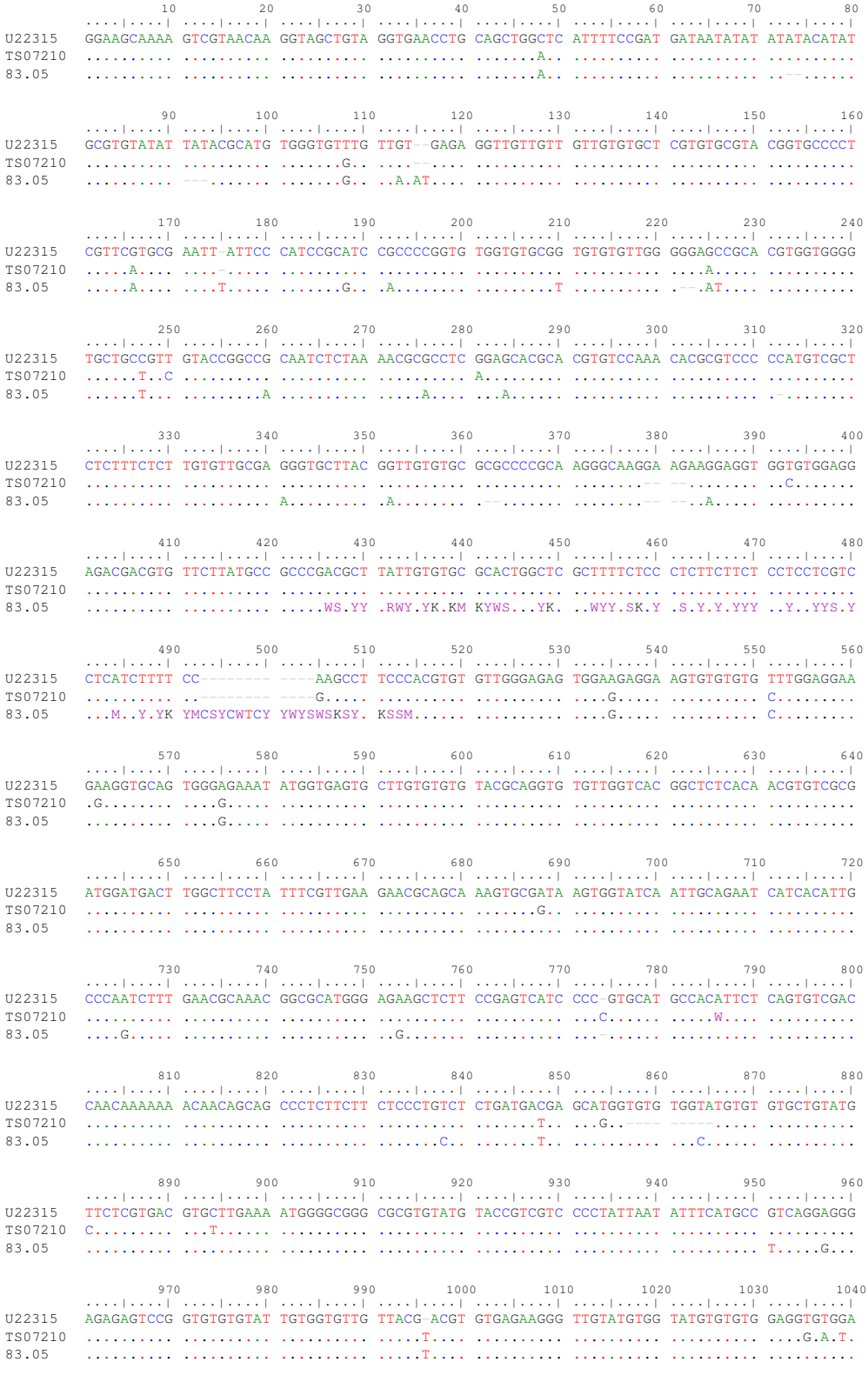

10 20 30 40 50 60 70 80
X05682 GGAAGCAAAA GTCGTAACAA GGTAGCTGTA GGTGAACCTG CAGCTGGATC ATTTTCTGAT ATCCATTATA CAAAAAAGAG
AC159414 ....
64.05 ....
TS07112 .... .A........ ....
90 100 110 120 130 140 150 160
X05682 CATATTTATG TGCATGTATA ATTGCACAGT ATGCAACCAA AAATATACAT ATATGTTTTA CATGTATGTG TTTCTATATG
AC159414 ....
64.05 ....
TS07112 ....--- -.........
170 180 190 200 210 220 230 240
X05682 CCGTTTGACA TGGGAGATGA GGGATGTTAT ATATAGTTCT GTTATTTTCT AATATGTATG TGTGTTAGAG TGTCTGTGTT
AC159414 ....
64.05 ....AA... ....
TS07112 ....G. ....
250 260 270 280 290 300 310 320
X05682 AATATACTTT TTAATGCGTG CTCTACATAA TATACAGTAG TAATAACTCA GAGAATACGT ATGTAATGCG TATCTCTCTA
AC159414 ....A.. ....AA. ...G...... ....
64.05 ....A.. ....AAG ....G...... ....-
TS07112 ....A.. ....A.. ...G...... ...G...... ....-
330 340 350 360 370 380 390 400
X05682 TATCTATATA TATATGTATA TATGCTATGT GTATATAAAC CTCGCATATT TTCTCCCTGT TGACCACGGC TCCCACAACG
AC159414 ~~.A.C.... ....C... ....
64.05 ---....... ....C... ....
TS07112 ---A...... ....
410 420 430 440 450 460 470 480
X05682 TGTCGCGATG GATGACTTGG CTTCCTATTT CGTTGAAGAA CGCAGCAAAG TGCGATAAGT GGTATCAATT GCAGAATCAT
AC159414 ....
64.05 ....
TS07112 ....
490 500 510 520 530 540 550 560
X05682 TTCATTGCCC AATCTTTGAA CGCAAACGGC GCATGGGAGA AGCTCTCTCG AGCCATCCCC GTGCATGCCA CATTTCTCAG
AC159414 ....
64.05 ....
TS07112 ....G.... ....
570 580 590 600 610 620 630 640
X05682 TGTCGAATAT AAAAACAAAA CACACACCTA TTTTTTGTGT TGTTCAACGC ACGCACAAAA TTCCGCCACC TCTTCTCCTC
AC159414 ....G.... .... .C........ ....
64.05 ....G ....
TS07112 ....T... .... .C........ .....C....
650 660 670 680 690 700 710 720
X05682 GTGTGGTGCA TATTCATGTT TGTGAGTGTG CACATATACG ATATCATTCA ACTCTTTCTA CTCGCACGAT TGTATATGTC
AC159414 ....A.. G..G......
64.05 ....
TS07112 ....T.... ....
730 740 750 760 770 780 790 800
X05682 ACGCATGTAC GTGTGTGTAG TGAGTGATAT GGAAGAGAAA TGGGAAAGGC ATATATATGT ATATGTATAT ACGTGATATA
AC159414 ......A... ....~~~. ....A.A.....
64.05 ....
TS07112 ......A... ....G.... ....---- ----...... ..A.A.....
810 820 830 840 850 860 870 880
X05682 TATGTGTGTG GATTTGTGTG TTGAGCACAC ATAAGGAAAA AGGTTGTGTG TATATACAGA GAGTCTGTGG CGGTTGGGAC
AC159414 ....T ....
64.05 ....
TS07112 ....C. ....T ....C..... ....A...

Figure 3-10: Sequence alignment for sequences 64.05 (99% identity) and TS07112 (97% identity) and *T. brucei* reference sequences X05682 and AC159414



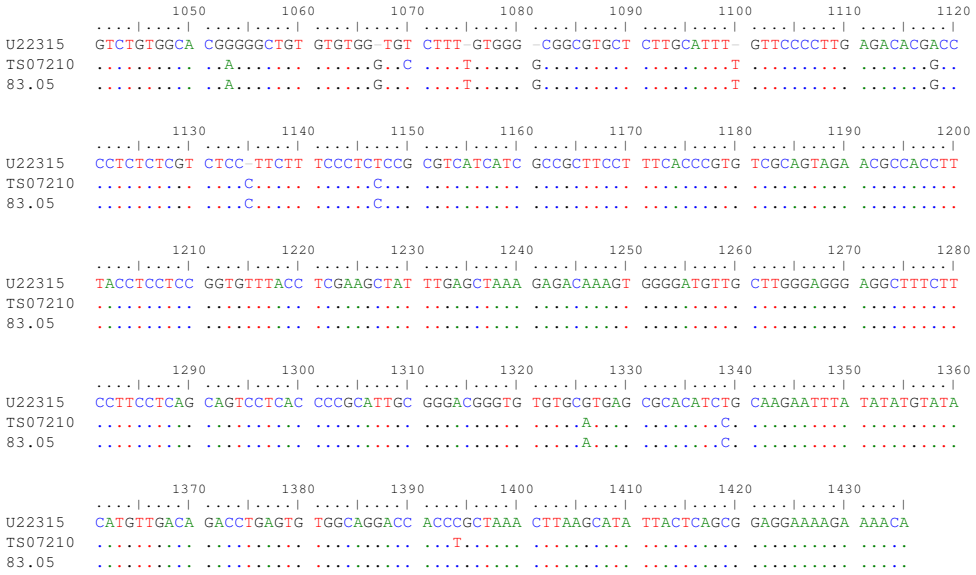


Figure 3-11: Sequence alignments for sequences TS07210 (97% identity) and 83.05 (92% identity) with *T. congolense* reference sequence (U22315)

3.5 Discussion

PCR primers based on internal transcribed spacer regions have the potential to reduce the time and cost of identifying trypanosomes compared to specific-species PCR primers. This is the first time their use has been assessed in wildlife. In this study, analysis of the sequences amplified by ITS primers suggested that *T. brucei* s.l., *T. congolense*, *T. simiae*, *T. simiae* Tsavo, *T. godfreyi* and *T. vivax* are circulating in wildlife in the Serengeti. This is the first potential identification of *T. godfreyi* and *T. simiae* Tsavo in wildlife hosts. Considerable sequence variation observed may indicate more strain variation than previously suspected. This was particularly evident for *T. vivax*; sequences obtained from waterbuck and giraffe were termed *T. vivax*-like sequences.

However, whilst the sequences generated by ITS primers provided interesting results about the diversity of trypanosomes present, the results of this study raised some concerns regarding their sensitivity and specificity.

3.5.1 Comparative sensitivity of ITS and TBR primers

ITS detected significantly less *T. brucei* s.l. positive samples than the species-specific primers, TBR. This is perhaps not surprising. The analytic sensitivity of ITS is 55 pg/ml (Cox *et al.*, 2005), compared to 0.2pg/ml for TBR primers (Moser *et al.*, 1989). The higher analytic sensitivity of the TBR primers is thought to be related to the abundance of the target sequence. The TBR primers target a highly repetitive element with a copy number of around 10 000 (Moser *et al.*, 1989), whereas the internal transcribed spacers are part of the ribosomal RNA genes, with 100-200 copies per trypanosome (Hernandez *et al.*, 1993). However, both of these analytic sensitivities correspond to less than one trypanosome per volume sampled. Since samples are unlikely to contain less than one trypanosome, for practical purposes there should be no difference in sensitivity (Cox *et al.*, 2005). There is little published data on the diagnostic sensitivity of these two tests which is directly comparable because diagnostic sensitivity is affected not only by analytic sensitivity but by factors such as methods of sample collection and storage. However, initial field validation of ITS yielded similar results to TBR (ITS 40/245 *T. brucei* positive, TBR 37/245 positive) (Cox *et al.*, 2005).

This does not explain the low number of samples testing positive using the ITS primers in this study. Since no data is available for the diagnostic sensitivity and specificity of TBR, there are two potential explanations for this result, indicating either low sensitivity of the ITS primers, or low specificity of TBR primers. However, the high specificity found for TBR primers in other studies makes the second explanation unlikely (Desquesnes & Davila, 2002).

The only difference between Cox *et al.*'s (2005) comparison of ITS and species-specific primers and that carried out in this study is the host species from which the samples were collected. Protocols followed for sample collection, storage, preparation and analysis were the same. *T. brucei* s.l. parasitaemia is usually low in wildlife (Mulla & Rickman, 1988b). Measurements of prevalence are particularly affected by host parasitaemia, with prevalence likely to be underestimated in populations with widespread sub patent infections (Cox, 2007). Njiru *et al.* (2005) tested their ITS primers on two different groups of cattle: in samples which tested positive by HCT or BCT, the sensitivity of ITS primers and species-specific primers was comparable; however, in samples where no trypanosomes were observed by microscopy but species-specific primers tested positive, only 63% were also positive using ITS primers.

Njiru *et al.* (2005) also found that the sensitivity of ITS primers decreased dramatically in mixed infections with more than three trypanosome species. Of the *T. brucei* s.l. infections identified by ITS in this study, six out of 10 were found in mixed infections with other trypanosome species. Whilst all these contained only two or three trypanosome species, it is possible that the sensitivity to detect mixed infections even when only two species are present is lower than expected.

3.5.2 Comparison of two sample preparation protocols

Using the species-specific TBR primers, the eluted DNA protocol detected more positive samples than the washed disc protocol. Methods for eluting DNA from matrices such as FTA cards have been widely used in pathogen identification. The use of chelex as an elution agent, first described by Walsh *et al* (1991), has been shown to produce high quality DNA for PCR analysis. The increased PCR sensitivity obtained using the eluted DNA protocol is most likely to be explained by the distribution of trypanosomes within the sample. Whilst

fixed onto the FTA matrix trypanosomes are heterogeneously distributed on the card. The probability that an individual disc includes trypanosome DNA is influenced by factors such as host parasitaemia. However, although the effective volume of blood tested using the eluted DNA protocol is lower (approximately 0.56µl per PCR, compared to 1µl per PCR for washed discs), trypanosome DNA has been removed from an area of card 11.25 times larger than the 2mm disc used in the washed disc protocol, and distributed homogeneously in solution. The probability of detecting trypanosome DNA is therefore increased.

For PCR using ITS primers, the same pattern is seen for detection of *T. simiae* and *T. vivax* (when these species were classified according to band size, as per Table 3-5). However for *T. congolense, T. brucei* s.l. and *T. theileri*, more positive samples were detected using the washed disc protocol. It is possible that this reflects variations in host parasitaemia between trypanosome species. If the parasitaemia is high, each disc is likely to contain a trypanosome, and elution of trypanosome DNA from a larger area is unlikely to increase the diagnostic sensitivity of the PCR. However, this is not supported by the results of TBR PCR, and there is no evidence to suggest that infections with *T. brucei* s.l. *, T. congolense* and *T. theileri* result in higher parasitaemia than other trypanosome species. In fact the parasitaemia of *T. brucei* s.l. is usually lower than for other species (Van den Bossche *et al.*, 2004). FTA card samples of blood spiked with known concentrations of trypanosomes could be tested by both protocols to disprove this hypothesis.

The feature that is shared by the PCR products of these species is large size (998-1408bp, compared to 177-847 for *T. simiae, T. vivax* and TBR PCR). A potential explanation for this difference is that whilst eluted DNA protocols will usually increase sensitivity, larger DNA fragments are more likely to be damaged in the elution process. Similar patterns have been observed in other DNA extraction protocols (Mharakurwa *et al.*, 2006).

3.5.3 Sequencing

There were three reasons for conducting sequence analysis to investigate the bands generated by the ITS primers.

1. Results obtained using the ITS primers were sometimes surprising. For example, a band size consistent with *T. vivax* was found in a baboon, a species usually

considered refractory to trypanosome species (Ashcroft *et al.*, 1959; Lambrecht, 1985). Whilst it was of course possible that results such as this simply represented new findings, further investigation was obviously necessary.

2. Bands were obtained at sizes not consistent with the expected sequence lengths, raising questions as to their identity.

3. Band sizes close to those expected for trypanosome species often showed variation around the exact size, making classification difficult.

Assessment of ITS sequences, obtained successfully for 16 bands, explored some of these concerns. Sequencing results are discussed below.

T. vivax was identified in buffalo, confirming previous findings of *T. vivax* in buffalo by microscopy (Drager & Mehlitz, 1978). In addition to transmission by tsetse, *T. vivax* can be mechanically transmitted by other biting flies, and is found in Central and South America as well as Africa (reviewed by Gardiner, 1989). Phylogenetic analysis indicates that whilst *T. vivax* isolates from West Africa and South America are very similar, the one reference sequence from East Africa was more diverse (Cortez *et al.*, 2006). This supports previous results of isoenzyme, satellite DNA, kDNA minicircles and karyotype analysis (Fasogbon *et al.*, 1990; Dirie *et al.*, 1993). This divergence is also evident from the use of specific *T. vivax* primers. *T. vivax* isolates from East Africa were not amplified by primers based on West African *T. vivax* sequences, eventually resulting in the development of a second primer set for *T. vivax*, targeting a sequence common to all *T. vivax* isolates (Masake *et al.*, 1997; Morlais *et al.*, 2001). This consistency between West African and South American isolates is hypothesized to indicate that *T. vivax* was introduced into South America via the import of infected cattle from West Africa (Dirie *et al.*, 1993). The sequence found in buffalo in this study most closely matched the East African reference sequence.

Sequences from a giraffe and a waterbuck were most closely related to *T. vivax*. *T. vivax* has been identified before in giraffe and waterbuck by microscopy (Ashcroft, 1959). However, these sequences shared only 81-89% identity with any of the references sequences and to each other. A divergent *T. vivax* sequence identified previously in tsetse in Tanzania (using a partial SSU sequence) was then assumed to represent the East African *T. vivax*, since at the

time only West African reference sequences were available (Malele *et al.*, 2003). However, subsequent analysis found this sequence separated from all other *T. vivax* isolates on phylogenetic analysis, including an East African *T. vivax* from Kenya, whilst still clearly clustering in the *T. vivax* clade (Cortez *et al.*, 2006). Trypanosomes isolated from a nyala in Mozambique showed a similar relationship – clustering in the *T. vivax* clade but divergent to other *T. vivax* sequences (Rodrigues *et al.*, 2008).

Following the convention of other authors, it appears that the sequences identified in giraffe and waterbuck represent *T. vivax*-like trypanosomes. Further work to compare these sequences to the other *T. vivax*-like trypanosomes, found in tsetse in Tanzania (Malele *et al.*, 2003) and in nyala in Mozambique (Rodrigues *et al.*, 2008) would be interesting to determine if any are consistent with each other. References sequences for these isolates are not currently available in the NCBI database. Whilst it is clear that distinct genetic variants of *T. vivax* exist in East Africa, the reasons for this are uncertain. The discovery of these within one ecosystem suggests that these cannot be explained simply be geographic distribution. It has been hypothesized that divergence may reflect more than one route of transmission, since in East Africa *T. vivax* can be transmitted both cyclically by tsetse and mechanically by biting insects, compared to consistent sequences in South America where only mechanical transmission occurs (Rodrigues *et al.*, 2008). This hypothesis has not yet been tested.

The sequence found in one warthog, sized 650bp and expected to be *T. vivax*, may actually represent the first identification of *T. godfreyi* in a mammalian host. The only reference sequence available in the NCBI database includes only the ITS 1 region. Over this region, the sequences share 87% identity, and are the closest match on BLAST search. *T. godfreyi* was identified as a new species when found in tsetse, but has never been identified in mammalian hosts before. Experimental infection of domestic pigs resulted in chronic disease and it was hypothesized that *T. godfreyi* may naturally circulate in warthogs, but this has never been confirmed. Interestingly, the sequence found in a warthog in this study shared 96% identity to sequences obtained from two warthogs in Zambia (N. Anderson pers. comm.).

A sequence identified from a warthog is likely to be *T. simiae*. Although only sharing 86% identity with the one reference sequence, the sequence was very similar (97%) to a sequence

from a warthog in Zambia (N. Anderson pers. comm.). A similar situation has been described in tsetse, where a sequence was identified that was 93% similar to the *T. simiae* reference sequence, but shared 99% identity with other sequences found in the same study (Adams *et al.*, 2006). The source of the reference sequence for *T. simiae* is not published, but these differences perhaps suggest strain variation, for example from different geographical areas. *T. simiae* has been found before in warthogs using the specific *T. simiae* primers (Kaare *et al.*, 2007).

A sequence similar to *T. simiae* Tsavo was found in warthogs. Although only sharing 90-91% identity with the one reference sequence, sequences from two warthogs shared 97% identity with each other, and 98% identity with sequences from two warthogs in Zambia (N. Anderson pers. comm.). *T. simiae* Tsavo was first identified in tsetse in Tsavo National Park, Kenya (Majiwa *et al.*, 1993). It was later confirmed as a sub group of *T. simiae*, rather than *T. congolense* as had first been thought (Haag *et al.*, 1998; Stevens *et al.*, 1999; Gibson *et al.*, 2001) (and is referred to as *T. simiae* Tsavo throughout this thesis). If this trypanosome is confirmed as *T. simiae* Tsavo, this represents the first identification in mammalian hosts.

Sequences consistent with *T. brucei* s.l. were identified in hyaena and zebra. This is the first identification of *T. brucei* s.l. in zebra by molecular analysis, although it has been reported once previously in zebra by microscopy (Baker, 1968). Bands of size 1407 and 1420bp had sequences consistent with *T. congolense*, as expected.

Unidentified bands

Sequences from five samples did not closely match any existing reference sequences. Unidentifiable sequences found in only one clone may arise from contamination or errors in sequencing. However, the accuracy of these sequences is supported by finding the same sequence in more than one individual; for example, two unidentified sequences from wildebeest shared 87% identity. Whilst not identifiable by the reference sequences available, these may represent trypanosome sequences since they were associated either with *Trypanosoma* spp or with the very closely related genera of *Bodo* or *Leptomonas*.

Studies in tsetse have revealed divergent sequences which suggested the presence of previously unidentified trypanosomes, including a *T. godfreyi*-like trypanosome (Malele *et al.*, 2003) and a *T. brucei*-like trypanosome (Hamilton *et al.*, 2008). Since reference sequences are not available for these potential new species, comparison with sequences in this study was not possible. Further investigation into the identity of the unknown sequences would be interesting since the trypanosomes found in tsetse populations must reflect those circulating in the vertebrate hosts.

Further characterisation

Sequences identified in this study were represented by only one clone, and further work is necessary both to confirm sequences found, and to identify the unknown sequences. Analysis of further clones per band improves the accuracy of sequences, and allows sequences to be uploaded to NCBI. The use of species-specific primers for *T. godfreyi* and *T. simiae* Tsavo could confirm the potential identification of these species in wildlife hosts. The high variability of the ITS regions, whilst ideal targets for PCR primers dependent on variability in length, makes them unsuitable for constructing phylogenetic trees above the species level. The 18S ribosomal small subunit is often used for phylogenetic analysis of the trypanosomes (see for example Malele *et al.*, 2003; Cortez *et al.*, 2006). It is a more conserved region, making it more suitable for this type of analysis than the variable ITS regions. In addition more reference sequences are available for this region. For further investigation of the trypanosomes circulating in this wildlife population, the use of SSU sequences would be valuable to confirm species identifications and allow analysis of the phylogenetic relationships of these trypanosomes.

Classification of trypanosomes has traditionally been based on factors such as morphology, host range, pathogenicity and distribution (Gibson, 2007). The inconsistencies of this system revealed by genetic analysis have provoked discussion on trypanosome classification. This has lead to reclassification of some species (Gibson *et al.*, 2001) and prompted suggestions that the system for trypanosome classification needs to be reconsidered (Rodrigues *et al.*, 2008). In this study, the discovery of sequences which either match existing sequences whilst showing some divergence, or cannot be matched to any existing trypanosome reference sequences, confirms the diversity of trypanosomes which cannot be explained by the current classification system.

3.5.4 Implications for further use of ITS primers

The ITS technique relies on PCR amplification of each trypanosome resulting in a band size which is unique and distinguishable from others. The presence of a band at an unexpected size, although raising questions as to its origin, does not affect the specificity of the protocol for other trypanosomes unless it cannot be differentiated from others. The lengths of sequences obtained are shown in Table 3-7.

Species	Expected band size from NCBI database (bp)	Band sizes obtained by Cox et al (bp)	Band sizes obtained in this study (bp)
T. congolense	1413	1408	1407, 1420
T. simiae (Tsavo)	954	951	967, 968
T. brucei	1207-1224	1215	1207, 1220
T. simiae	850	847	878
T. vivax	611	620	594, 596, 654
T. theileri	988	998	none detected
T. godfreyi	NA	NA	650

Table 3-7: Sequence lengths from reference sequences in NCBI database, bands obtained by Cox *et al.* (2005), and bands obtained in this study, in base pairs (bp)

It is evident that some variation in sequence length occurs, even within individual trypanosome species. Differentiation of *T. brucei* s.l. and *T. congolense* was never problematic. The size differences in the sequences obtained were slight, and the band sizes are sufficiently different to other species that they are easily identified. Cox *et al* (2005) report the differentiation of the three *T. congolense* groups - savannah, Kilifi and forest, by slightly differing band sizes (savannah: 1408; Kilifi: 1430; forest: 1501bp). In previous studies in Serengeti, the majority of *T. congolense* have been identified as savannah-type, with Kilifi occasionally identified (Adams *et al.*, 2006; Kaare *et al.*, 2007). *T. congolense* forest has never been identified in Serengeti (Adams *et al.*, 2006). In this study, no bands consistent with *T. congolense* forest were observed. Differentiation of *T. congolense* savannah and *T. congolense* Kilifi, which is difficult due to their similar sequence length, was not done in this study. Variation in sequence length in the two *T. congolense* sequences obtained, both of the savannah type, confirmed the difficulties of differentiating these two groups.

However, differentiation of other species was complex. For example, *T. vivax* or *T. vivax*-like trypanosomes were identified at 594, 596 and 654bp, whilst a putative *T. godfreyi* sequence was 650bp and a sequence of 646bp could not be identified. For *T. simiae*, the sequence length obtained was 28-31bp different to previous sequences.

Other authors have found evidence of size variation in ITS sequences within species. For example the length of the ITS1, 5.8 and ITS2 regions in *T. vivax* from South America and West Africa was 490bp. However, *T. vivax* sequences from East Africa were not only longer (varying between 525 and 534bp), but varied between gene copies of the same isolate (Cortez *et al.*, 2006).

Clearly these results have implications for the specificity of ITS. There are two solutions to this issue. Firstly, the number of bands sequenced in this study is quite small, and although sequencing is expensive and time-consuming, the important issues raised by this study suggest further sequence analysis is essential. Studies on the trypanosome populations circulating in free ranging wildlife are logistically difficult to conduct and this substantial sample set has the potential to reveal much more information. At the same time this will reveal if there are trypanosomes which consistently give bands which cannot be differentiated, as for example *T. vivax* and *T. godfreyi* appear to do. Potentially, single species primers can then be used to differentiate individual species on positive samples. This has been the case for other ITS primers sets; the TRYP primers cannot differentiate *T. simiae* and *T. simiae* Tsavo so a single species PCR is conducted to tell these species apart when positive bands are obtained (Adams *et al.*, 2006). Establishing the length of other *T. simiae* sequences would reveal whether the band size found in this study is consistent with other isolates, perhaps reflecting a different strain to the reference sequence.

It could be argued that if additional single species PCRs are necessary, the benefits of ITS are diminished. However, the potential scope of ITS primers provide an important opportunity for identifying new trypanosomes. It is unlikely that sequences that do not closely match existing trypanosomes would be identified as positive by PCRs designed for individual trypanosome species or groups. Depending on the degree of variation in the target sequence, even sequences that match reference sequences, but not very closely, may not be detected, as is illustrated by the lack of detection of East African *T. vivax* isolates when using *T. vivax* primers designed for *T. vivax* isolated in West Africa. The use of species-specific

primers in this study, whilst perhaps resulting in data that was easier to interpret, would not have revealed information on the diversity of trypanosomes in wildlife.

In light of the results of sequence analysis, Table 3-5 should be interpreted carefully, and identifications of species such as *T. vivax* in a baboon require further investigation. Unfortunately this particular band was one of the four bands for which clonal sequence analysis was not successful. However, the discovery of other sequences not consistent with *T. vivax* but of similar length make this finding unlikely.

Band sizes for *T. congolense* and *T. brucei* s.l. appear to be specific: two sequences from each species were consistent with reference sequences and the host range and prevalence detected was consistent with other studies (bar the low sensitivity of ITS for *T. brucei*). The results obtained for *T. brucei* s.l. and *T. congolense*, together with the TBR PCR results, will therefore be used for further analysis in Chapter 4.

Discussion of wildlife as reservoirs has focused on the transmission of HAT. However, *T. congolense, T. simiae* and *T. vivax*, all identified in wildlife in this study, are pathogenic to livestock. *T. simiae* Tsavo and *T. godfreyi* may also be important as pathogens of pigs, since they infect pigs in experimental infections (McNamara *et al.*, 1994; Zweygarth *et al.*, 1994). No data is available on the prevalence of these species in livestock around SNP, but their identification in wildlife indicates the potential for transmission between livestock and wildlife, and further work to quantify the importance of these trypanosome species in livestock disease would be helpful.

3.6 Conclusions

In recent years genetic analysis has revealed the complexity of trypanosome classification. Previous studies have found a diverse range of trypanosomes in tsetse populations, some of which cannot easily be explained by current classification. The use of ITS primers on samples from wildlife for the first time has confirmed that trypanosomes circulating in wildlife are equally diverse.

The use of ITS primers on samples collected from wildlife revealed interesting and important observations on trypanosome host range and diversity, but also showed that sensitivity was

low compared to the species-specific primers (for *T. brucei* s.l.) and band sizes needed further characterisation. Is ITS a good choice for further studies identifying trypanosome infections in wildlife? If the aim is to explore the diversity of trypanosomes in wildlife, techniques based on size variation of the ITS region, combined with sequence analysis, have more potential for identifying new species and subspecies, and exploring variation than species-specific PCRs. The potential identification of two species never before reported in mammalian hosts illustrates the importance of characterising trypanosomes of wildlife. However, for generating prevalence data for epidemiological studies on trypanosome transmission in wildlife, species-specific primers are more sensitive (in the case of *T. brucei* s.l.) and potentially more specific, and are likely to produce results which are easier to interpret. Further characterisation of ITS bands may resolve some of the issues of assigning band sizes to species experienced in this study, improving its specificity.

Comparison of two sample preparation protocols did not reveal an overall advantage of one protocol. The number of samples testing positive for *T. brucei* s.l. was significantly lower using ITS primers compared to TBR primers. Low diagnostic sensitivity of ITS may be associated with the low parasitaemia common in wildlife, or to a tendency of *T. brucei* s.l. to be found in mixed infections, both factors which are shown to reduce the diagnostic sensitivity of ITS primers.

Chapter 4: Trypanosomes in wildlife: assessment of prevalence and risk factors associated with infection

4.1 Introduction

The importance of wildlife as a reservoir of *Trypanosoma brucei rhodesiense*, the causal agent of human African trypanosomiasis (HAT) has been a subject of discussion for many years. In particular, the roles that different wildlife species play in transmission are unclear (reviewed in Chapters 1 and 2). In this chapter, data is presented on the prevalence of trypanosome infections in wildlife in the Serengeti Mara ecosystem (SME), using laboratory and statistical approaches that overcome some of the limitations of earlier studies.

The primary focus of most previous studies has been assessing which wildlife species carry trypanosome infections, usually with a view to identifying potential reservoirs. Whilst natural infection is obviously a prerequisite for being a reservoir, the importance of a species as a reservoir of infection is not related only to prevalence. Prevalence is a dynamic quantity; it is not specific to a species but is influenced by many other factors as well. These may include host factors such as age, sex and health status and ecological factors such as vector density. In addition it is almost impossible to avoid some bias when collecting samples from free-ranging wildlife, for example animals immobilised may be those that are easiest to dart, potentially selecting for animals with compromised fitness. Without understanding the influence that other factors have on prevalence, erroneous conclusions may be reached. However, the availability of statistical software and methods which allow computationally intense analysis of multiple risk factors and their interactions mean that is it increasingly becoming possible to account for the ecological factors determining trypanosome prevalence. The risk factors for infection considered in this study are discussed below.

4.1.1 Risk factors for trypanosome infection – host factors

Host species

Previous studies have identified *Trypanosoma brucei* sensu lato in over 20 species (reviewed in Chapter 1). In the first study to use PCR to identify *T. brucei* s.l. in wildlife in SME, Kaare *et al* (2007) identified *T. brucei* s.l. in 29% of warthogs in the SME in 2001. This is much higher than previously reported and combined with the importance of warthogs as a

food source for both *Glossina swynnertoni* and *Glossina pallidipes*, suggests warthogs may be particularly important in *T. brucei* s.l. epidemiology. If this is the case, options for targeted control can be considered. However since the sample size in this study was small (n=21) and other factors which may affect prevalence, such as age, were not considered, further investigation of the relationship between warthogs and trypanosomes is essential.

T. b. rhodesiense has been identified in lion, hyaena, hartebeest, bushbuck, warthog, waterbuck and reedbuck (Heisch, 1952; Geigy *et al.*, 1971; Robson *et al.*, 1972; Geigy & Kauffman, 1973; Geigy *et al.*, 1973b; Awan, 1979). However earlier techniques available for differentiating *Trypanosoma brucei brucei* from *T. b. rhodesiense* were limited by the ethical and logistical concerns of the use of human volunteers, and the inconsistent results obtained using the blood incubation infectivity test (BIIT) (reviewed in Chapter 1). New PCR protocols based on the presence of the serum-resistance-associated (SRA) gene in *T. b. rhodesiense*, but not *T. b. brucei*, allow more specific identification. To date, two studies have used the SRA PCR protocol to identify *T. b. rhodesiense* in wildlife. Njiru *et al* (2004b) found *T. b. rhodesiense* in hyaena and reedbuck from trypanosome stocks in Kenya. In the SME, Kaare (2007) identified *T. b. rhodesiense* in warthogs only.

Sex

Sex differences in parasite infections are widely documented, with males frequently showing higher prevalence of disease than females (reviewed by Zuk & McKean, 1996). These differences can arise for both ecological reasons, for example behavioural differences which affect exposure to a pathogen, and intrinsic physiological reasons, such as differences in immune function (Moller *et al.*, 1998). Some studies report a higher prevalence of trypanosomiasis in male cattle, thought to result from exposure factors such as preference of tsetse for larger animals (Torr *et al.*, 2006), or management practices increasing exposure to tsetse (Rowlands *et al.*, 1993). It is unclear whether immune function also plays a role in these differences. No differences in prevalence have been reported in wildlife species.

Age

Differences in prevalence by age are common for many pathogens. This may result from variation in exposure with age (such as differing vector feeding preferences for larger animals) or variation in response to exposure with age (for example if maternally-derived

antibodies or an acquired immune response is protective). Several studies have examined the relationship between trypanosome prevalence and the age of the host. Buffalo were observed to show a peak infection rate (when considering all trypanosome species) between 1 and 3 years using microscopy, and antibody titres peaked at 4 years then persisted at a high level (Drager & Mehlitz, 1978). A similar pattern of age stratification was observed in lions in Serengeti, with peak infection by microscopy between 2 and 3 years (although this was not statistically significant) (Sachs *et al.*, 1971). This has been confirmed by recent studies on lions in Serengeti, which hypothesised that an acquired immune response may explain the peak in *T. brucei* s.l. prevalence seen in two to three year-old lions (Welburn *et al.*, 2008).

Health status

Trypanosome infections cause mortality in some wildlife species in experimental studies (Ashcroft *et al.*, 1959), reviewed in Chapter 1. However sick animals are generally not observed in the field. It is feasible that trypanosomiasis confers a fitness disadvantage, but in an ecosystem such as SME with high densities of large carnivores, infected animals are likely to be predated before mortality is observed. Therefore it might be expected that animals which have been caught by predators are more likely to carry trypanosome infections.

Trypanosome infections cause immune suppression which increases susceptibility to other pathogens (Holmes *et al.*, 1974), causing reduced immune response to helminth infections (Urquhart *et al.*, 1972) and lack of response to bacterial and viral vaccines (Rurangirwa *et al.*, 1983). Conversely, subsequent infection with another pathogen increases the parasitaemia of an already present trypanosome infection (Tosas, 2007). These two factors mean that any animals that are observed to be sick are more likely to carry trypanosome infections. Collection of samples from sick or dead animals may therefore introduce positive bias into the dataset. However this also provides the opportunity to study this relationship, incorporating health status into the analysis, and comparing the prevalence of trypanosome infections in animals that were sick or found dead, compared to routine sampling.

4.1.2 Sample factors

The logistical difficulties of collecting blood samples from free-ranging wildlife means that sampling is largely opportunistic and samples are collected by a variety of methods.

Concurrent research projects allow collection of samples from live animals immobilised for fitting of radio telemetry collars or for disease surveillance. However, it is difficult to obtain samples from a sufficient range of species and number of individuals using samples from live animals only. Animals in SME are often found dead, for example killed by predators or in road traffic accidents, and collection of post mortem samples provides a way of increasing sample size considerably, in addition to providing an opportunity to study the importance of health status, as discussed in Section 4.1.1. However any change in the probability of detecting parasites could influence prevalence. Studies on detection of other pathogens in post mortem samples indicate that the likelihood of detection decreases as time between death and sample collection increases (Panella *et al.*, 2005; Gal *et al.*, 2008), but there are no data available specific to trypanosome detection.

4.1.3 Vector factors

Density

The density of tsetse follows the vegetation gradient across the SME, from the grassland plains of the south east where tsetse cannot persist, to the woodlands and savannah further north and west which support high density tsetse populations (see maps in chapter 2).

The relationship between trypanosome prevalence in wildlife hosts and the density of *G. swynnertoni* and *G. pallidipes* in SME is likely to be complex. Vector density is a component of vector challenge, which also incorporates prevalence of trypanosomes in the tsetse population, and tsetse feeding preferences. There may be spatial variation in both these factors. For example variation in wildlife species composition in different areas may influence both the species that tsetse are feeding on and the prevalence of trypanosomes in tsetse. By looking at the relationship between trypanosome prevalence and tsetse density in individual wildlife species, or using statistical models which control for the effect of species, it is possible to start exploring these factors. However the confounding effects of many related factors must be considered.

4.2 Objectives

This chapter describes a cross sectional study of *T. brucei* s.l. and *T. congolense* in wildlife with the following objectives:

- To assess the distribution of trypanosome infections between wildlife species and whether this can be explained by taxonomic grouping, habitat, diet or tsetse fly host choice

- To determine whether the prevalence of trypanosome infections in warthogs is significantly higher than in other species

- To assess the importance of other host level factors (sex, age, health status) on the prevalence of trypanosome infections

- To assess whether post mortem samples represent a useful method for assessing trypanosome prevalence by comparing prevalence in post and ante mortem samples

- To explore the relationship between the density of *G. swynnertoni* and *G. pallidipes* and the prevalence of *T. brucei* s.l. and *T. congolense* in wildlife

4.3 Methodology

4.3.1 Research Clearance and Documentation

Wildlife research in Tanzania is coordinated and regulated by Tanzania Wildlife Research Institute (Tawiri), and all fieldwork must be approved by the Tawiri board of directors before permission is given for research to be conducted. All field work for this thesis was approved by Tawiri and conducted under Tawiri research permits following discussion of research priorities in this field with personnel at both Tawiri and Tanzania National Parks. Ethical clearance is not required unless research contains medical components, which this thesis does not. As required by Tawiri, all handling or immobilisation of wildlife was carried out in conjunction with the Tawiri Messerli Wildlife Veterinary Programme. Samples were transported to the UK under Tanzania Ministry of Livestock Zoosanitary Department export certificates for biological samples (which also require Tawiri approval), Scottish Executive Environment and Rural Affairs Department import permits, and for species listed on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) appendices, CITES export and import permits (which also require Tawiri approval).

4.3.2 Sample collection

Samples were obtained from wildlife within Serengeti National Park (SNP) and Grumeti, Ikorongo and Maswa Game Reserves. Two types of sampling were carried out: (a) opportunistic sampling of a range of wildlife species; and (b) targeted sampling of warthogs. Sampling was carried out between January 2005 and December 2007. In addition archived samples collected by Dr Sarah Cleaveland between 2002 and 2004 as part of a research programme on carnivore viral diseases were analysed.

(a) Opportunistic sampling

The number of research projects operating in the SME means some species of wildlife are regularly immobilised. Reasons for immobilisation include disease surveillance, fitting or removal of radio collars and removal of snares. Collaborations were established with projects carrying out wildlife immobilisations during the study period (Tawiri-Messerli Foundation Wildlife Veterinary Programme, Tanapa Veterinary Unit, Serengeti Lion Project, Serengeti Cheetah Project, Viral Transmission Project and Antelope Conservation Project), and

samples collected whenever animals were immobilised. In addition, samples were collected from any animals found dead during the study period, and from animals shot by commercial hunting companies that operate within Maswa, Grumeti and Ikorongo Game Reserves. All sampling was either carried out by the author, or if it was not possible for the author to be present, sampling kits were supplied to other personnel. Kits comprised one Flinders Technology Associates (FTA®) classic card (Whatman Biosciences, Cambridge, UK), syringe, needle, scalpel blade, gloves, foil envelope, sachet of dessicant and a sampling protocol. Archived samples included samples collected from immobilised animals and from animals which had been found dead.

(b) Warthog sampling

Two protocols have previously been described for warthog capture. Animals can be captured physically be netting burrows at dawn to catch them as they leave their burrows (Cumming, 1975). Chemical immobilisation of warthogs is reported to be difficult, due to the close range needed for darting, risks of darted animals entering burrows, and the predisposition of warthogs for heat related complications and respiratory and cardiac depression (Burroughs, 1993).

In this study, two protocols were established for immobilisation of warthogs for sample collection, adapted from the protocols reported previously. Within SNP protocols were developed for chemical immobilisation in collaboration with Tawiri-Messerli Foundation Wildlife Veterinary Programme. Warthogs were darted from a vehicle using a combination of 3-4mg etorphine and 30 to 60mg of azaperone per animal, combined in a 1ml Pneu-dart disposable dart and using a Pneu-dart cartridge-fired projector (Pneu-dart Inc., Pennsylvania, USA), at a distance of less than 25m (Photograph 4-1 & Photograph 4-2).

Photograph 4-1: Recently darted warthog

Photograph 4-2: Blood sampling of chemically immobilised warthog

In addition warthogs were sampled in Grumeti Game Reserve, where they were not approachable for darting due to previous commercial hunting. A method of physical capture was developed. A warthog or group of warthogs was pursued by vehicles until they sought refuge in burrows. Game capture nets were used to cover the hole, preventing the warthog(s) from exiting the burrow. Holes were then excavated and warthogs extracted by their hind legs and restrained for sampling. Some burrows were too deep and it was not possible to reach the warthog, in which case the attempt was abandoned.

When warthog were immobilised using either protocol, a notch was removed from the dorsal ear margin to mark the animal and prevent accidental resampling of the same individuals.

4.3.3 Sample processing

For all samples, heparinised or whole blood was collected from the jugular, cephalic or saphenous veins (depending on species) in immobilised animals, or from the heart for post mortem samples, and applied to FTA cards. One card was used per animal to reduce the risk of contamination between samples. Cards were allowed to dry thoroughly, sealed in foil envelopes with desiccant sachets and stored at room temperature.

4.3.4 Data recording

The following information was recorded for each sample where possible:

- **Date** of sample collection

- The coordinates of the **location** where the sample was collected, using a handheld Garmin Global Positioning System (GPS).

- **Wildlife species** If sample collection was carried out by anyone other than the author, it was ensured that they were confident in species identification.

- **Sex** was recorded where possible. In some cases sexes could not be differentiated, for example in post mortem samples from scavenged carcasses or in strongly monomorphic species such as hyaena.

- **Age category** Accurate aging of free ranging wildlife is difficult, and at risk of subjectivity if assessed by different people. Therefore each animal was assigned to a basic age category only (juvenile, sub-adult, adult or old). For some species more detailed information was available. Lions belonging to study prides of the Serengeti Lion Project are individually identified by morphological features such as ear notches and the pattern of whisker spots on the muzzle. Known individuals can be aged to an accuracy of one month.

- **Reason for sampling** such as immobilisation to fit a radio collar or post mortem examination.

- **Additional information** such as results of post mortem examination or diagnostic tests for other diseases. For samples collected post mortem, the time elapsed between death and sampling was estimated.

4.3.5 Laboratory analysis

T. brucei s.l. and *T. congolense* were identified using the TBR and ITS protocols described in Chapter 3. Only the results obtained for *T. brucei* s.l. and *T. congolense* were included in the analysis as the species could not be identified with certainty. In addition, all samples which tested positive for *T. brucei* s.l. were analysed using the PLC/SRA multiplex PCR to differentiate *T. b. brucei* and *T. b. rhodesiense*.

SRA / PLC multiplex PCR

T. b. rhodesiense is differentiated from *T. b. brucei* by detection of the serum-resistance-associated (SRA) gene. Simultaneous amplification of another single copy gene, a phospholipase C (PLC) sequence specific to *T. brucei* s.l., confirms whether there is sufficient *T. brucei* s.l. material present in the sample to detect the presence of *T. b. rhodesiense.* PCR was carried out in a 25µl reaction volume containing 3mM MgCl, 1.25µl

of Rediload dye (Invitrogen), 1.5 Units Hot StarTaq, 0.2µM of each primer and one washed disc. Cycling conditions and primer sequences can be seen in Table 4-1. SRA gives a 669bp product, with a PLC band at 324bp, shown in Figure 4-1.

For all PCRs, one negative control (water) and one positive control (genomic DNA) were run for every 16 samples, in addition to negative control blank discs. Thermal cycling was carried out in a DNA Engine DYAD^{TM} Peltier thermal cycler. All primers were sequenced by MWG Biotech. PCR products were run on a 1.5% (w/v) agarose gel at 100V, stained with ethidium bromide and visualised under an ultraviolet transilluminator (Gel-Doc 2000, Bio-Rad).

PCR	Primer Sequence
SRA/PLC Multiplex	SRA-F: 5'- GAA GAG CCC GTC AAG AAG GTT TG -3'
(Picozzi *et al.*, 2008)	SRA-R: 5'- TTT TGA GCC TTC CAC AAG CTT GGG -3'
	PLC-F: 5' - CGC TTT GTT GAG GAG CTG CAA GCA -3'
	PLC-R: 5' - TGC CAC CGC AAA GTC GTT ATT TCG -3'
	Cycling Conditions: 95ºC for 15min, then 45 cycles: 94ºC for 30sec, 63ºC for 90sec, 72ºC for 70s, final extension 72 ºC for 10min. Duplicate samples also run using 50 cycles.

Table 4-1: Primer sequences and cycling conditions for SRA/PLC PCR

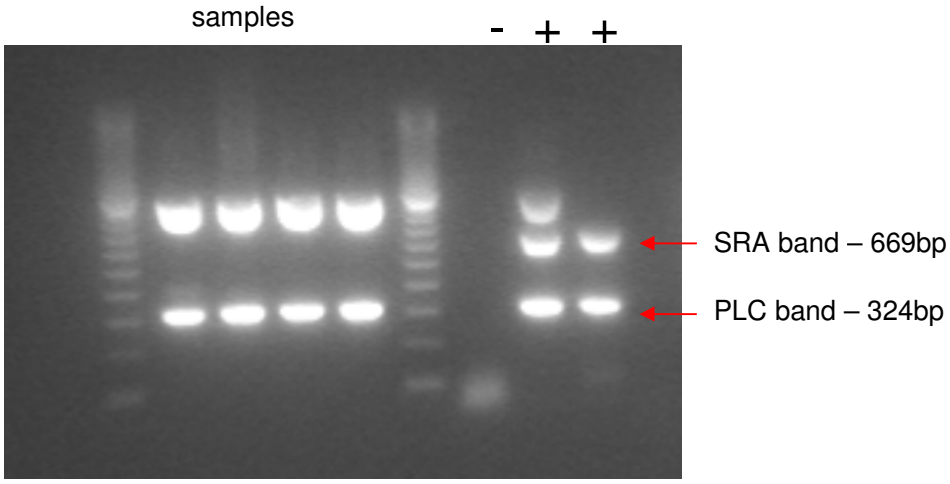


Figure 4-1: Agarose gel showing SRA and PLC PCR products

The SRA/PLC PCR is used to differentiate *T. b. brucei* and *T. b. rhodesiense*. Gel shows four sample lanes (2-5), which all show PLC bands but test negative for SRA. *T. brucei rhodesiense* positive controls in lanes 8 and 9 show both PLC and SRA bands. A third band of size >1kbp results from amplification of a VSG whose sequence shows similarity with the SRA target sequence, but also includes a deletion sequence not found in SRA. Lanes 1 and 6 contain 100bp ladder with double strength band at 1kbp.

4.3.6 Statistical analysis

This study relied almost entirely on opportunistic sampling. Whilst often used in wildlife studies out of necessity (for example Guberti & Newman, 2006), opportunistic sampling strategies do not result in a randomly selected and unbiased dataset. Potentially positive bias may have been introduced if those individuals most likely to be sampled (for example those easiest to catch, more likely to be hit by cars, or found dead) are also those most likely to be carrying trypanosome infections (if trypanosome infections impair health to any degree). However, inclusion of reason for sampling as a risk factor enabled this effect to be considered in analysis.

Statistical analysis was carried out in two stages. Initial data inspection suggested that species would be an important variable in determining infection status. However, the data set included samples from 37 different species, and small sample sizes for some of these species precluded analysis at the species level. Therefore it was necessary to group species for analysis. The first part of the analysis uses classification tree models to choose criteria for grouping species which explains the most variance, by looking at which attributes of a species most explain its likelihood of carrying trypanosome infections.

The second part of the analysis comprises univariate analysis of risk factors at an individual level, including host, sample and environmental risk factors, and bivariate analysis of each factor after controlling for species (grouped according to results of part one). Finally statistically significant variables were included in a multivariate model. All models were carried out for both *T. brucei* s.l. and *T. congolense*, and where sample size permitted, for *T. b. rhodesiense.*

For all statistical analyses, a sample was designated positive for *T. brucei* s.l. if found positive by either or both the TBR and ITS protocols. A sample was designated positive for *T. congolense* if found positive using the ITS protocol (either from eluted DNA or with PCR directly from punches). Samples positive with the SRA PCR were designated *T. b. rhodesiense* positive. The other trypanosome species were not included in statistical analysis, due to the difficulties of accurate trypanosome species identification in wildlife identified in chapter 3. Species which contained two or fewer samples were excluded from the analysis since inclusion of groups with small sample sizes resulted in very high standard errors and unstable models.

For all analysis, confidence limits for prevalence were exact binomial 95% confidence intervals, and a p-value of 0.05 was used for statistical significance unless otherwise stated.

All statistical analyses were carried out using R 2.7.2 (The R Foundation for Statistical Computing, http://www.r-project.org).

4.3.7 Species level analysis - Tree models

Tree models provide a simple method for visualising the structure of data. Tree models are fitted using binary recursive partitioning to split the data at points which maximally distinguish the response variable between the right and left branches. At each point, the data is divided according to the explanatory variable which explains the largest amount of the deviance. Tree models are ideal for assessing classification issues (Breiman *et al.*, 1984; Crawley, 2002).

Tree analysis was used in this study to assess which species level criteria best explained the deviance in prevalence of trypanosome infections. Species could potentially be assigned to categories in several ways, for example based on taxonomy, habitat preferences, diet, and importance as feeding sources for tsetse. The criterion which explained the most deviance was used to assign species to groups for further analysis.

The prevalence of trypanosomes in each species was used to generate a tree model. Separate trees were generated for *T. brucei* s.l. and *T. congolense*, using a binary response variable (positive or negative). The explanatory variables were the potential criteria for classification, described below:

- **Taxonomy** Species were grouped according to taxonomic classification, shown in Table 4-2. To summarise, three families of the order Carnivora were represented (Canidae, Felidae and Hyaenidae). Of the order Artiodactyla, the Giraffidae and Suidae families were included, along with the family Bovidae which divided into four subfamilies (Bovinae, Alcelaphinae, Antilopinae and Reduncinae) (reviewed by Matthee & Davis, 2001). Zebra were the only members of the Equidae family. Also included were the orders Primates and Rodentia.

- **Habitat** Species were divided into those predominantly found on the grassland plains, those found predominantly in woodland, and those either found across both habitats, or in the intermediate areas (Kingdon, 1997) (Table 4-3), as a indicator of tsetse exposure.

- **Diet** It has been hypothesized that carnivores can become infected by consumption of infected prey, suggesting diet could be an important risk factor in determining trypanosome prevalence. Species were divided into herbivores and those whose diets include at least a proportion of meat (Kingdon, 1997) (Table 4-4).

- **Tsetse host preferences** Different tsetse species have differing preferences for host selection. *G. swynnertoni* feed consistently more on warthog, buffalo and giraffe (Moloo *et al.*, 1971; Rogers & Boreham, 1973) which were therefore classified as high; all other species were classified as low. *G. pallidipes* feed predominantly on warthog, buffalo and bushbuck, with other species identified in less than 1% of blood meals (Weitz, 1963; Clausen *et al.*, 1998). Bushbuck were excluded from the analysis due to small sample size (n=2) so the high category comprised warthog and buffalo only (Table 4-5).

Below is an example of the code used shows to generate a tree model in R, including all potential criteria:

```
plot(tree(congopos~taxonomy+habitat+bloodmealswyn+
bloodmealpall+diet))
```

Table 4-2: Classification of species by taxonomy

(Overleaf) Species could be grouped for analysis based on four different criteria. This table shows the allocation of species into groups based on taxonomy, with the number of samples in each group. Groups were chosen at the order, family or sub-family level.

Classification			Number sampled	Group total
Order Carnivora	Family Canidae			14
	Bat-eared fox	*Octocyon megalotis*	4	
	Jackal (black-backed)	*Canis mesomelas*	10	
	Family Felidae			152
	Cheetah	*Acinonyx jubatus*	3	
	Leopard	*Panthera pardus*	4	
	Lion	*Panthera leo*	145	
	Family Hyaenidae			78
	Spotted hyaena	*Crocuta crocuta*	78	
Order Artiodactyla	Family Bovidae			
	Subfamily Bovinae			31
	Buffalo	*Syncerus caffer*	25	
	Eland	*Taurotragus oryx*	6	
	Subfamily Alcelaphinae			86
	Hartebeest	*Alcephalus buselaphus*	11	
	Topi	*Damaliscus korrigum*	18	
	Wildebeest	*Connochaetes taurinus*	57	
	Subfamily Antilopinae			72
	Dikdik	*Rynchotragus kirkii*	6	
	Grant's gazelle	*Gazella granti*	21	
	Thomson's gazelle	*Gazella thomsoni*	45	
	Subfamily Reduncinae			26
	Impala	*Aepyceros melampus*	17	
	Reedbuck	*Redunca redunca*	3	
	Waterbuck	*Kobus ellipsiprymnus*	6	
	Family Giraffidae			11
	Giraffe	*Giraffa camelopardalis*	11	
	Family Suidae			37
	Warthog	*Phacochoerus africanus*	37	
Order Perissodactyla	Zebra	*Equus burchelli*	62	62
Order Primates	Olive baboon	*Papio anubis*	7	10
	Vervet monkey	*Cercopithecus aethiops*	3	
Order Rodentia	Rodents	*Mastomys natalensis,*	45	45
	Total			624

Species	Number sampled	Group total
Plains		24
Cheetah	3	
Grant's gazelle	21	
Mixed/Intermediate		562
Bat-eared fox	4	
Buffalo	25	
Eland	6	
Hartebeest	11	
Jackal (black-backed)	10	
Lion	145	
Olive baboon	7	
Reedbuck	3	
Rodents	45	
Spotted hyaena	78	
Thomson's gazelle	45	
Topi	18	
Vervet monkey	3	
Warthog	37	
Waterbuck	6	
Wildebeest	57	
Zebra	62	
Woodland		38
Dikdik	6	
Giraffe	11	
Impala	17	
Leopard	4	
Total		624

Table 4-3: Classification of species by habitat

Species were assigned to groups based on their habitat preferences. Plains species spend all or most of their time on grassland plains. Woodland species are found predominantly in woodland. Mixed or intermediate species are either found in both habitats, or spend most of their time in areas containing a combination of grassland plains and woodland.

Species	Number sampled	Group total
Carnivorous/omnivorous		251
Cheetah	3	
Bat-eared fox	4	
Jackal (black-backed)	10	
Leopard	4	
Lion	145	
Olive baboon	7	
Spotted hyaena	78	
Herbivorous		373
Buffalo	25	
Dikdik	6	
Eland	6	
Giraffe	11	
Grant's gazelle	21	
Hartebeest	11	
Impala	17	
Reedbuck	3	
Rodents	45	
Thomson's gazelle	45	
Topi	18	
Vervet monkey	3	
Warthog	37	
Waterbuck	6	
Wildebeest	57	
Zebra	62	
Total		624

Table 4-4: Classification by diet

Species were allocated to groups based on their dietary preferences. Species in which consumption of prey comprised all or part of their diet formed one group, indicating potential exposure to trypanosomes via consumption of prey species carrying trypanosome infections. The second group comprised herbivores where no such exposure could occur.

Species	Number sampled	Group total
G. swynnertoni		
High		73
Buffalo	25	
Giraffe	11	
Warthog	37	
Low		551
Bat-eared fox	4	
Cheetah	3	
Dikdik	6	
Eland	6	
Grant's gazelle	21	
Hartebeest	11	
Impala	17	
Jackal (black-backed)	10	
Leopard	4	
Lion	145	
Olive baboon	7	
Reedbuck	3	
Rodents	45	
Spotted hyaena	78	
Thomson's gazelle	45	
Topi	18	
Vervet monkey	3	
Waterbuck	6	
Wildebeest	57	
Zebra	62	
Total		624

Species	Number sampled	Group total
G.pallidipes		
High		62
Buffalo	25	
Warthog	37	
Low		562
Bat-eared fox	4	
Cheetah	3	
Dikdik	6	
Eland	6	
Giraffe	11	
Grant's gazelle	21	
Hartebeest	11	
Impala	17	
Jackal (black-backed)	10	
Leopard	4	
Lion	145	
Olive baboon	7	
Reedbuck	3	
Rodents	45	
Spotted hyaena	78	
Thomson's gazelle	45	
Topi	18	
Vervet monkey	3	
Waterbuck	6	
Wildebeest	57	
Zebra	62	
Total		624

Table 4-5: Classification of species by tsetse feeding preferences

Species were allocated to groups based on the feeding preferences of the two main tsetse species in Serengeti, *G. swynnertoni* and *G. pallidipes*. Numbers of samples in each group are indicated.

4.3.8 Individual level analysis: Univariate and bivariate analysis of risk factors

Only taxonomic groups which included at least one positive for either *T. brucei* s.l. or *T. congolense* were included in further analysis. Therefore Canidae, Giraffidae, Primates and Rodentia were not included in risk factor analysis. The effect of host species, sex, age, reason for sampling, length of time between death and sample collection and tsetse density on the presence of *T. brucei* s.l. and *T. congolense* were analysed. Logistic regression with binomial errors was used to calculate odds ratios and 95% confidence intervals. Odds ratios (OR) calculate the odds of disease in the group exposed to a risk factor compared to the non-exposed group (Dohoo *et al.*, 2003). OR over the value 1.0 indicate increased risk, whilst OR below 1.0 indicate reduced risk. However, if the confidence intervals of the OR span the value 1.0, the difference is not statistically significant. Likelihood ratio tests (LRTs) were used to assess the contribution of each variable. LRTs compare the likelihood of the model including the selected variables to a null model, and have an approximate χ^2 distribution (Dohoo *et al.*, 2003). LRTs assess the overall significance of a risk factor but do not assess the differences in prevalence between each level of the factor. Tukey post-hoc multicomparison tests, which consider all possible pairwise comparisons of mean prevalence, were therefore used to identify statistically significant differences in prevalence between factor levels.

Analysis was conducted for each risk factor individually (univariate analysis) then with species group included first in each model (bivariate analysis).

Response variable

Samples were designated as positive as described in section 4.3.6. Trypanosome infection status was analysed as a binary response variable (positive or negative), with separate models constructed for *T. brucei* s.l. and *T. congolense*.

Explanatory variables

- **Host species** were grouped according to the results of the tree model analysis. The choice of reference level for calculation of odds ratios was essentially arbitrary. Alcelaphinae was chosen since the number of samples in this group was high

(therefore giving more certain prevalence estimates) and the prevalence of *T. brucei* s.l. and *T. congolense* in the Alcelaphinae was not one of the extreme values, which could have resulted in odds rations that were very high or low.

- **Gender** was analysed as a factor with two levels – male and female.

- **Age** was analysed as a factor with four levels – juvenile, sub-adult, adult and old. Previous literature values are predominantly derived from adult animals so the adult group was used as the reference level for calculation of odds ratios.

- **Reason for sampling** an animal was analysed as an indicator of health status. The reasons given for sampling were grouped into seven categories (Table 4-6). Analysis was carried out as a factor with seven levels, and the routine samples were used as the reference level when calculating odds ratios.

Reason for sampling	Description
Routine sampling	Immobilised to put on, remove or adjust radiocollar or immobilised for disease surveillance
Killed by predator species	Killed by black backed jackal, golden jackal, cheetah, lion or unknown predator
Found dead	Found dead, not associated with road
Road traffic accident	Found dead, circumstances or post mortem examination results suggested hit by vehicle
Commercial hunting	Shot by hunting company, post mortem sample collected
Sick	Observed to be sick, immobilised and samples collected, or observed to be sick then euthanased, or observed to be sick then found dead
Snare removal	Live animals observed with snares and immobilised for snare to be removed

Table 4-6: Summary of reasons for sampling

For each sample collected, the reason that the animal was available for sampling was recorded. These reasons were grouped into seven categories for analysis.

- **Density of *G. swynnertoni* and *G. pallidipes*** The effect of tsetse density on prevalence of trypanosome infections was assessed using the predicted values for tsetse density generated in chapter 2. Analysis was performed using density of *G. swynnertoni* as a continuous variable in a generalised linear model with binomial errors, and repeated for *G. pallidipes*. Initial data inspection suggested that the relationship between tsetse density and prevalence may follow a quadratic trend. The relationship between tsetse density and prevalence is illustrated using kernel smoothing lines. Linear and quadratic functions were fitted and the model fit assessed. Analysis was also performed using logged values of tsetse density. This did not significantly affect the results so only the simpler analysis is reported.

4.3.9 Multivariate analysis

Variables were selected for inclusion in a multivariate model on the basis of the p value generated by likelihood ratio tests (LRT) of the univariate analysis (for species group) and bivariate analysis (for all other variables), using a liberal p value of 0.15 to reduce the risk of accidentally eliminating important variables (Dohoo *et al.*, 2003). Stepwise regression was carried out to eliminate any variables that did not remain significant in the multivariate model. Interactions between variables were assessed but limited by the small sample size of some groups. Code used to generate logistic regression models in R is shown below: (a) univariate analysis with explanatory variable of species group; (b) bivariate analysis with explanatory variable of age, and species group also included; (c) multivariate model with all significant variables included.

```
(a)modelspecies<-glm(congopos~speciesgrouped,
data=wildlife, family=binomial)

(b)modelagespecies<-glm(congopos~speciesgrouped+age,
data=wildlife, family=binomial)

(c)modelmulti<-glm(congopos~speciesgrouped+age+tsetse,
data=wildlife, family=binomial)
```

4.4 Results

4.4.1 Summary of data

Laboratory analyses were carried out on 641 samples. This included 495 samples collected during this study and 146 archived samples. Maps to show the distribution of sample collection can be seen in Figure 4-2 to Figure 4-4. The maps show the distribution of sample sites compared to vegetation, the distribution of samples that tested positive, and the distribution of samples collected from each species.

Samples were obtained from 31 species. The number of samples and the prevalence of *T. brucei s.l.* and *T. congolense* in each species are summarised in Table 4-7. The largest numbers of samples were collected from lion (n=145) and hyaena (n=78). Species where two or fewer samples were collected were excluded from further analysis. Of these, it is worth noting that samples from bushbuck (n=2) were both positive for both *T. brucei* s.l. and *T. congolense*.

Samples were predominantly collected from SNP and Maswa and Grumeti Game Reserves. The large number of samples from lions within the Serengeti Lion Project study area (in the south east of the SNP, broadly covering the grassland plains) is evident. Broadly speaking, samples testing positive for *T. brucei* s.l. and *T. congolense* are widely distributed and occur in all areas samples were collected from. *T. b. rhodesiense* positive samples were located on the grassland plains and in the west of the park.

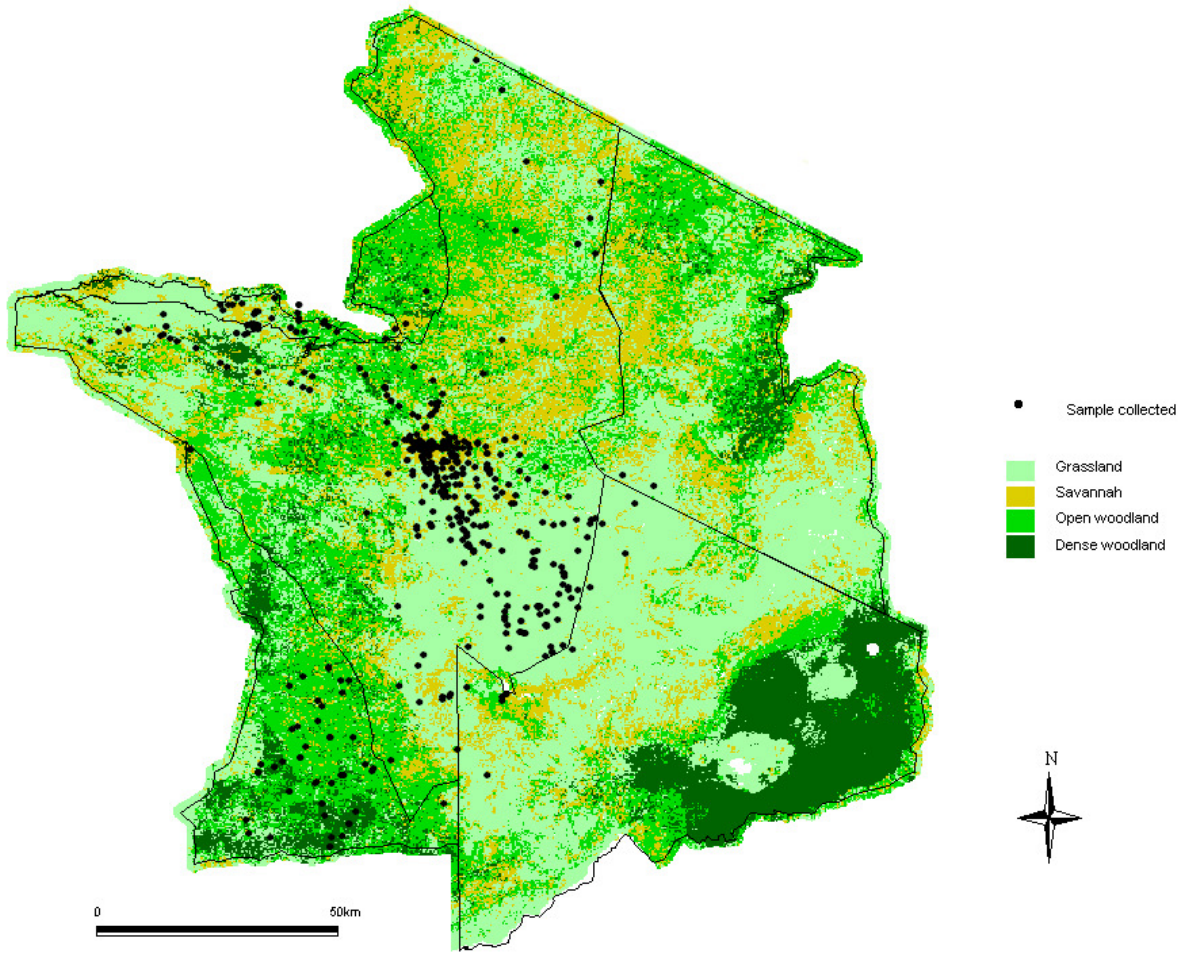


Figure 4-2: Distribution of sample sites and associated vegetation type in Serengeti

Samples were collected from wildlife species in the Serengeti ecosystem. The locations of all samples for which sample location was recorded are indicated (n=545). The distribution of predominant vegetation types is shown.

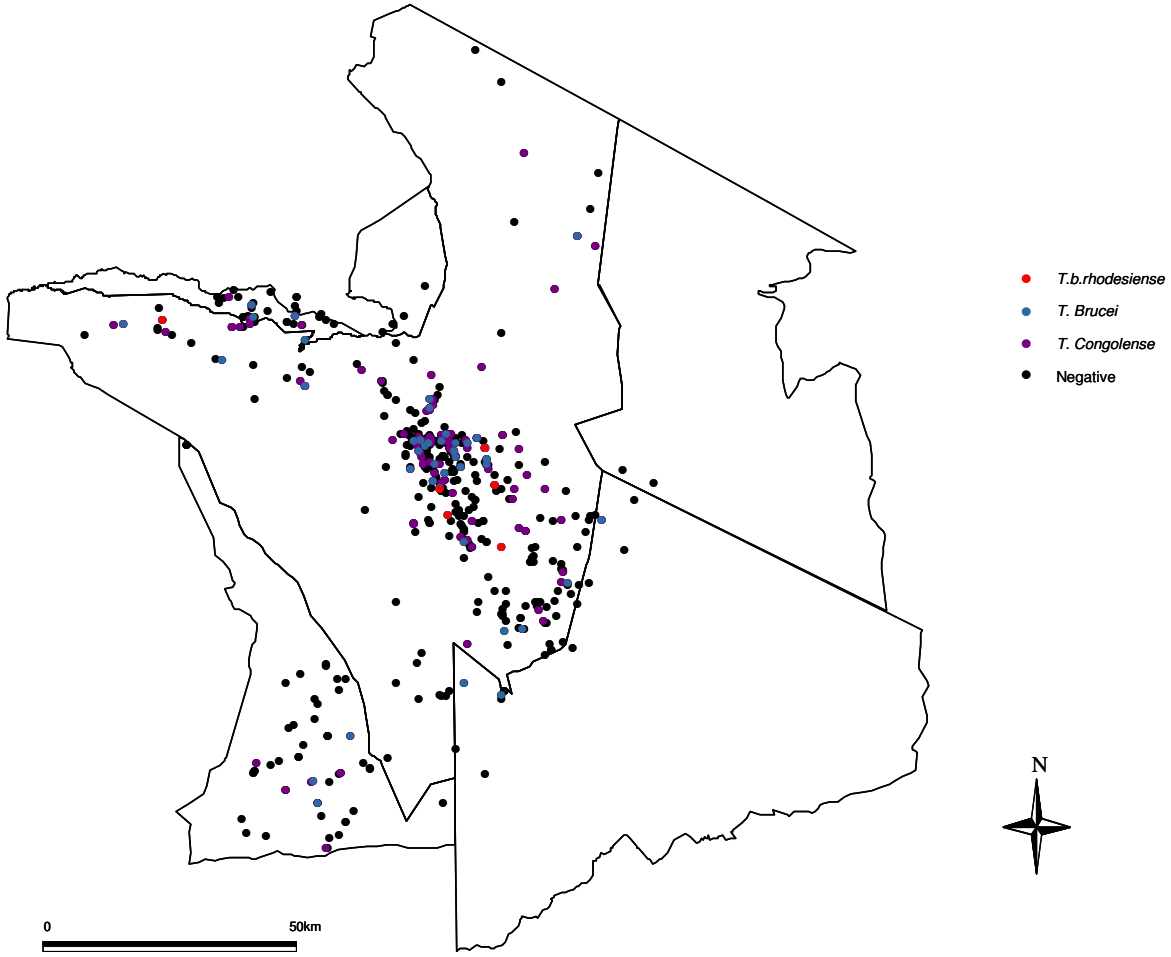


Figure 4-3: Map showing spatial distribution of samples by trypanosome species identified

Of the samples for which location was recorded, the distribution of samples testing positive by PCR for *T. b. rhodesiense* (n=6), *T. brucei* (excluding those positive for *T. b. rhodesiense*) (n=4) and *T. congolense* (n=116) is shown.

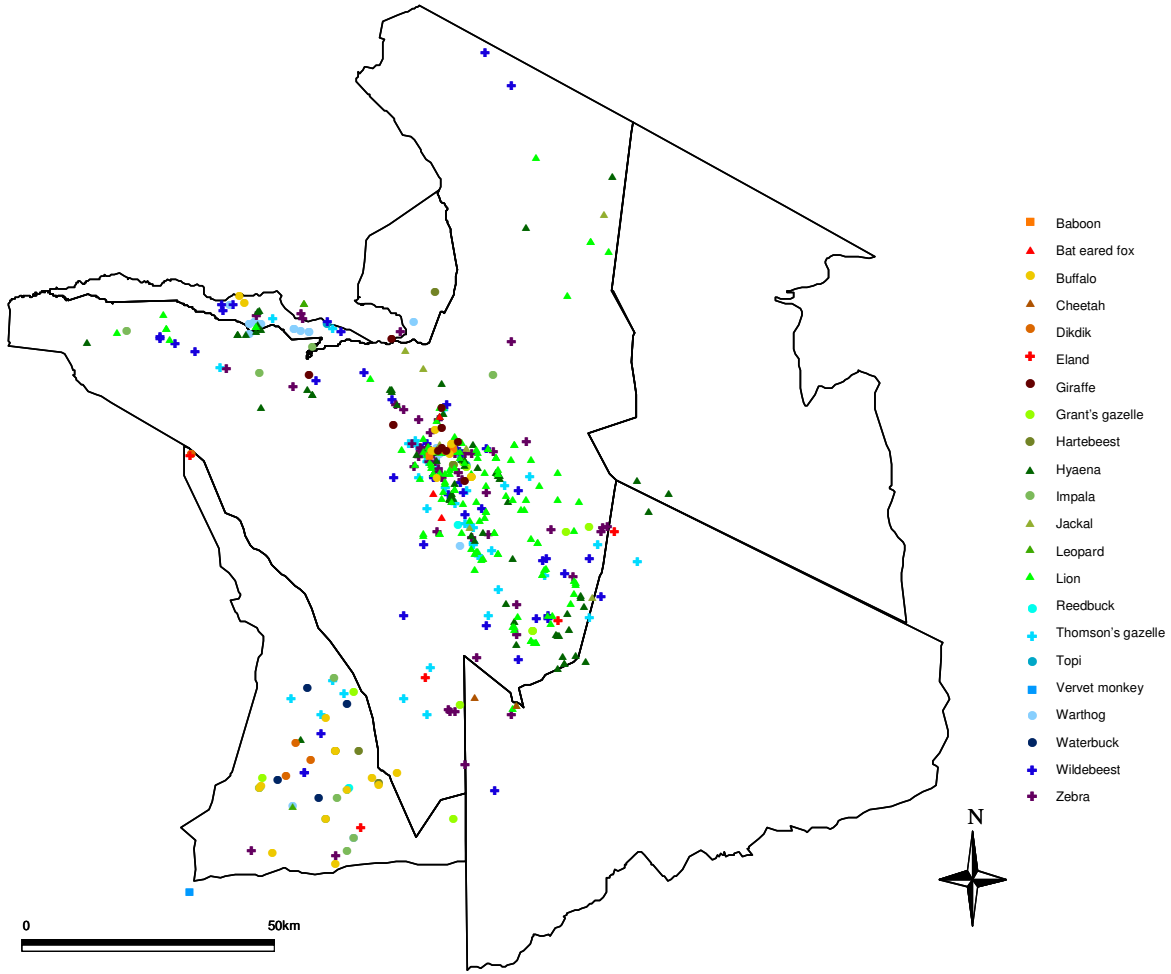


Figure 4-4: Map showing spatial distribution of samples by wildlife host species

The wildlife species of each sample is shown.

Species	Number sampled	Number positive forTb	Prevalence Tb % (CI)	Number positive for Tc	Prevalence Tc % (CI)
Aardwolf	1	0	0 (0-98)	0	0 (0-98)
Baboon	7	0	0 (0-41)	0	0 (0-41)
Bat eared fox	4	0	0 (0-60)	0	0 (0-60)
Buffalo	25	1	4 (0.1-20)	3	12 (2.5-31)
Bushbuck	2	2	100 (16-100)	2	100 (16-100)
Cheetah	3	1	33 (0.8-91)	0	0 (0-71)
Civet	2	0	0 (0-84)	0	0 (0-84)
Dikdik	6	0	0 (0-46)	0	0 (0-46)
Duiker	1	0	0 (0-98)	0	0 (0-98)
Eland	6	1	17 (0.4-64)	2	33 (4.3-78)
Elephant	1	0	0 (0-98)	0	0 (0.98)
Genet	1	0	0 (0-98)	0	0 (0-98)
Giraffe	11	0	0 (0-29)	0	0 (0-29)
Grants gazelle	21	1	5 (0.1-24)	1	5 (0.1-24)
Greater kudu	1	0	0 (0-98)	1	100 (2-100)
Hare	1	0	0 (0-98)	0	0 (0-98)
Hartebeest	11	2	18 (2.3-52)	0	0 (0-29)
Hyaena	78	10	13 (6.3-22)	20	26 (16-37)
Hyrax (tree)	1	0	0 (0-98)	0	0 (0-98)
Impala	17	4	24 (6.8-50)	2	12 (1.5-36)
Jackal (black backed)	10	0	0 (0-31)	0	0 (0-31)
Jackal (golden)	2	0	0 (0-84)	0	0 (0-84)
Leopard	4	0	0 (0-60)	0	0 (0-60)
Lion	145	21	14 (9.2-21)	77	53 (45-61)
Mongoose (white tailed)	1	0	0 (0-98)	0	0 (0-98)
Mongoose (banded)	1	0	0 (0-98)	0	0 (0-98)
Reedbuck	3	1	33 (0.8-91)	0	0 (0-71)
Roan antelope	1	0	0 (0-98)	0	0 (0-98)
Rodents	45	0	0 (0-7.9)	0	0 (0-7.9)
Serval	1	0	0 (0-98)	0	0 (0-98)
Thomsons gazelle	45	1	2 (0.06-12)	2	4 (0.5-15)
Topi	18	1	6 (0.1-27)	0	0 (0-19)
Vervet monkey	3	0	0 (0-71)	0	0 (0-71)
Warthog	37	2	5 (0.7-18)	2	5 (0.7-18)
Waterbuck	6	0	0 (0-46)	2	33 (4.3-78)
Wildebeest	57	0	0 (0-6)	2	4 (4.3-12)
Zebra	62	2	3 (3.9-11)	4	6 (1.8-16)
Total	641	50	7.8 (5.8-10.2)	120	18.7 (15.8-22.0)

Table 4-7: Prevalence of *T. brucei* s.l. and *T. congolense* in each species of wildlife

All wildlife samples were analysed by PCR for the presence of *T. brucei* and *T. congolense*. The number of samples testing positive and the prevalence (with exact binomial 95% confidence intervals (CI)) of *T. brucei* (Tb) and *T. congolense* (Tc) is shown for each wildlife species.

4.4.2 Assessment of criteria for categorizing species

Tree models were used to assess four potential sets of criteria for categorisation of species for further analysis (taxonomy, habitat, predominant food source and tsetse feeding preferences) (Figure 4-5 and Figure 4-6).

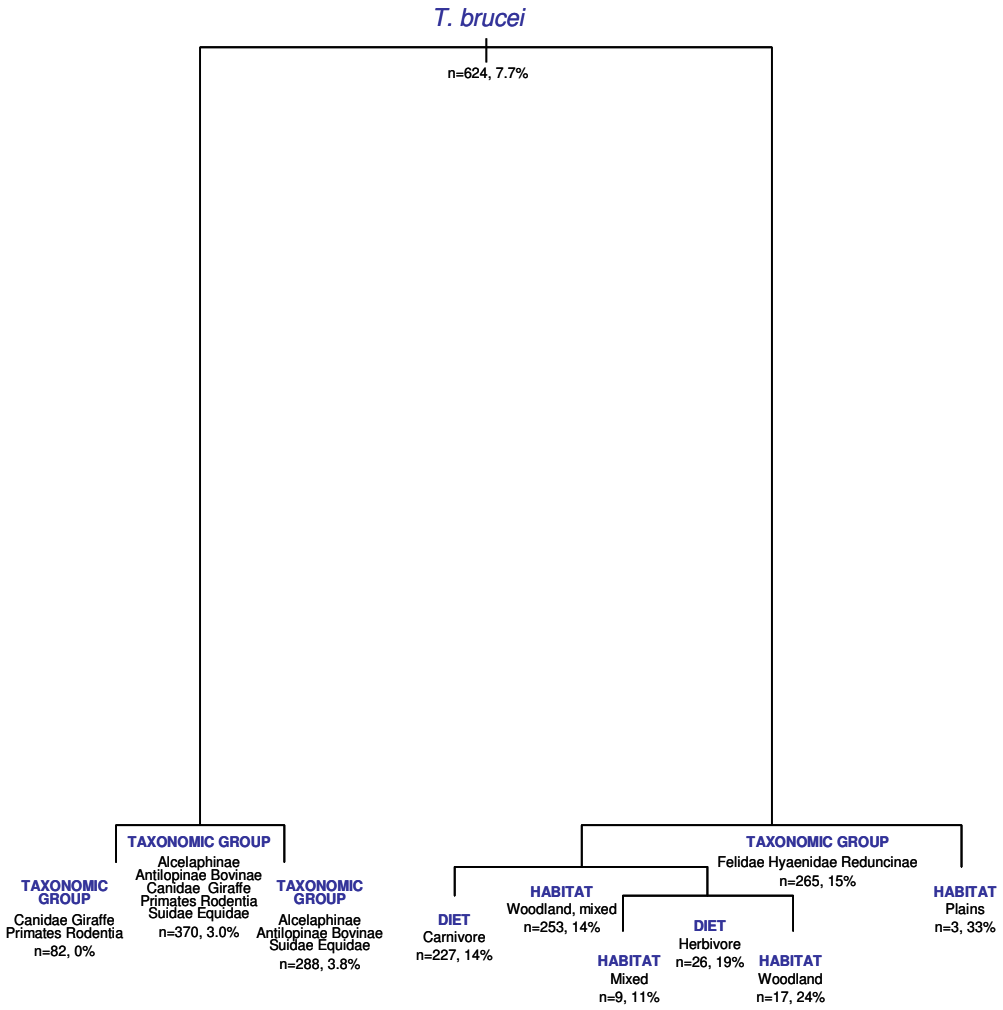


Figure 4-5: Tree model for *T. brucei* s.l.

Tree model shows the analysis of the prevalence of *T. brucei* s.l. in each wildlife species, with explanatory variables of taxonomic group, habitat, diet and tsetse feeding preferences. Number of samples (n) and prevalence (%) are shown for each node.

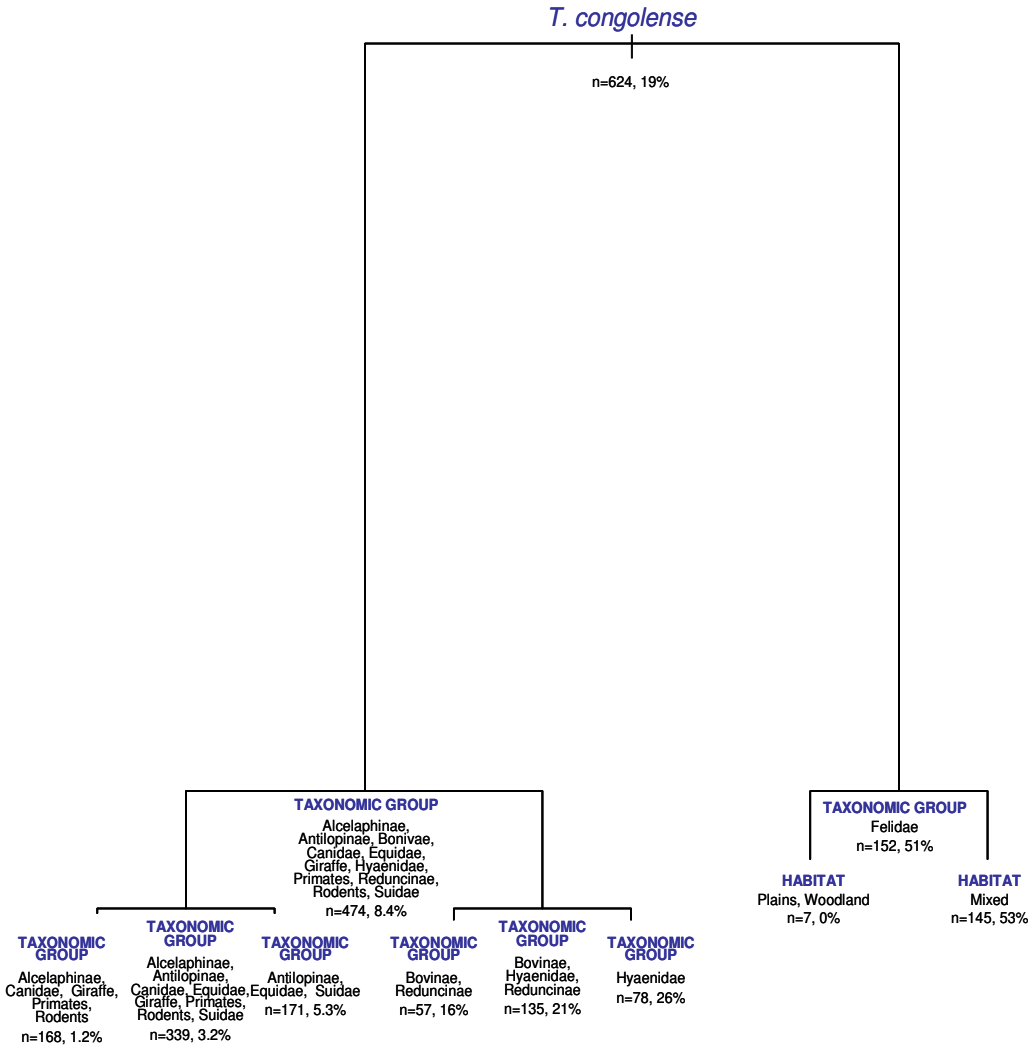


Figure 4-6: Tree model for *T. congolense*

Tree model shows the analysis of the prevalence of *T. congolense* in each wildlife species, with explanatory variables of taxonomic group, habitat, diet and tsetse feeding preferences. Number of samples (n) and prevalence (%) are shown for each node.

Most of the variance can be explained by taxonomic classification with the first level of binary partition depending on taxonomy. For *T. brucei* s.l.., Felidae, Hyaenidae and Reduncinae separate from the other species. Within this branch, diet and habitat then become important, indicating that taxonomic group does not solely explain the variance of the model. Similarly for *T. congolense*, all branches divide by taxonomic group except for within the Felidae, the group with highest prevalence, where habitat becomes important. Therefore, for further analysis, species were grouped according to taxonomic group.

4.4.3 Risk factors at an individual level: Taxonomic group

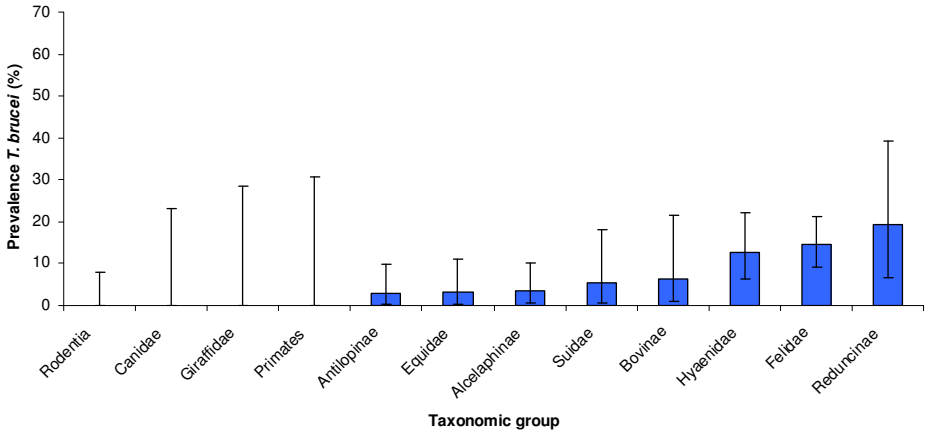
The numbers of species and samples included in the taxonomic groups were listed in Table 4-2. The prevalence of *T. brucei* s.l. and *T. congolense* by taxonomic group is shown in Figure 4-7. Results of univariate analysis of taxonomic group on the prevalence of *T. brucei* s.l. and *T. congolense* are shown in Table 4-8. The prevalence of *T. brucei* s.l. was significantly higher in Felidae, Hyaenidae and Reduncinae compared to Alcelaphinae. The prevalence of *T. congolense* was significantly higher in Felidae, Hyaenidae, Bovinae and Reduncinae compared to Alcelaphinae.

	Taxonomic group	LRT	p- value	OR	CI
T. brucei		21.4	**0.003**		
	Alcelaphinae			NA	
	Antilopinae			0.79	0.13-4.9
	Felidae			4.68	1.4-16.1
	Hyaenidae			4.07	1.08-15.4
	Bovinae			1.91	0.3-12.0
	Reduncinae			6.59	1.46-29.8
	Suidae			1.58	0.25-9.88
	Equidae			0.92	0.15-5.69
T. congolense		128	**<0.001**		
	Alcelaphinae			NA	
	Antilopinae			1.83	0.3-11.2
	Felidae			43.1	10.2-182
	Hyaenidae			14.4	3.26-64.4
	Bovinae			8.08	1.48-44.1
	Reduncinae			7.64	1.31-44.4
	Suidae			2.4	0.33-17.7
	Equidae			2.9	0.51-16.3

Table 4-8: Summary of analysis of the effect of taxonomic group on prevalence of *T. brucei* s.l. and *T. congolense*

The effect of taxonomic group on the prevalence of *T. brucei* s.l. and *T. congolense* was assessed using univariate logistic regression with binomial errors. Likelihood ratio tests (LRT), p-values, odds ratios (OR) and 95% confidence intervals (CI) are shown. Odds ratios were calculated compared to the reference level, Alcelaphinae.

(a)



(b)

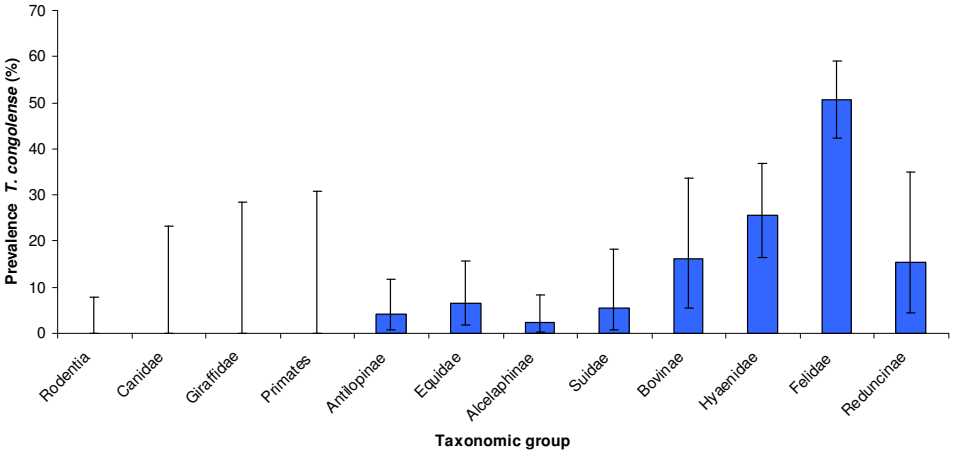


Figure 4-7: Prevalence of (a) *T. brucei* s.l. and (b) *T. congolense* by taxonomic group

Error bars show exact binomial 95% confidence intervals.

4.4.4 Risk factors at an individual level: Sex

The distribution of samples between the sexes by taxonomic group is shown in Figure 4-8. Females are overrepresented in the Felidae family. This is due to the large number of samples obtained from the Serengeti Lion Project, who immobilise lions to fit radio collars. Radio collars are predominantly fitted to female lions to enable reliable location of each pride. The relatively high proportion of samples from male animals of the Alcelaphinae, Antilopinae, Bovinae and Reduncinae sub families is due to samples collected during commercial hunting operations, which target males only.

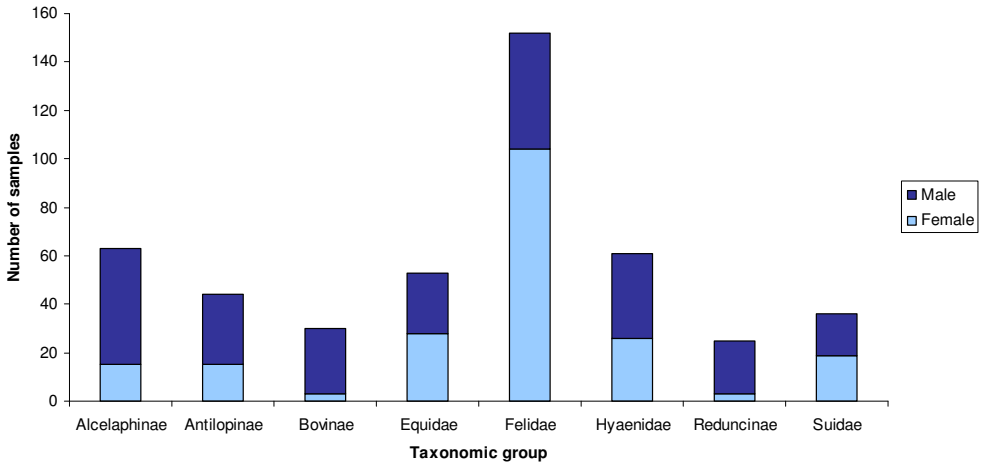


Figure 4-8: Distribution of samples by sex and taxonomic group

The proportion of samples from male and female animals varies with taxonomic group, with sample bias associated with the reasons for sampling, such as hunting of male animals only.

In univariate analysis the prevalence of trypanosome infections was statistically significantly lower in males compared to females for *T. congolense*, but not for *T. brucei* s.l.. When taxonomic group was included in the model, the difference in *T. congolense* prevalence between the sexes was no longer significant confirming that it was confounded by taxonomic group (Table 4-9). There was no evidence of a significant interaction between sex and taxonomic group. Univariate analysis was repeated excluding Felidae, since the large

number of samples from female lions had the potential to cause confounding. The difference in *T. congolense* prevalence between sexes were no longer significant (χ^2_1=0.72, p=0.40), and there was no statistically significant difference in *T. congolense* prevalence between sexes within Felidae (χ^2_1=0.012, p=0.91), confirming that the difference in prevalence which initially appeared to be significant was due only to the confounding effect of many female lions.

	Univariate (sex)				Bivariate (tax. group and sex)			
	LRT	***p-value***	***OR***	***CI***	***LRT***	***p-value***	***OR***	***CI***
T. brucei	0.12	0.73			0.46	0.5		
Female			NA					NA
Male			0.89	0.5-1.7			1.3	0.6-2.6
T.congolense	8.2	**0.004**			0.42	0.52		
Female			NA					NA
Male			0.54	0.4-0.8			2.1	0.7-2.0

Table 4-9: Summary of analysis of the effect of sex on prevalence of *T. brucei* s.l. and *T. congolense*

The effect of sex on the prevalence of *T. brucei* s.l. and *T. congolense* was assessed using logistic regression with binomial errors, with univariate analysis of the effect of sex only, and bivariate analysis which also included taxonomic group in the model. Likelihood ratio tests (LRT), p-values, odds ratios (OR) and 95% confidence intervals (CI) are shown. Odds ratios were calculated compared to the reference level, female.

4.4.5 Risk factors at an individual level: Age

The distribution of samples between age categories is shown in Figure 4-9. The majority of samples were taken from adult animals (63%) regardless of taxonomic group.

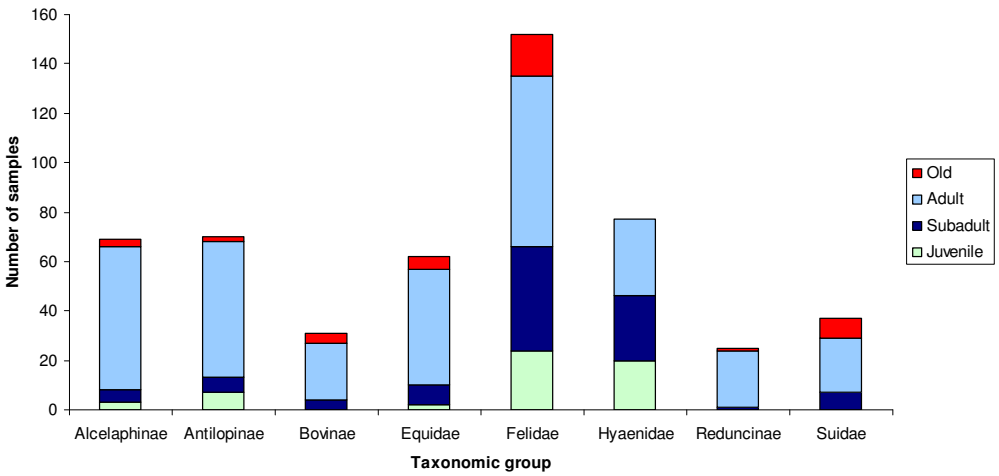


Figure 4-9: Distribution of samples by age category and taxonomic group

Each animal sampled was classified as old, adult, sub adult or juvenile. Age distribution of samples collected varied between taxonomic groups.

The prevalence of trypanosome infections differed significantly between age groups for *T. brucei* s.l.. Analysis by post-hoc multiple comparisons showed prevalence to be significantly higher in sub adults compared to adults ($p<0.001$) and juveniles ($p=0.050$). There was also a significant difference between age categories for *T. congolense* with prevalence higher in sub adults compared to adults ($p<0.001$) and juveniles ($p=0.012$) on post hoc analysis. If taxonomic group was also included in the model, the difference in prevalence between age groups remained significant for both *T. brucei* s.l. and *T. congolense*. This analysis is summarised in Table 4-10.

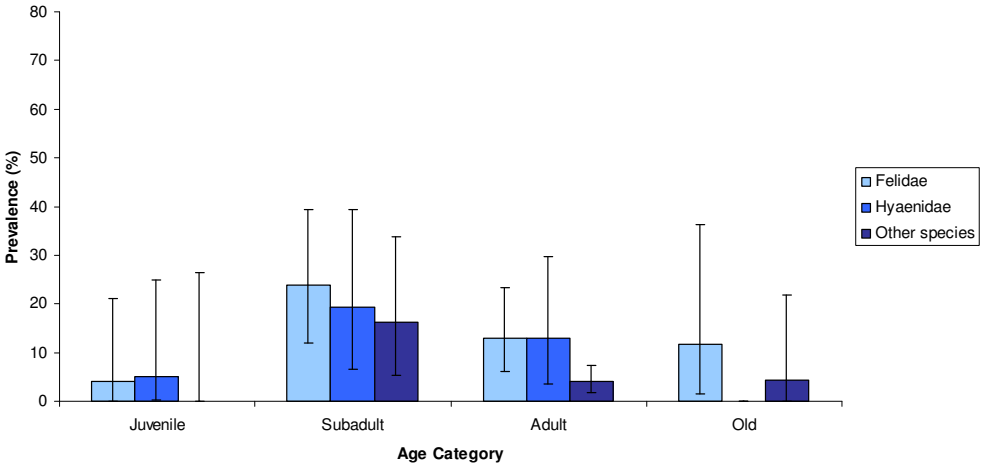
		Univariate (age)				Bivariate (tax. group and age)			
		LRT	***p-value***	***OR***	***CI***	***LRT***	***p-value***	***OR***	***CI***
T. brucei		16.6	**<0.001**			13.07	**0.004**		
	Juvenile			0.5	0.1-2.3			0.36	0.08-1.7
	Sub adult			3.5	1.8-6.8			2.7	1.3-5.5
	Adult			NA				NA	
	Old			1.1	0.3-4.0			1.01	0.27-3.7
T.congolense		26	**<0.001**			14.9	**0.002**		
	Juvenile			1.1	0.5-2.2			0.45	0.2-1.0
	Sub adult			3.6	2.2-5.9			2.1	1.2-3.7
	Adult			NA				NA	
	Old			1.4	0.6-3.1			0.84	0.34-2.1

Table 4-10: Summary of analysis of the effect of age on prevalence

The effect of age on the prevalence of *T. brucei* s.l. and *T. congolense* was assessed using logistic regression with binomial errors, with univariate analysis of age only, and bivariate analysis which also included taxonomic group in the model. Likelihood ratio tests (LRT), p-values, odds ratios (OR) and 95% confidence intervals (CI) are shown. Odds ratios were calculated compared to the reference level, adult.

Analysis of interactions was precluded by insufficient sample numbers in each category. However, separate models were constructed for Felidae, Hyaenidae and other species to examine the consistency of these finding across taxonomic groups. Prevalence of *T. brucei* s.l. and *T. congolense* for Felidae, Hyaenidae and other species are shown in Figure 4-10. In the model containing all taxonomic groups except Felidae and Hyaenidae, the difference in prevalence between age categories was no longer statistically significant (juveniles excluded since no positive juveniles in other species) (*T. brucei* χ^2_2=7.05, p=0.07, *T. congolense* χ^2_2=2.19, p=0.53). Univariate analysis of prevalence with age in Felidae using the same groups showed the prevalence of *T. congolense* to differ significantly with age (χ^2_3=8.0, p=0.046) but not *T. brucei* s.l. (χ^2_2=5.5, p=0.14). Age was also a significant factor for *T. congolense* prevalence in hyaenas (χ^2_2=6.7, p=0.036), though not for *T. brucei* s.l. (χ^2_2=2.2, p=0.33) (old group excluded because there were no hyaenas in this category).

(a)



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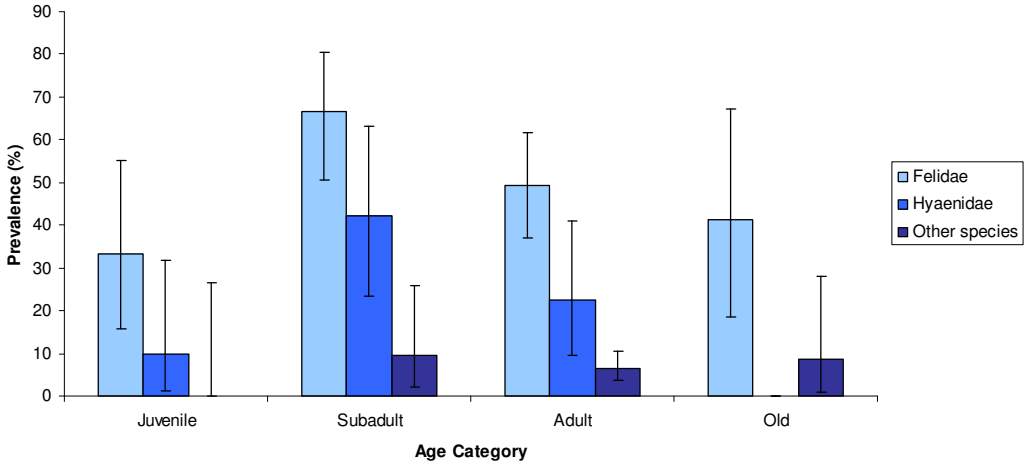


Figure 4-10: Prevalence of (a) *T. brucei* s.l. and (b) *T. congolense* by age category for Felidae, Hyaenidae and other species

Error bars are exact binomial 95% confidence intervals.

Analysis of age prevalence in lions

Out of a total of 145 samples collected from lions, 108 samples came from Serengeti Lion Project study animals, where the exact ages of each animal were known (+/- one month). The graphs below show the prevalence of trypanosome infections in these animals only. Overall the prevalence of *T. brucei* s.l. and *T. congolense* peaks between two and four years, then declines in older lions. However if lions found on the plains and in the woodlands are considered separately the relationship is less clear. In plains lions, the prevalence of *T. congolense* is highest at six to seven years before declining, where as the prevalence of *T. brucei* s.l. peaks between two and three years. In contrast in woodland lions, peak prevalence is seen between three and four years for *T. congolense*.

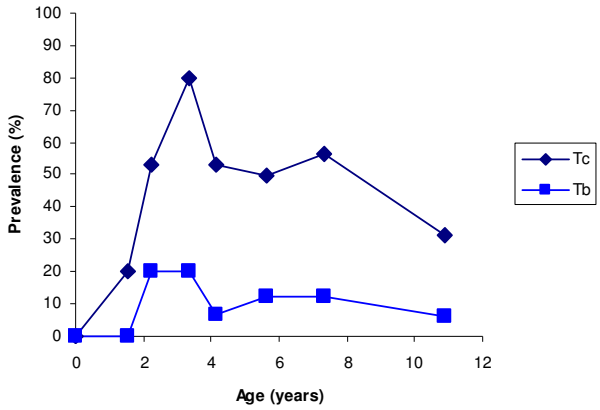


Figure 4-11: Prevalence of *T. brucei* s.l. and *T. congolense* with age in lions

The graph shows the prevalence of *T. brucei* s.l. (Tb) and *T. congolense* (Tc) for all lions with known ages (n=108), divided into seven age categories of equal sample size.

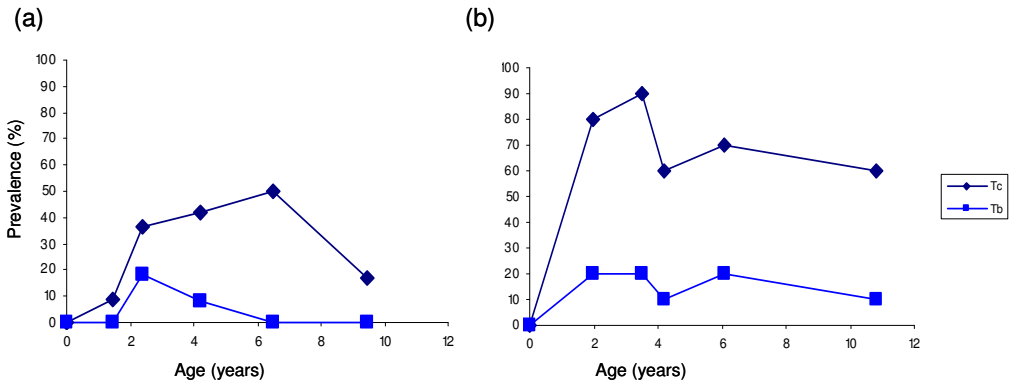


Figure 4-12: Prevalence of *T. brucei* s.l. and *T. congolense* with age in lions, (a) on the plains and (b) in the woodlands

The graphs show the prevalence of *T. brucei* s.l. (Tb) and *T. congolense* (Tc) for all lions with known ages living predominantly on the grassland plains ((a), n=58) or in the woodland areas ((b), n=50), divided into five age categories of equal sample size.

4.4.6 Risk factors at an individual level: Reason for Sampling

The distribution of reasons for sampling by taxonomic group is shown in Figure 4-13. Whilst some routine samples were obtained from all taxonomic groups, for the Alcelaphinae, Felidae, Hyaenidae and Suidae routine sampling predominated, because these groups include species which are frequently immobilised. In particular, the number of routine samples from lions was very high. Samples obtained from commercial hunting companies were mainly from Alcelaphinae, Antilopinae, Bovinae and Reduncinae. Samples were obtained from Alcelaphinae, Antilopinae and Equidae killed by predators. Small numbers of samples came from animals immobilised for snare removal, sick animals, and animals found dead.

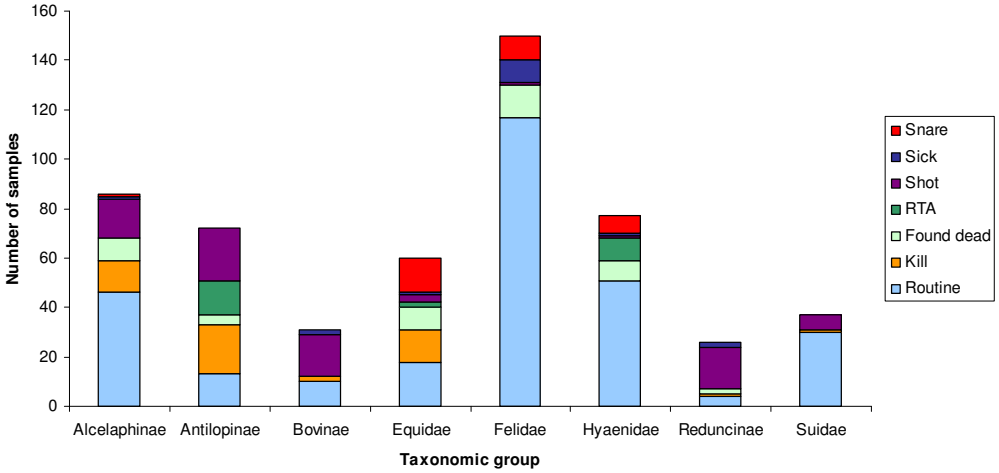


Figure 4-13: Distribution of reasons for sampling by taxonomic group

For each animal sampled, the reason for sampling was recorded. The graph shows the number of samples in each category for each taxonomic group.

On univariate analysis there were no significant differences in the prevalence of *T. brucei* s.l., but the prevalence of *T. congolense* was significantly different between groups. When taxonomic group was also included in the model, the difference in prevalence between the reasons for sampling was not significant for *T. brucei* s.l. or for *T. congolense* ($p>0.55$). The p-values and odds ratios are listed in Table 4-11.

	Univariate (reason)				Bivariate (tax. group and reason)			
	LRT	***p-value***	***OR***	***CI***	***LRT***	***p-value***	***OR***	***CI***
T. brucei	3.44	0.75			4.96	0.55		
Routine			NA				NA	
Found dead			0.6	0.2-2.2			0.73	0.2-2.6
Killed by predators			0.8	0.3-2.3			2.8	0.7-11
Road traffic accident			0.8	0.2-3.5			1.5	0.3-7.7
Commerical hunting			0.5	0.2-1.4			0.49	0.1-2.0
Sick			1.3	0.3-5.9			0.9	0.2-4.4
Snare removal			1.3	0.4-3.9			1.6	0.5-5.2
T.congolense	30.66	**<0.001**			2.43	0.88		
Routine			NA				NA	
Found dead			0.5	0.2-1.1			0.55	0.21-1.4
Killed by predators			0.2	0.1-0.5			1.2	0.28-4.71
Road traffic accident			0.5	0.2-1.4			1.4	0.4-5.09
Commerical hunting			0.2	0.1-0.5			0.77	0.25-2.36
Sick			1.5	0.5-4.3			1.1	0.33-3.38
Snare removal			0.8	0.4-2.0			0.98	0.37-2.58

Table 4-11: Summary of analysis of the effect of reason for sampling on the prevalence of *T. brucei* s.l. and *T. congolense*

The effect of the reason for sampling on the prevalence of *T. brucei* s.l. and *T. congolense* was assessed using logistic regression with binomial errors, with univariate analysis of reason for sampling only, and bivariate analysis which also included taxonomic group in the model. Likelihood ratio tests (LRT), p-values, odds ratios (OR) and 95% confidence intervals (CI) are shown. Odds ratios were calculated compared to the reference level, routine sampling.

4.4.7 Risk factors at an individual level: Status of animal/carcass at time of sampling

The distribution of samples collected by time of sampling for each taxonomic group are shown in Figure 4-14. For the Alcelaphinae, Equidae, Felidae, Hyaenidae and Suidae, the majority of samples were collected from live animals. These represent the species which are commonly immobilised in SME, as shown in Figure 4-13. Antilopinae, Bovinae and Reduncinae comprised many animals shot for commercial hunting, which were sampled

soon after death. The number of samples collected more than 4 hours after death was relatively small.

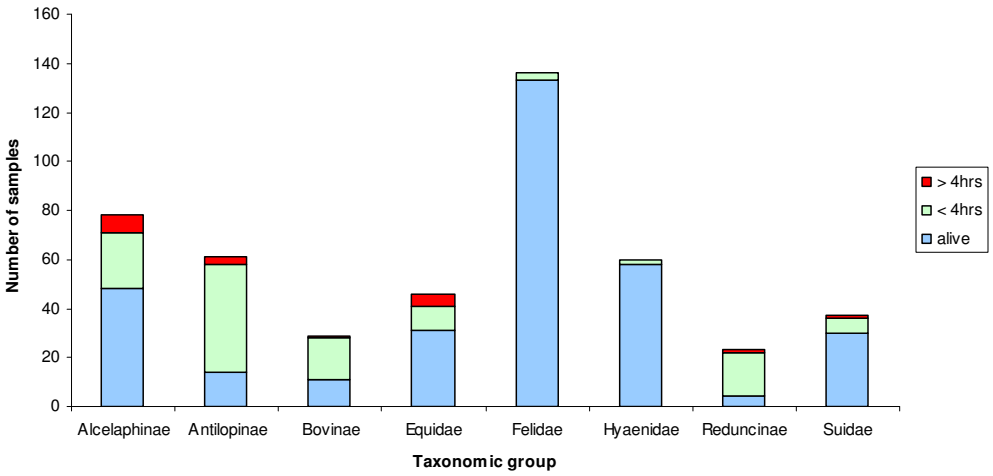


Figure 4-14: Distribution of samples by time of sampling and taxonomic group

For each sample, it was recorded whether the animal was alive, had died less than four hours ago, or had died more than four hours ago. The graph shows the number of samples in each category for each taxonomic group.

Univariate analysis showed the prevalence of trypanosome infections to be significantly different between samples from animals live at the time of sampling and different time points after death for *T. congolense*, although not for *T. brucei* s.l. . When taxonomic group was also included in the model, there were no statistically significant differences in prevalence for *T. brucei* s.l. or *T. congolense* ($p>0.95$) (Table 4-12).

	Univariate (carcass)				Bivariate (tax. group and carcass)			
	LRT	***p-value***	***OR***	***CI***	***LRT***	***p-value***	***OR***	***CI***
T. brucei	2.54	0.28			0.1	0.95		
Alive			NA				NA	
Less than four hours			0.5	0.2-1.3			1.1	0.35-3.7
Over four hours			0.5	0.1-4.1			1.4	0.15-12
T. congolense	26.31	**<0.001**			0.05	0.98		
Alive			NA				NA	
Less than four hours			0.3	0.1-0.5			1.1	0.42-3.0
Over four hours			0.2	0.02-1.1			1.1	0.21-9.5

Table 4-12: Summary of analysis of the effect of the status of animal/carcass on the prevalence of *T. brucei* s.l. and *T. congolense*

The effect of status of the animal or carcass on the prevalence of *T. brucei* s.l. and *T. congolense* was assessed using logistic regression with binomial errors, with univariate analysis of status of the animal/carcass only, and bivariate analysis which also included taxonomic group in the model. Likelihood ratio tests (LRT), p-values, odds ratios (OR) and 95% confidence intervals (CI) are shown. Odds ratios were calculated compared to the reference level, alive.

4.4.8 Risk factors at an individual level: Tsetse density

The density of *G. swynnertoni* and *G. pallidipes* at the location where each animal was sampled was obtained from the predicted tsetse density maps in Chapter 2. Tsetse density was analysed as a continuous variable. Distribution of tsetse density by taxonomic group is shown in Figure 4-15. For *G. swynnertoni*, the mean density of tsetse at sample sites is lowest for Felidae and Hyaenidae (because many were sampled on the grassland plains). Suidae and Equidae were sampled in areas of highest *G. swynnertoni* density. For *G. pallidipes*, most species were found at moderate densities. Again, the density of *G. pallidipes* was low for Felidae and Hyaenidae. The high density shown for the Reduncinae illustrates the different habitat requirements of the two tsetse species. Reduncinae are found in thickets and woodland, where *G. pallidipes* predominates, whilst the density of *G. swynnertoni* is moderate.

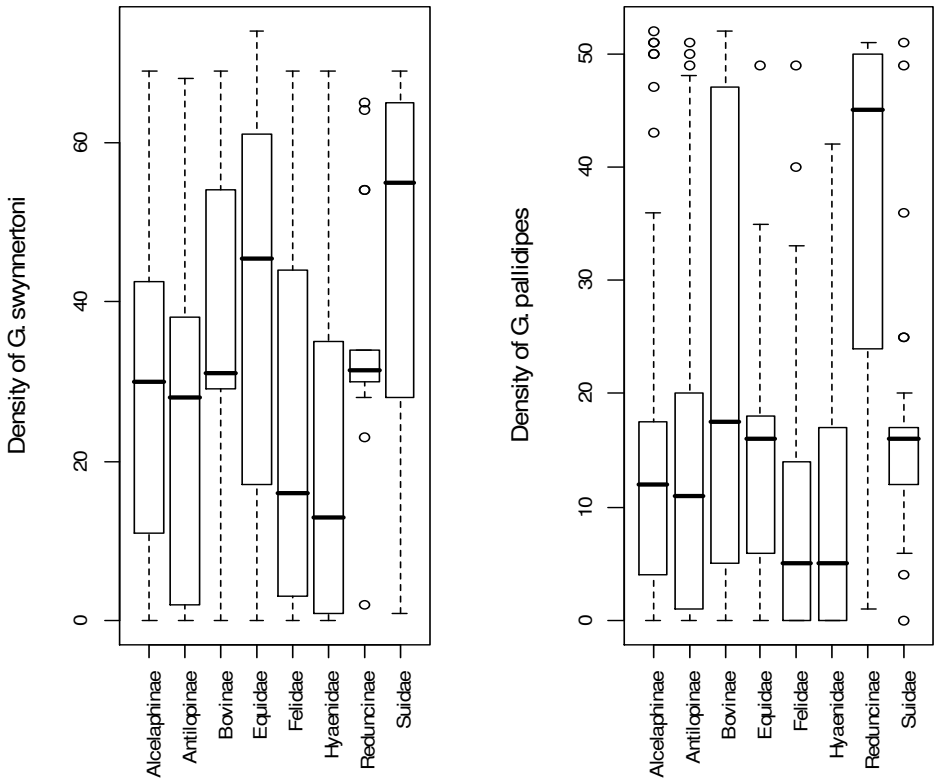


Figure 4-15: Density of *G. swynnertoni* and *G. pallidipes* by taxonomic group

The density of *G. swynnertoni* and *G. pallidipes* was estimated using the predicted values generated by TsetsePlan in section 2.7 for the location that each sample was collected. Box and whisker plots indicate tsetse density at which samples from each taxonomic group were collected, and show median, interquartile range, minimum and maximum values and outliers (outside 1.5 times interquartile range).

Univariate analysis of tsetse density showed no significant relationship between prevalence of *T. brucei* s.l. and the density of *G. swynnertoni* or *G. pallidipes*, with either the linear or quadratic expression. The prevalence of *T. congolense* showed a significant linear increase with the density of *G. swynnertoni*, which was not improved by inclusion of a quadratic term. However with *G. pallidipes*, the quadratic term was most significant.

Inclusion of taxonomic group improved the fit of the model. For *T. brucei* s.l., the relationship with the density of *G. pallidipes* became significant with the quadratic term. For *T. congolense*, the relationship with *G. swynnertoni* remained as before, with the linear term

only significant, while for *G. pallidipes* both linear and quadratic terms were significant. A summary of analysis with and without inclusion of taxonomic group is shown in Table 4-13.

	Univariate (tsetse density)		**Bivariate (tax. group and tsetse density)**	
	LRT	***p-value***	***LRT***	***p-value***
T. brucei				
Density of *Gs*	0.32	0.58	1.46	0.23
Density of *Gs* plus quadratic term	0.19	0.67	0.22	0.64
Density of *Gp*	0.69	0.55	0.92	0.34
Density of *Gp* plus quadratic term	1.1	0.29	4.34	**0.04**
T. congolense				
Density of *Gs*	8.96	**0.003**	32.19	**<0.001**
Density of *Gs* plus quadratic term	0.51	0.48	3.63	0.06
Density of *Gp*	0.22	0.64	9.89	**0.002**
Density of *Gp* plus quadratic term	14.39	**<0.001**	23.45	**<0.001**

Table 4-13: Summary of analysis of the effect of tsetse density on the prevalence of *T. brucei* s.l. and *T. congolense*

The effect of the density of *G. swynnertoni* (Gs) and *G. pallidipes* (Gp) on the prevalence of *T. brucei* s.l. and *T. congolense* was assessed using logistic regression with binomial errors, with univariate analysis of tsetse density only, and bivariate analysis which also included taxonomic group in the model. The models were also carried out with a quadratic term included. Likelihood ratio tests (LRT) and p-values are shown.

To explore this result of significant quadratic relationships between trypanosome prevalence and density of *G. pallidipes*, the data was analysed separately for Felidae, Hyaenidae and other species, since the large number of samples in the Felidae and Hyaenidae groups, which have high prevalence, may confound the relationships between tsetse density and prevalence. This analysis is summarised in Table 4-14.

	Felidae only (univariate)		Hyaenidae only (univariate)		Other species (bivariate)	
	LRT	***p-value***	***LRT***	***p-value***	***LRT***	***p-value***
T. brucei						
Density of *Gs*	5.7	**0.017**	0.085	0.77	0.76	0.38
Density of *Gs* plus quadratic term	0.004	0.95	0.23	0.64	0.21	0.65
Density of *Gp*	1.7	0.19	0.63	0.43	3.4	0.06
Density of *Gp* plus quadratic term	2.7	0.1	0.74	0.39	0.09	0.76
T. congolense						
Density of *Gs*	28.4	**<0.001**	7.3	**0.007**	0.68	0.41
Density of *Gs* plus quadratic term	0.1	0.75	2.6	0.1	0.53	0.47
Density of *Gp*	21.4	**<0.001**	2.7	0.1	0.047	0.83
Density of *Gp* plus quadratic term	10.1	**0.001**	6.5	**0.01**	0.39	0.53

Table 4-14: Summary of analysis of the effect of tsetse density on the prevalence of *T. brucei* s.l. and *T. congolense* for Felidae, Hyaenidae and other species

Analysis was performed separately for Felidae, Hyaenidae and other species to investigate the significance of quadratic terms. Likelihood ratio tests (LRT) and p-values are shown.

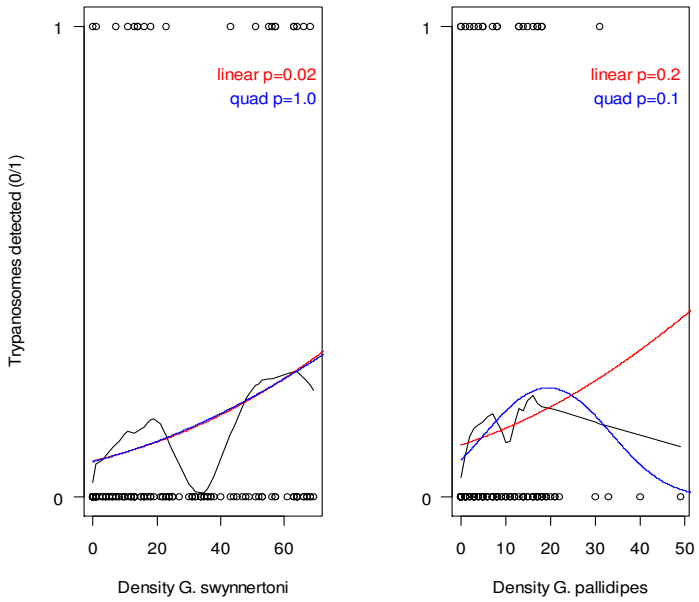
Felidae

If only the Felidae are considered, there is a significant positive linear relationship between *T. brucei* s.l. prevalence and *G. swynnertoni* density. For *T. congolense* the linear relationship with *G. swynnertoni* density is significant, whilst for *G. pallidipes* both linear and quadratic are significant (Figure 4-16).

Hyaenidae

A similar pattern is seen for the Hyaenidae. There was a statistically significant positive linear relationship between density of *G. swynnertoni* and prevalence of *T. congolense*. The linear relationship between *G. pallidipes* density and *T. congolense* prevalence was not significant but addition of a quadratic term significantly improved the fit of the model (Figure 4-17).

(a)



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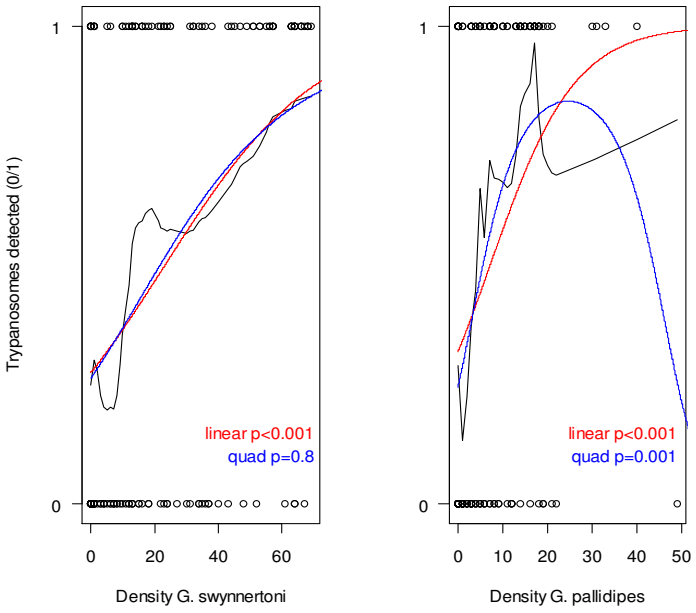


Figure 4-16: Distribution of (a) *T. brucei* s.l. and (b) *T. congolense* infections in Felidae with tsetse density, using a supersmooth line, with linear and quadratic relationships and associated p-values

Black lines show an illustration of the relationship between tsetse density and prevalence by plotting Friedman's supersmoother between values of 0 (negative) and 1 (positive). Red and blue lines show model predicted values for linear and quadratic variables.

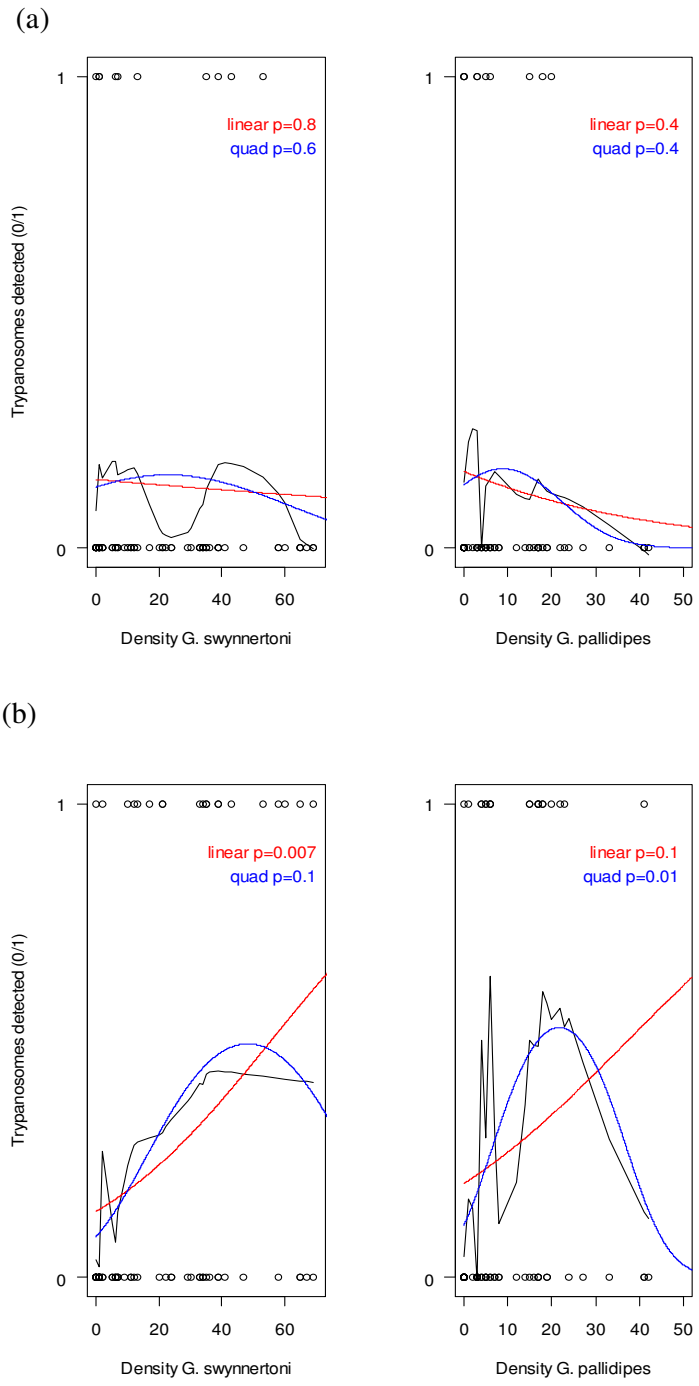


Figure 4-17: Distribution of (a) *T. brucei* s.l. and (b) *T. congolense* infections in Hyaenidae with tsetse density, using a super smooth line, with linear and quadratic relationships and associated p-values

Black lines show an illustration of the relationship between tsetse density and prevalence by plotting Friedman's supersmoother between values of 0 (negative) and 1 (positive). Red and blue lines show model predicted values for linear and quadratic variables.

Other species

When Felidae and Hyaenidae were excluded, there was no statistically significant relationship between trypanosome prevalence and tsetse density.

4.4.9 Multivariate model

Of the risk factors assessed, taxonomic group, age category and density of *G. swynnertoni* and *G. pallidipes* showed p values of less than 0.15 and were selected for inclusion into a multivariate model.

The densities of *G. swynnertoni* and *G. pallidipes* showed co linearity (Figure 4-18) and inclusion of both factors in a multivariate model resulted in instability. Therefore for multivariate analysis, the densities of *G. swynnertoni* and *G. pallidipes* were summed to give one measure of tsetse density.

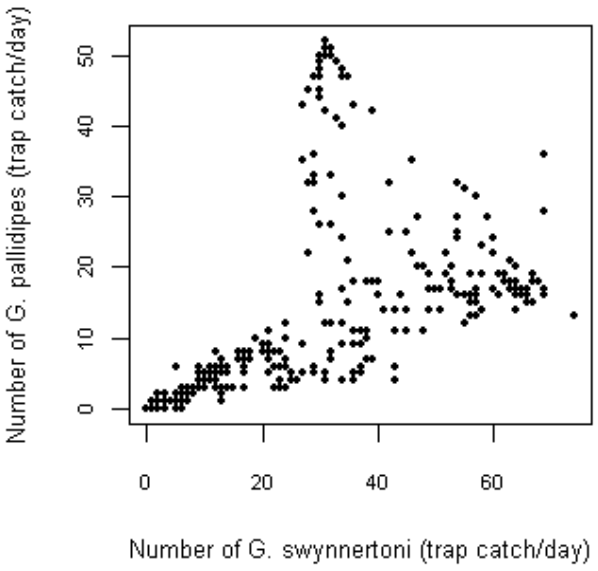


Figure 4-18: Relationship between density of *G. swynnertoni* and *G. pallidipes*

Predicted values of tsetse density from Tsetse Plan show co linearity between density of *G. swynnertoni* and *G. pallidipes*.

For *T. brucei* s.l., the final model included only taxonomic group and age category (Table 4-15). Tsetse density did not significantly affect prevalence of *T. brucei* s.l. in the multivariate model with either the linear relationship or with the inclusion of a quadratic term and was therefore excluded. It was not possible to assess the interaction between age and taxonomic group as small sample numbers in some groups resulted in very high standard errors.

For *T. congolense*, the final model included taxonomic group, age and tsetse density as a linear function (Table 4-16). It was not possible to assess the interaction between taxonomic group and age. There were no significant interactions between age and tsetse density, or tsetse density and taxonomic group.

Variable	Level	LRT	p-value	OR (CI)
Taxonomic Group		21.6	**0.003**	
	Alcelaphinae			NA
	Antilopinae			1.0 (0.14-7.3)
	Bovinae			2.1 (0.28-16)
	Felidae			4.8 (1.1-22)
	Hyaenidae			4.1 (0.83-20)
	Reduncinae			8.7 (1.6-49)
	Suidae			1.6 (0.21-12)
	Equidae			1.0 (0.14-7.6)
Age		13.1	**0.004**	
	Juvenile			0.36 (0.08-1.7)
	Subadult			2.7 (1.3-5.5)
	Adult			NA
	Old			1.0 (0.3-3.7)

Table 4-15: Summary of analysis for multivariate model of prevalence of *T. brucei* s.l. in wildlife

The final multivariate model for *T. brucei* s.l. included taxonomic group and age. Likelihood ratio tests (LRT), p-values, odds ratios (OR) and 95% confidence intervals (CI) are shown. Odds ratios were calculated compared to the reference levels, Alcelaphinae and adult.

Variable	Level	LRT	p-value	OR (CI)
Taxonomic Group		111	**<0.001**	
	Alcelaphinae			NA
	Antilopinae			1.8 (0.29,12)
	Bovinae			3.5 (0.59,21)
	Felidae			50 (11,225)
	Hyaenidae			15 (3.2,71)
	Reduncinae			4.2 (0.63,28)
	Suidae			1.1 (0.15,8.7)
	Equidae			1.6 (0.28,9.4)
Age		14	**0.004**	
	Juvenile			0.53 (0.22,1.3)
	Subadult			2.3 (1.3,4.4)
	Adult			NA
	Old			1.1 (0.42,3.0)
Tsetse density		29	**<0.001**	
				1.02 (1.01,1.03)

Table 4-16: Summary of analysis for multivariate model for prevalence of *T. congolense* in wildlife

The final multivariate model for *T. congolense* included taxonomic group, age and tsetse density. Likelihood ratio tests (LRT), p-values, odds ratios (OR) and 95% confidence intervals (CI) are shown. Odds ratios were calculated compared to the reference levels, Alcelaphinae and adult.

4.4.10 Differentiation of T. b. brucei and T. b. rhodesiense

The number of samples testing positive for PLC and SRA are shown in Table 4-17. SRA positives were found in hyaena, lion and reedbuck. The small number of positive samples limited statistical analysis, with no statistically significant differences in prevalence with taxonomic group, sex, age, reason for sampling, age of carcass or tsetse density on univariate analysis (all p>0.09).

	Number of samples positive		
Species	***T. brucei* s.l. (TBR/ITS)**	**PLC**	**SRA**
Buffalo	1	0	
Bushbuck	2	1	
Cheetah	1	1	
Eland	1	1	
Grants gazelle	1	0	
Hartebeest	2	0	
Hyaena	10	6	2
Impala	4	1	
Lion	21	9	3
Reedbuck	1	0	1
Thomson's gazelle	1	0	
Topi	1	0	
Warthog	2	0	
Zebra	2	2	

Table 4-17: Summary of *T. brucei* s.l. positive samples analysed with PLC and SRA

All samples testing positive for *T. brucei* s.l. with ITS or TBR primers were also analysed using PLC/SRA primers to differentiate *T. b. brucei* from *T. b. rhodesiense*. SRA positives are interpreted in conjunction with PLC results, as PLC primers are included to confirm the presence of sufficient DNA for detection of the SRA sequence.

4.4.11 Summary of Results

- At a species level, prevalence was predominantly explained by taxonomic group.

- At an individual level, taxonomic group had a statistically significant effect on prevalence of *T. brucei* s.l. and *T. congolense*.

- There was no statistically significant difference between males and females in the prevalence of *T. brucei* s.l. or *T. congolense*.

- Age had a statistically significant effect on the prevalence of *T. brucei* s.l. and *T. congolense*, with sub adults most likely to be infected. However, when analysed separately for Felidae, Hyaenidae and other species, this effect was significant only for the prevalence of *T. congolense* in Felidae and Hyaenidae.

- Reason for sampling had no significant effect on prevalence of *T. brucei* s.l. or *T. congolense.*

- Whether an animal was alive, or the time between death and sampling had no significant effect on prevalence of *T. brucei* s.l. or *T. congolense.*

- The relationship between tsetse density and trypanosome prevalence was complex. It was generally best explained by a linear term in Felidae and Hyaenidae but addition of a quadratic term improved the fit for the relationship between *G. pallidipes* density and prevalence of *T. congolense*. In other species no statistically significant relationships were seen.

4.5 Discussion

This study represents one of the biggest wildlife data sets used to look at the prevalence of trypanosomes, and is the first study to consider the effects of multiple risk factors on prevalence. Whilst taxonomic group was a significant risk factor, the statistically significant effects of age and tsetse density on trypanosome prevalence illustrate the importance of other factors.

4.5.1 Differences at species level

The large number of species with small sample numbers necessitated categorising species for further analysis. Tree models provided a method of examining the data to find the most justifiable way to place species into categories. For both *T. brucei* s.l. and *T. congolense*, partitioning of data according to taxonomic group explained the largest portion of variance. At a species level, it appears that living in habitat conducive to tsetse, consumption of infected prey, or being a preferred host of *G. swynnertoni* or *G. pallidipes*, are not as important factors in determining trypanosome prevalence as taxonomic classification.

Taxonomic classifications reflect evolutionary history. Behavioural or ecological differences may result from similar evolution, so that the likelihood of a species being exposed to trypanosomes is confounded by taxonomic group. However by considering habitat, diet and blood meal preferences a reasonable measure of ecological differences has been included. Therefore it is more likely that differences predominantly result from inherent differences in the susceptibility and control of trypanosome infections, rather than differing exposure to tsetse (as would occur in different habitats, or due to tsetse preferences for particular species) or trypanosomes (in the case of differences in diet and oral transmission from infected hosts).

There is other evidence for intrinsic species differences. The experimental studies by Ashcroft *et al* (1959) identified differences between species in susceptibility to trypanosome infections, with 100% mortality in species such as Thomson's gazelle, compared to transient parasitaemia with no clinical signs in species such as warthog. Experimental studies on the effect of sera from a range of captive species on the replication of *T. brucei* s.l. found Thomson's gazelle, dikdik, zebra and hartebeest sera had no ability to restrict replication at

all, whilst sera from lion and leopard caused inhibition but only of one of two clones tested, and sera from buffalo, giraffe, kudu and warthog severely limited all trypanosome replication (Black *et al.*, 1999).

However, at lower branches of the tree variance partitioning according to diet and habitat shows that taxonomic group alone cannot explain the distribution of trypanosome infections. For example, for *T. congolense*, branching by taxonomic group places Felidae in one category. However further partitioning splits Felidae according to habitat. This results from separating cheetah (found in plains habitat) and leopard (found in woodlands) which were all negative for *T. congolense*, from lions (found in both habitats) which were frequently infected. It appears that the factors which make lions likely to carry *T. congolense* infections are not common to all members of the Felidae family (although small sample numbers for cheetah and leopard preclude definite conclusions).

4.5.2 Risk Factors at an individual level

The categorisation of species according to taxonomic group was used in further assessment of risk factors at an individual level. In addition to taxonomic group, the effect of age, sex, reason for sampling, age of carcass, and density of tsetse at the sampling site on trypanosome prevalence were assessed.

Species - warthogs

Warthogs were targeted for sampling because a previous study in the Serengeti Mara Ecosystem found surprisingly high prevalence of *T. brucei rhodesiense* of 9.5% (*T. brucei* s.l. prevalence 29%). In this study only 5% of warthogs carried *T. brucei* s.l. infections (CI 0.7-18), and no *T. brucei rhodesiense* was found in warthogs.

Many aspects of these two studies are consistent – they were both carried out in the Serengeti Mara Ecosystem, with some sampling for each study performed in Grumeti Game Reserve, and the same protocols for sample analysis were used (FTA card discs with TBR PCR). The prevalence of *T. brucei* s.l. and *T. congolense* in species other than warthogs are also relatively consistent between the two studies.

This leaves three potential explanations for these differences:

1. This study included warthogs of a range of ages. The ages of warthogs in Kaare's study are not recorded, and could have included more sub-adults, the age category with the highest prevalence in this study. Indeed, the 2 warthogs positive for *T. brucei* s.l. in this study were both sub adults, giving a prevalence of *T. brucei* s.l. in sub adult warthogs of 29% (CI 7-71).

2. Whilst both studies were carried out in the SME, differences in location at a smaller spatial scale, leading to differences in tsetse exposure, may account for differences in prevalence.

3. The third explanation is that prevalence in warthogs fluctuates temporally, either with season or over a longer period. Seasonal fluctuations in tsetse numbers may introduce a seasonal pattern in prevalence in wildlife (Rogers, 1988). Kaare's study was carried out in 2001, towards the end of a peak in the incidence of human cases though to originate from SME. This explanation is particularly intriguing, suggesting the possibility that longer term fluctuations in wildlife prevalence may be correlated with human disease incidence. Unfortunately there is no other information on longer term patterns of trypanosome prevalence in wildlife, since longitudinal studies of wildlife are difficult to conduct. Continuation of warthog sampling in this area would be very useful to test this hypothesis.

Other species

The prevalence of *T. brucei* s.l. and *T. congolense* in other species was not dissimilar to that found by previous studies. The relative consistency between the results of this PCR-based study with previous studies which have relied on microscopic identification of trypanosomes provides useful validation of earlier results.

Felidae, Hyaenidae and Reduncinae separated out with the highest *T. brucei* s.l. prevalence on classification tree analysis, and had significant odds ratios compared to the reference level (Alcelaphinae) on univariate analysis. The Reduncinae (reedbuck, waterbuck and impala) had the highest prevalence at 19% (CI 7-39). This is not surprising as all these species have

consistently tested positive for *T. brucei* s.l. in previous studies. The Felidae, in which the prevalence of *T. brucei* s.l. was 14% (CI 9-21), predominantly comprised lions. However, it is of interest that one cheetah, euthanased after exhibiting neurological clinical signs, also tested positive for *T. brucei brucei*. This is the first reporting of *T. brucei* s.l. in this species; it is so far unclear whether clinical signs may have related to trypanosome infection of the central nervous system. Investigations to confirm the present of *T. brucei* s.l. in the central nervous system, and to identify other potential pathogens that could be present in co infections is continuing.

The highest prevalence of *T. congolense* was found in Felidae with 51% (CI 42-59) testing positive. Similar prevalences have been found by earlier authors (Geigy *et al.*, 1971; Geigy & Kauffman, 1973; Kaare *et al.*, 2007). The prevalence was also high in the Hyaenidae (26% CI 16-37), Bovinae (16% CI 6-34) and Reduncinae (15% CI 4-35).

The high prevalence of *T. brucei* s.l. and *T. congolense* in lion and hyaena found in this study is consistent with other studies but perhaps surprising since neither species represent important hosts for tsetse. A potential explanation is that these species, whilst not often fed on, maintain high parasitaemia for long periods of time, and therefore frequently test positive for trypanosomes. However it also lends support to the hypothesis that carnivores can become infected through oral transmission of trypanosomes via consumption of infected prey animals. Oral transmission has been documented before (reviewed in chapter 1). In this study, trypanosomes were observed in lions which live on the plains, where there are no tsetse. Other authors have found the force of infection in the woodlands and plains to be similar (Welburn *et al.*, 2008). This could be explained by oral transmission since migratory prey species move from tsetse habitat towards the plains. Vector-independent transmission provides an additional challenge in understanding transmission dynamics, since its importance can be difficult to quantify (Smith, 2008).

For some species the small numbers of samples analysed precluded precise prevalence estimates. For example, two bushbuck samples in this study both tested positive for both *T. brucei* s.l. and *T. congolense*. Bushbuck are frequently cited as important hosts of HAT. The main reason for this is that the first identification of *T. b. rhodesiense* involved infection of a human volunteer with a trypanosome isolated from bushbuck, and although other studies have also identified *T. brucei* s.l. and *T. brucei rhodesiense* in bushbuck, their importance in

transmission is unclear. Within SME the density of bushbuck is low. However, in contrast to most other antelope species bushbuck are able to persist in habitats modified by human settlement and therefore have the potential to be important in transmission of *T. b. rhodesiense* to man. Analysis of further bushbuck samples from SME and the surrounding areas would be beneficial, although potentially difficult, given their low numbers.

This study included samples from a wide range of mammalian hosts, including rodents which had never been assessed before in SME as potential reservoirs of HAT. Studies in West Africa found *T. brucei* s.l. in several rodent species, although their importance in disease transmission was unclear (Njiokou *et al.*, 2004). In this study no trypanosomes were identified in rodents. Together with the fact that blood meal analysis indicates that rodents are not an important food source for either of the tsetse species in Serengeti (less than 0.5% of meals identified as Rodentia) (Clausen *et al.*, 1998), it seems unlikely that rodents play an important role in trypanosome transmission in SME. However, rodents were sampled over a small geographic area and may not be representative of all areas.

In this study, no samples were obtained from avian or reptilian hosts. The identification of *T. brucei* s.l. in a monitor lizard in Uganda (Njagu *et al.*, 1999), and evidence that tsetse do feed on avian and reptilian hosts (Weitz, 1963; Clausen *et al.*, 1998) indicates that these species may also be important. The studies conducted in SNP where many alternative host species are available showed only small numbers of blood meals taken from non-mammalian hosts (Moloo *et al.*, 1971; Rogers & Boreham, 1973), suggesting they only play a minor role in trypanosome transmission in complex ecosystems, but confirmation would be valuable.

Sex

Once taxonomic group was included in the model, and the confounding effect of a large number of female lion samples controlled for, the prevalence of *T. brucei* s.l. and *T. congolense* did not differ between sexes. No differences in the prevalence of trypanosome infections between sexes have been reported before in wildlife. Although effects of sex have been reported in trypanosomiasis in cattle, it has been suggested that these result from differing management of male and female livestock (Rowlands *et al.*, 1993).

Reason for sampling

As with sex, the reason for sampling appeared significant on univariate analysis due to the large number of lion samples which were predominantly collected at routine sampling for either disease surveillance or fitting of radio collars. When taxonomic group was included in the model, there was no significant difference in prevalence between animals sampled for different reasons. This study was therefore unable to reject the hypothesis that reason for sampling, as a potential indicator of health status, has no effect on trypanosome prevalence. This remains an interesting question. Experimental studies reported high mortality in some wildlife species infected with trypanosome infection but there is no evidence of this mortality in the field, perhaps because animals showing clinical signs are likely to be predated before disease is evident. Continued sampling would be useful; differences in prevalence are likely to be small and the sample size in this study may have been insufficient to detect subtle differences.

Age

The prevalence of trypanosome infections differed significantly between age groups, with sub adults most likely to be infected with *T. brucei* s.l. and *T. congolense*. Felidae, Hyaenidae and other species were analysed separately to assess whether this relationship was consistent over different taxonomic groups.

In both Felidae and Hyaenidae sub-adults were most likely to be infected with *T. brucei* s.l. and *T. congolense*. This effect was statistically significant for *T. congolense* though not for *T. brucei* s.l.. Lions from study prides in SNP are unique because their birth dates are known, allowing more detailed analysis of age relationships. Previous studies on age prevalence in Serengeti lions identified a peak in prevalence of *T. brucei* s.l. at 2-3 years of age, followed by a decline, which was more pronounced in woodland lions than plains lions. *T. congolense* prevalence showed a monotonic increase with age. A modelling approach was used to suggest that this provides evidence of acquired immunity, potentially due to the rapid exposure of lions to many VSGs through consumption of infected prey, leading to the development of immunity against *T. brucei* s.l., but not *T. congolense*, which has a larger VSG repertoire (Welburn *et al.*, 2008).

In this study some similarities were found. *T. brucei* s.l. prevalence peaked at 2-3 years, although prevalence increased again at 6 years in woodland lions. However, *T. congolense* showed a decrease in prevalence after about 7 years, in both plains and woodland lions. This could support the hypothesis of acquired immunity, with immunity to *T. congolense* also developing, but over a longer period of time to reflect the larger VSG repertoire. Welburn *et al.* (2008) reported no *T. b. rhodesiense* infections in lions older than 6 years, whereas in this study the oldest lion which tested positive for *T. b. rhodesiense* was aged 9 years and 4 months. However the low number of *T. b. rhodesiense* positive-lions precluded detailed analysis, and this may not be typical of the normal pattern of age prevalence.

In this study the age prevalence curves for *T. brucei* s.l. and *T. congolense* follow similar patterns. This is not surprising as the proportion of mixed infections was very high. In fact 86% of lions which tested positive for *T. brucei* s.l. were also carrying *T. congolense* infections. The use of molecular tools to identify trypanosome species has made it easier to detect mixed infections. However, the level of mixed infections found in lions in this study was unusually high even when compared to other PCR-based studies. Kaare (2007) found no mixed infections in lions, although 4 out of 12 *T. brucei* s.l. positives in other species were present in mixed infections. In the lion age prevalence study described above (Welburn *et al.*, 2008), 32 out of 72 *T. brucei* s.l. infected lions also carried *T. congolense* (44%) (K. Picozzi, pers. comm.).

Around a third of tsetse have been found to be carrying more than one trypanosome species (Lehane *et al.*, 2000; Malele *et al.*, 2003) and up to four species have been detected in one tsetse (Lehane *et al.*, 2000; Njiru *et al.*, 2004a). Mixed infections in wildlife may arise from simultaneous infection with several trypanosome species from a fly carrying multiple trypanosome species, or from subsequent infections. An increase in *T. brucei* parasitaemia has been observed after subsequent infection with *T. congolense* in cattle chronically infected with *T. brucei* s.l. (Van den Bossche *et al.*, 2004). However, the proportion of mixed infections in lions was much higher than in other species (in which only 26% of *T. brucei* s.l. positive animals also tested positive for *T. congolense*) suggesting that this cannot be explained by exposure to tsetse only. The difference in lions may arise from inherent differences in species susceptibility, or may reflect a different transmission route, such as ingestion of infected prey.

For species other than Felidae and Hyaenidae, sub-adults had highest prevalence of *T. brucei* s.l. and *T. congolense* infections, but this effect was not statistically significant. Buffalo have previously been found to have highest prevalence in the sub-adult group (Drager & Mehlitz, 1978). Given the potential explanation of acquired immunity for the pattern found in lions, it is important to know if this is also seen in other species. There are two reasons why an age effect, if present, may not have been detected in this study. Firstly, the limitations of opportunistic sampling mean that (other than for lions) samples were not sufficiently representative of a range of ages to accurately assess the effect of age in all species. Combining species remained the only option, but has disadvantages, since there may be inconsistencies in applying age categories to different species, and potential species differences in the age prevalence relationship may be masked. Secondly, age estimation is difficult in free ranging species and even the placement of animals into categories is likely to introduce unreliability. In order to specifically address this question, a study design is necessary which incorporates stratification of sampling by age, in a small number of species in which age can be estimated reliably, such as buffalo.

Status of animal / age of carcass

This study found no significant difference in prevalence between animals sampled when alive, animals sampled within four hours of death, and animals sampled more than four hours after death. In two cases, trypanosomes were identified from carcasses estimate to be 8 hours old. However, the small number of samples from carcasses more than four hours old meant it was not possible to make more accurate estimates of how long trypanosomes continued to be detected.

Under certain conditions DNA can persist for a very long time; *Trypanosoma cruzi* has been identified by PCR in Chilean mummies between 600 and 2000 years old (Ferreira *et al.*, 2000). However, usually pathogen detection decreases as DNA is degraded by DNAses released from lysed cells during post mortem autolysis. *Ehrlichia canis* could be detected up to 4 days post mortem in blood and lymph node tissue, but not in liver kidney or spleen (Gal *et al.*, 2008). Detection of West Nile Virus in carcasses from experimentally infected birds maintained for 4 days at 20^{O}C decreased daily, and WNV was frequently undetectable on day four (Panella *et al.*, 2005). Current guidelines in WNV detection advise testing of carcasses less than 24 hours old (Gubler *et al.*, 2000).

It was not possible to estimate time since death with sufficient accuracy to include it as a continuous variable in this study, so although no significant differences in detection were observed, there may have been subtle changes in the likelihood of detection with time that were masked by the categories used or the samples obtained. The first stages of autolysis degrade cell walls and causes DNA to be released. Potentially this could lead to increased detection shortly after death. With increasing time after death, detection is likely to decrease, but older carcasses are rarely found in SME due to the high densities of predators and scavengers.

Post mortem sampling provides a useful way of looking at trypanosome infections and increasing sample size in logistically difficult wildlife studies. This study concludes that trypanosome detection is not impeded up to four hours after death, and potentially longer. However further research to quantify the likelihood of parasite detection over longer time periods is important. This would probably be best achieved by protecting carcasses in a natural environment and carrying out repeated sampling over a period of time.

Tsetse density

The relationships between tsetse density and prevalence of *T. congolense* and *T. brucei* s.l. are complex, and the number of factors which have the potential to affect them means careful interpretation is necessary to avoid misinterpretation of confounding factors.

Felidae, Hyaenidae and other species were analysed separately. The large numbers of samples from Felidae and Hyaenidae, the high prevalence found in these groups, and the fact that Felidae and Hyaenidae were sampled in sites that had on average, low density of either tsetse species (Figure 4-7, Figure 4-15), indicated that separate analysis was necessary to avoid confounding.

For Felidae, significant positive linear relationships between prevalence of *T. brucei* s.l. and density of *G. swynnertoni*, and prevalence of *T. brucei* s.l. and *T. congolense* and density of *G. pallidipes* were found. This is not unexpected; tsetse density is a component of tsetse challenge, and if the other two components (prevalence of trypanosomes in the tsetse population and feeding preferences of tsetse) remain constant (a topic which is explored further in Chapter 5), a positive linear relationship between tsetse density and host

trypanosome prevalence would be expected. However, the significance of the quadratic relationship between the prevalence of *T. congolense* and the density of *G. pallidipes* was unexpected. The small number of Felidae samples at high density of *G. pallidipes* could suggest this analysis is not very robust. However the same pattern was seen in Hyaenidae.

There are a number of potential explanations for this pattern: (i) There may be differences in host response to exposure to trypanosomes in high tsetse density areas, for example an acquired immune response may lead to lower prevalence in areas where exposure is very high, as evidenced by the lower prevalence of *T. brucei* s.l. in woodland lions compared to plains lions in previous studies (Welburn *et al.*, 2008). However the lack of significant quadratic relationship for trypanosome prevalence with the density of *G. swynnertoni* does not support this hypothesis; (ii) If the main route of transmission for carnivores is indeed through consumption of infected prey, different species consumption in the woodlands where the highest density of *G. pallidipes* is found could influence the exposure of carnivores to trypanosomes, if the species found in woodlands are those which are less likely to be carrying trypanosome infections. (iii) The third potential explanation is that the relationship between prevalence and tsetse density is linear, but the co-linearity between the density of *G. pallidipes* and *G. swynnertoni* (Figure 4-18) is affecting the analysis for *G. pallidipes*. The prevalence of *T. congolense* is higher in *G. swynnertoni*. At low densities of *G. pallidipes*, the prevalence of *T. congolense* in host species appears to increase with density of *G. pallidipes* but is actually reflecting the density of *G. swynnertoni*. At the high *G. pallidipes* densities found in the woodlands, there are few *G. swynnertoni* and the prevalence in wildlife hosts decreases.

In other species, there was no statistically significant relationship between trypanosome prevalence and tsetse density. Whilst a linear relationship might be expected within individual species, the lack of pattern here may simply reiterate that the likelihood of a species carrying trypanosome infections is not related simply to tsetse exposure, but to intrinsic species differences, and result from differing species composition in areas of higher tsetse density.

It is clear that the number of factors which can potentially affect the relationship between tsetse density and trypanosome prevalence makes understanding these patterns difficult. The

data collected in this study suggests potential explanations but further work will be necessary to understand transmission in the complex system.

4.5.3 Identification of T. b. rhodesiense

T. b. rhodesiense was identified in lions, hyaenas and reedbuck. This confirms previous identifications of *T. b. rhodesiense* in these species in SME using the BIIT (Geigy & Kauffman, 1973). The SRA/PLC multiplex PCR protocol amplifies the single copy PLC band in conjunction with SRA, to confirm whether sufficient genetic material is present to detect the SRA gene. The number of samples testing positive for either PLC or SRA was low in this study, probably reflecting low parasitaemia in trypanosome infections in wildlife.

The proportion of *T. brucei* s.l. samples testing positive for *T. b. rhodesiense* can be calculated by using a denominator of the total number of PLC positive samples. Using this calculation, *T. b. rhodesiense* was identified in one third of *T. brucei* s.l. infections in lions (3/9) and hyaenas (2/6). One reedbuck was positive for SRA only, with no PLC band, which gives an overall ratio of SRA to PLC of 6/15. A ratio of 1 to 3 is often seen in livestock and wildlife populations, thought to reflect the fitness costs to the trypanosome of human serum resistance (Coleman & Welburn, 2004).

A consistent ratio between *T. b. brucei* and *T. b. rhodesiense* suggests that studying the risk factors associated with *T. brucei* s.l. infection yields information which is indicative also of the prevalence of *T. b. rhodesiense*. This is particularly useful as the prevalence of *T. b. rhodesiense* is so low that risk factor analysis will rarely reveal significant relationships, as illustrated in this study. However this ratio may not hold true for all factors, for example the ratio of *T. b. brucei* to *T. b. rhodesiense* changed from 1:3 in lions on the grassland plains to 1:7 in lions in the woodland (when considering lions aged less than six years) (Welburn *et al.*, 2008). Unfortunately the low number of samples testing positive for *T. b. rhodesiense* in this study precluded further analysis of this ratio, and it is not possible to conclude whether identification of *T. b. rhodesiense* in lions, hyaenas and reedbuck is simply a reflection of the prevalence of *T. b. brucei*, or if these species are even more important in HAT transmission than their *T. brucei* s.l. prevalence suggests.

4.5.4 Study design

This study shared issues common in other wildlife studies: the logistical, financial and ethical considerations in obtaining samples from free ranging wildlife; political issues associated with working with potentially endangered species in an important tourist area; and difficulties of collecting and maintaining samples in a field situation. These limitations results in a sample set which is not randomly selected and has high inherent variation. However, analysis incorporated as many of these variables as possible so that important factors could be assessed.

Some factors which were not included in this study may have important effects on trypanosome prevalence. In particular, the pattern of prevalence over time, both on a seasonal level, and over longer time periods, should be assessed. The importance of longer term factors such as climate has been illustrated for other diseases in SME (Munson *et al.*, 2008).

4.6 Conclusions

Understanding the factors which affect prevalence of trypanosome infections in wildlife is important. Prevalence is one of the parameters used to assess reservoir competence of wildlife species but it is a dynamic parameter which is a function of many other factors. Species is the most important risk factor in determining the prevalence of *T. brucei* s.l. and *T. congolense* in wildlife. This effect appears to be better explained by intrinsic factors, rather than differing exposure to trypanosomes, since taxonomic group explains more variation in prevalence differences than diet, habitat or tsetse feeding preferences. However trypanosome prevalence was also affected by host factors (age) and vector factors (density). The difficulties in elucidating the true relationship between trypanosome prevalence and tsetse density illustrate the difficulties of understanding pathogen transmission in a complex ecosystem.

Chapter 5: Prevalence of *T. brucei* sensu lato in tsetse

5.1 Introduction

The proportion of a vector population that carry transmissible infections is an essential component in understanding vector-borne pathogen transmission dynamics. Used as an indicator of transmission intensity, prevalence of a pathogen in its vector is important for several reasons. It is used to assess spatiotemporal heterogeneity in risk, for example to target intervention measures to address West Nile Virus (WNV) transmission (Gu *et al.*, 2008), in the monitoring of spatiotemporal patterns, for example to predict the effects of climate change on the incidence of leishmaniasis (Martin-Sanchez *et al.*, 2006), and in the evaluation of control programs, for example to assess the effects of mass treatment with anthelmintics on transmission of *Onchocerca volvulus* (Rodriguez-Perez *et al.*, 2006).

The low prevalence of *Trypanosoma brucei* sensu lato and *Trypansoma brucei rhodesiense* in tsetse populations, combined with diagnostic tests where interpretation is challenging, mean that obtaining meaningful estimates of trypanosome prevalence can be difficult. In addition, a number of factors are known to influence the prevalence of trypanosome infections in tsetse. These include endogenous factors such as tsetse species, sex, population age structure, host choice, concurrent infections and physiological state, ecological factors such as climate, availability of infected hosts and hosts available for subsequent feeds, and host factors which influence the prevalence of trypanosome infections in hosts, such as susceptibility, immune response and concurrent infection.

This chapter assesses the prevalence of *T. brucei* s.l. and *T. b. rhodesiense* infections in tsetse in Serengeti National Park (SNP) using the tools currently available, microscopy and PCR, and explores how the resulting data can be interpreted. Heterogeneities in prevalence will be examined by assessing the effects of tsetse species, sex and habitat. Current knowledge on the prevalence of trypanosomes in tsetse in SNP and the potential effects of tsetse species, sex and habitat on prevalence are summarised below.

5.1.1 Prevalence of trypanosomes in G. swynnertoni *and* G. pallidipes

The majority of studies carried out in SNP have relied on dissection and microscopy. The results of these studies is summarised in Table 5-1. Large scale studies in 1970 and 1971 failed to identify any salivary gland infections (Moloo *et al.*, 1971; Rogers & Boreham, 1973). In 1972, in order to confirm the role of *G. swynnertoni* in transmission of *T. brucei* s.l., a pooled rodent inoculation technique was used to analyse over 10 000 flies, giving a prevalence of 0.08% (Moloo & Kutuza, 1974).

	No. of flies examined	Prevalence (%)			Reference
		Duttonella	*Nannomonas*	*Trypanozoon*	
G. swynnertoni	6348	12.6	2.0	0	(Moloo *et al*., 1971; Moloo, 1973)
	3550	17.0	2.3	0	(Rogers & Boreham, 1973)
	677	4.3	2.1	3.0	(Malele *et al*., 2007)
G. pallidipes	623	*	*	0	(Moloo *et al*., 1971)
	199	0	0	0	(Malele *et al*., 2007)

Table 5-1: Prevalence of mature trypanosome infections by dissection and microscopy in tsetse in the Serengeti Mara Ecosystem

Table indicates the prevalence of *Duttonella* (mouthpart only), *Nannomonas* (mouthpart and midgut) and *Trypanozoon* (salivary gland) infections as the proportion of *G. swynnertoni* and *G. pallidipes* found infected. *Combined prevalence of *Duttonella* and *Nannomonas* mature infections 8.7%, separate prevalence not reported

This low number of salivary gland infections is a common finding in the *morsitans* tsetse group, with infection rates of less than 0.1% consistently reported for *Glossina swynnertoni* and *Glossina pallidipes* (Vanderplank, 1947; Robson *et al.*, 1972; Wilson *et al.*, 1972). However, a study recently carried out in the Serengeti Mara Ecosystem (SME) found 20 out of 667 *G. swynnertoni* carrying salivary gland infections, reporting a prevalence of 3.0% for *T. brucei* s.l. (Malele *et al.*, 2007). The authors gave no suggestions for this unusually high prevalence.

Studies in SME that have carried out PCR on dissection-positive midguts found *T. brucei* s.l. in 5 out of 63 samples, all of which were identified as *T. b. rhodesiense* (Malele *et al.*, 2007), and in 12 out of 144 samples (includes samples from Msumbugwe as well as SNP, *T. b.*

brucei and *T. b. rhodesiense* not differentiated) (Adams *et al.*, 2006). However, as discussed in Chapter 1, methods of integrating microscopy and PCR data are inconsistent, and it is not clear how results relate to the prevalence of transmissible infections.

5.1.2 Heterogeneity in prevalence

Species

As can be seen above, previous studies have focussed predominantly on *G. swynnertoni*. The reasons for this are two-fold: *G. swynnertoni* are reported to have formed the majority of trap catches; and dissection and microscopy have consistently shown a higher prevalence of trypanosome infections in *G. swynnertoni*. However, in other ecosystems *G. pallidipes* is an important trypanosome vector and plays a role in *T. b. rhodesiense* transmission (Njiru *et al.*, 2004a). Both *Glossina brevipalpis* and *Glossina longipennis* have been reported in SNP in small numbers (Moloo *et al.*, 1971; Mlengeya *et al.*, 2003), but the trypanosome infection rate in these species was not assessed. Both species are relatively refractory to infection with *T. brucei* s.l. in experimental studies (Moloo *et al.*, 1998). In field studies in Kenya infection rates of 3.4% for *T. vivax* and 0.9% for *T. congolense* were found in *G. brevipalpis*, and 2.5% for *T. vivax* and 4.8% for *T. congolense* in *G. longipennis*; no *T. brucei* s.l. infections were found (Owaga, 1981).

Sex

Whilst several field studies have found a higher prevalence of trypanosome infections in female flies by dissection and microscopy (Waiswa *et al.*, 2003; Njiru *et al.*, 2004a; Malele *et al.*, 2007), others have found sex to have no effect on prevalence (Woolhouse *et al.*, 1994). In experimental infections, male flies produce significantly more mature *T. brucei* s.l. infections than female flies (Welburn & Maudlin, 1999).

Habitat

Whilst climatic factors such as temperature are thought to affect the prevalence of trypanosomes in tsetse over a larger spatial scale (reviewed by Ford & Leggate, 1961), within an ecosystem the degree of heterogeneity has not been quantified. Host diversity and density, tsetse density and tsetse population age structure may differ between habitats within an ecosystem, all of which can affect the prevalence of trypanosome infections. Assessing

how the prevalence of trypanosomes in the tsetse population varies across with habitat type and exploring potential reasons for variation is likely to help in understanding the transmission of trypanosomes in an ecosystem. However it is also an important question because any heterogeneity in the prevalence of transmissible infections in tsetse will affect the risk of disease transmission to man, and may have implications for effective distribution of the limited resources available for control programs.

5.2 Objectives

The objectives of this study were to:

- Assess the prevalence of trypanosome infections in tsetse in Serengeti National Park by dissection and microscopy, and by PCR

- Assess the effect of species, sex and habitat on

    - prevalence of trypanosome infections by dissection and microscopy

    - proportion of dissection positives also testing positive for *T. brucei* s.l. by PCR

5.3 Materials and methods

5.3.1 Selection of study sites

All field work was conducted between August and October 2006. Six sites were selected for tsetse trapping within SNP. Sites were stratified by vegetation category and randomly selected. Stratified random sampling usually improves the precision of estimates, since between-stratum variation is removed. In addition, stratum specific estimates can be produced (Dohoo *et al.*, 2003). The vegetation map used for stratification was a classified satellite image with $30m^2$ resolution in four categories: dense woodland, open woodland, savannah and grassland (Reed *et al.*, 2009), which was described further in Chapter 2.

Using the grid analyst extension in ArcView GIS 3.2 (ESRI), a $1km^2$ grid was overlaid on the map, extending in a circle with radius 40km and the centre in Seronera, and each square classified by the predominant vegetation type(s), i.e. grassland, savannah, open woodland or dense woodland, or combinations of two or three types. For one type to be classified as predominant, it comprised over 90% of the pixels in the grid square. For two predominant types, each type comprised more than 30% of the square, with no other type more than 10%. For three predominant types, each type comprised more than 20%, with no other type more than 10%. This meant that out of a total 3484 grid squares, 1622 were unclassified and excluded as potential study sites. A buffer was added to select only grid squares within 1km from a road, to allow quick transportation of flies back to the laboratory in Seronera. Although the proximity to roads may introduce bias into the sampling, it was logistically impossible to repeatedly visit sites less accessible than this.

Grid squares that fitted the criteria were then selected using a random number generator. Squares containing dense woodland were not selected since vehicle access was impossible, and access on foot considered too dangerous due to the presence of wildlife such as buffalo and lions. It was not possible to trap flies in grassland areas since this habitat is not suitable for tsetse. Tsetse sampling was attempted at two grassland sites during pilot work but only three flies were caught in six trapping days. Therefore two grid squares were chosen in each of the following vegetation categories: (a) open woodland (study sites 1WD, 2WD), (b) mixed open woodland and savannah (3WS, 4WS), and (c) savannah (5SV, 6SV). The

location of these study sites can be seen in Figure 5-1, and photographs of vegetation category in Photograph 5-1.

Trapping was also carried out in riverine vegetation to specifically target *G. brevipalpis*, which were not seen in any of the study sites.

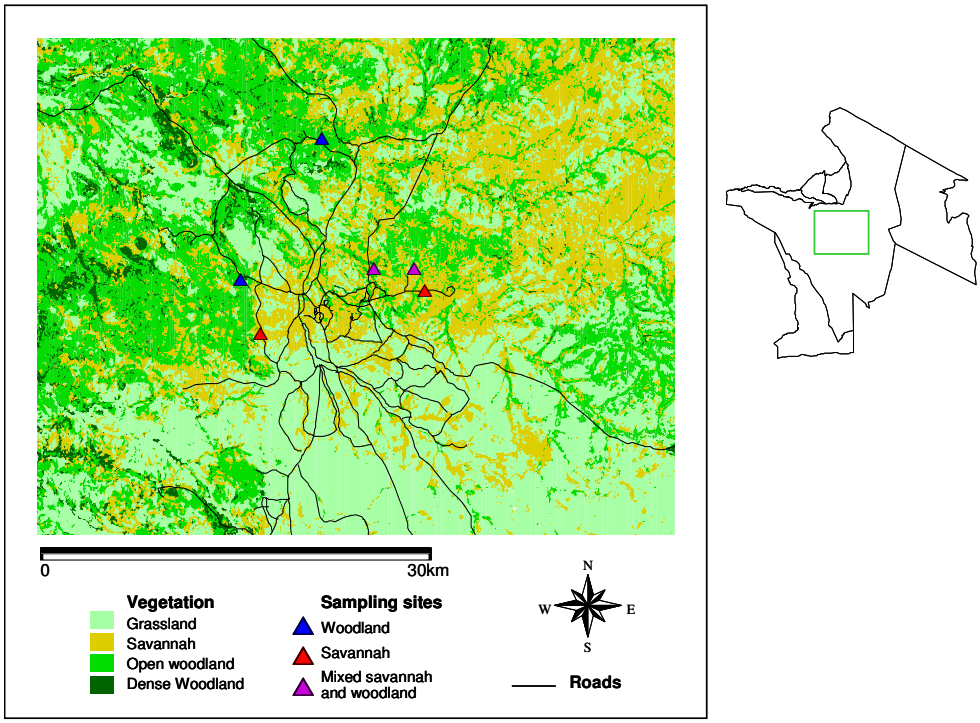


Figure 5-1: Map to show the location of sampling sites in Serengeti National Park

Sites for collection of tsetse for analysis of trypanosome prevalence were stratified by vegetation type and randomly selected. Sites were located within 1km of roads and within 40km of the laboratory in Seronera.

Woodland | Mixed savannah and woodland | Savannah

Photograph 5-1: Examples of each vegetation category

5.3.2 Tsetse collection

Tsetse sampling was carried out with the assistance of technicians from the Tsetse and Trypanosome Research Institute, Tanga. Epsilon traps were chosen (Photograph 5-2) since they are effective for savannah species of fly (Hargrove & Langley, 1990). In each study site, three epsilon traps were used. All three traps were sited within the chosen grid square, with each trap at least 200m from the next. Traps were erected in mottled shade to reduce fly mortality. When placing traps, areas with fallen trees were avoided and traps were placed so that the entrances were directed towards gaps in vegetation, measures known to maximise fly catches by following the natural patterns of tsetse flight (Vale, 1998). In addition, traps faced downwind, so that the bait odour plume directed flies into the trap entrance. Traps were baited with phenol, octenol and acetone and emptied twice daily.

Photograph 5-2: Epsilon trap in use in mixed woodland and savannah

5.3.3 Tsetse examination

All live non-teneral flies were dissected and labrum, hypopharynx, salivary glands and midgut examined for trypanosomes under 400x magnification. Teneral flies are excluded from the analysis because they have not yet taken a blood meal, and therefore have had no opportunity for exposure to trypanosomes. For each fly, species and sex and the presence or absence of trypanosomes in each organ were recorded. Dissection instruments were cleaned in 5% sodium hypochlorite, followed by rinsing in distilled water then phosphate buffered saline, between each organ, to prevent contamination both between flies and between different parts of the same fly. No evidence of contamination was seen in the sequence of dissection or PCR results.

All trypanosome-positive mouthparts, midguts and salivary glands were macerated in phosphate buffered saline and applied to FTA Whatman Classic cards for further analysis. A subset of trypanosome-negative mouthparts, midguts and salivary glands were also preserved on FTA cards for comparison. In each site, at least 500 *G. pallidipes* and 500 *G. swynnertoni* were dissected if possible. In some areas one species predominated and it was not possible to catch sufficient numbers of the second species.

Tsetse population age structure affects the prevalence of trypanosome infections. Age was therefore assessed so that it could be considered when evaluating the influence of other variables. The wings of the first 100 male flies of each species caught in each site were removed and preserved for wing fray analysis, a method which considers the degree of wear on the hind margin of each wing to estimate the average population age (Jackson, 1946). The protocol described by Jackson (1946) was followed. Briefly, both wings were examined using a dissection microscope and a score assigned between one and six, depending on the degree of wear. Average population age for each site and species was then assessed according to the following calculation: The number of flies in each category was recorded. The number of flies in category 1 was multiplied by 1, number in category 2 multiplied by 2, category 3 by 3, category 4 by 4.4, category 5 by 5.5 and category 6 by 6.9. The mean wing fray value is calculated from sum of these products divided by the total number of flies. This value is used to indicate the estimated average age in days of flies in the sample, using the reference table reported by Jackson (1946).

5.3.4 Laboratory analysis

All mouthparts, midguts and salivary glands in which trypanosomes were observed by microscopy were preserved on FTA card and analysed by PCR. Mouthparts, midguts and salivary glands from dissection-negative flies were also analysed by PCR. After FTA sample preparation, TBR PCR was carried out first to identify *T. brucei* s.l.. SRA was then performed on those samples positive for *T. brucei* s.l. to differentiate *T. b. rhodesiense* from *T. b. brucei*.

FTA card preparation

Discs were cut from the FTA card using a Harris Micro Punch™ tool. Between sample discs, 10 punches were taken from clean FTA paper, to prevent contamination between samples. One disc of diameter 2mm was cut out from each sample. Discs were prepared according to the washed disc protocol described in Chapter 3. Briefly, discs were washed for two washes of 15 minutes each with FTA purification reagent (Whatman Biosciences, Cambridge, UK), followed by two washes of 15 minutes each with TE buffer (Sigma Aldrich, Dorset, UK). Each disc was dried at room temperature for 90 minutes, and then used to seed a PCR reaction. After every seven sample discs, a negative disc was included and the punch tool and mat cleaned, to reduce the risk of contamination between discs, and ensure that any potential contamination would be detected.

PCR protocols

PCR protocols were the same as described in previous chapters but are included here for clarity.

T. brucei s.l. (TBR)

TBR detects a 177bp satellite repeat sequence common to *T. b. brucei, T. b. rhodesiense* and *T. b. gambiense*. PCR was carried out in 25µl reaction volumes containing 16.0mM $(NH_4)_2SO_4$, 67mM Tris-HCl, 0.01% Tween 20 (NH_4 buffer, Bioline Ltd, London, UK) 1.5mM $MgCl_2$, 800µM total dNTP's, 0.4µM of each primer TBR1 and TBR2, 0.7 Units of BioTaq Red DNA polymerase (Bioline Ltd, London, UK) and one washed disc.

SRA / PLC Multiplex

T. b. rhodesiense is differentiated from *T. b. brucei* by detection of the serum-resistance-associated (SRA) gene. Simultaneous amplification of another single copy gene, a phospholipase C (PLC) sequence specific to *T. brucei* s.l., confirms whether there is sufficient *T. brucei* s.l. material present in the sample to detect the presence of *T. b. rhodesiense*. PCR was carried out in a 25µl reaction volume containing 3mM MgCl, 1.25µl of Rediload dye (Invitrogen), 1.5 Units Hot StarTaq, 0.2µM of each primer and one washed disc. The SRA gives a 669bp product, with a PLC band at 324bp.

For all PCRs, one negative control (water) and one positive control (genomic DNA) were run for every 16 samples, in addition to negative control blank discs. Thermal cycling was carried out in a DNA Engine DYAD™ Peltier thermal cycler. Cycling conditions and primer sequences can be seen in Table 5-2. All primers were sequenced by MWG Biotech.

PCR products were run on a 1.5% (w/v) agarose gel at 100V, stained with ethidium bromide and visualised under an ultraviolet transilluminator (Gel-Doc 2000, Bio-Rad).

PCR	Primer Sequence
TBR	TBR1 5'- CGA ATG AAT ATT AAA CAA TGC GCA GT-3'
(Moser *et al.*, 1989)	TBR2 5'- AGA ACC ATT TAT TAG CTT TGT TGC-3'
	Cycling Conditions: 94ºC for 3min, 30 cycles: 94ºC for 60sec, 55ºC for 60sec, 72ºC for 30s, final extension 72ºC for 5min
SRA/PLC Multiplex	SRA-F: 5'- GAA GAG CCC GTC AAG AAG GTT TG -3'
(Picozzi *et al.*, 2008)	SRA-R: 5'- TTT TGA GCC TTC CAC AAG CTT GGG -3'
	PLC-F: 5' - CGC TTT GTT GAG GAG CTG CAA GCA -3'
	PLC-R: 5' - TGC CAC CGC AAA GTC GTT ATT TCG -3'
	Cycling Conditions: 95ºC for 15min, then 45 cycles: 94ºC for 30sec, 63ºC for 90sec, 72ºC for 70s, final extension 72 ºC for 10min. Duplicate samples also run using 50 cycles.

Table 5-2: Primer sequences and cycling conditions for TBR and SRA/PLC PCR protocols

5.3.5 Statistical analysis

Calculations of prevalence and proportions

Prevalence by microscopy was calculated according to the classifications of trypanosome species by location within the tsetse fly (Lloyd & Johnson, 1924). Trypanosomes observed only in the mouthparts were classified as *Duttonella* (*T. vivax*-type), those found in both the midgut and mouthparts as *Nannomonas* (*T. congolense*-type), and those found in the salivary glands as *Trypanozoon* (*T. brucei*-type). Trypanosomes found only in the midgut are assumed to be immature infections with *Nannomonas* or *Trypanozoon*. Calculations on the proportion of samples testing positive for *T. brucei* s.l. by PCR, used the number of flies in which trypanosomes were observed by dissection as the denominator and were carried out for (a) flies in which trypanosomes were observed in mouthparts only, (b) flies in which trypanosomes were found in mouthparts and midgut and (c) flies in which trypanosomes were found in any location. Exact 95% binomial confidence intervals were calculated. Two sample tests for equality of proportions were used when comparing the proportion testing positive in two groups.

Risk factor analysis

Logistic regression was used to investigate the effect of tsetse species, sex and habitat on (a) the prevalence of trypanosomes by dissection and microscopy, and (b) the proportion of flies in which trypanosomes were observed by microscopy which then tested positive for *T. brucei* s.l. by PCR.

The univariate relationship for each variable was assessed first, by including each variable individually in a generalised linear model with binomial errors. Likelihood ratio tests (LRT) were used to compare this model to a null model, to determine the significance of each variable. Odds ratios (OR) with 95% confidence intervals were also calculated (LRTs and ORs were described fully in section 4.3.8). Variables were selected for inclusion in a multivariate model on the basis of the p-value generated by the LRT of the univariate analysis using a liberal p-value of 0.15 to reduce the risk of accidentally eliminating important variables.

Generalised linear models or generalised linear mixed effect models were used for multivariate analysis. Mixed effects models are often used to analyse data that is spatially or

temporally correlated, such as in hierarchical sampling strategies or longitudinal studies, by addressing both random and fixed effects (Paterson & Lello, 2003). Fixed effects influence only the mean of the response variable, and usually consist of the variables or treatment levels of interest. Random effects influence the variance of the response variable. Observations that contain the same random effect are correlated and therefore have non independent errors, which contravenes the assumptions of normal statistical modelling (Crawley, 2002). Mixed effects models address this non-independence of errors by modelling the covariance structure that the grouping introduces.

Analysis of prevalence of trypanosomes by dissection and microscopy

Models were constructed using trypanosome presence as a binary response variable with analysis conducted for:

a) Flies with trypanosomes observed in mouthparts only

b) Flies with trypanosomes observed in mouthparts and midgut

c) Flies with trypanosomes observed in any location (i.e. sum of (a) and (b) plus any flies with trypanosomes observed in midgut only)

Univariate analysis was carried out for the following explanatory variables: tsetse species (factor with two levels: *G. pallidipes* and *G. swynnertoni*); sex (factor with two levels: female and male) and habitat (factor with three levels: woodland, mixed woodland and savannah and savannah). Sampling site was also included as a factor with six levels.

Because two sampling sites were used in each habitat, effectively introducing pseudoreplication or spatial correlation, multivariate analysis was performed using generalised linear mixed effects models. Sampling site was included as a random effect to account for the variation associated with individual sampling sites, since the prevalence of individual sampling sites is not of interest, only the variability in prevalence between habitats.

Analysis of proportion of dissection-positive samples testing positive for *T. brucei* by PCR

For the second part of the analysis, the response variable was positive or negative by PCR. Only the flies which were tested by PCR were included in the model, e.g. for (a) below, only the flies in which trypanosomes were found by in the mouthparts by microscopy, which then went on to be analysed by PCR, were included in the model. Analysis was conducted for:

a) Flies testing PCR positive for *T. brucei* s.l. on mouthparts, out of those in which trypanosomes were found in the mouthparts only by microscopy.

b) Flies testing PCR positive for *T. brucei* s.l. on either mouthparts or midgut, out of those in which trypanosomes were found in the mouthparts and the midgut by microscopy.

c) Flies testing PCR positive for *T. brucei* s.l. on any organ, out of those in which trypanosomes were found in any location in the fly.

Analysis was carried out using the same explanatory variables described above.

All statistical analyses were carried out using R 2.7.2 (The R Foundation for Statistical Computing, http://www.r-project.org).

5.4 Results

5.4.1 Summary of flies examined

In total, 5428 tsetse were dissected and examined, comprising 1691 *G. pallidipes* and 3737 *G. swynnertoni*. The number of flies dissected in each site can be seen in Table 5-3, and the proportion of males and females in Table 5-4. *G. pallidipes* and *G. swynnertoni* were the predominant tsetse species in Serengeti National Park. In three study sites *G. swynnertoni* predominated and it proved impossible to collect the target number of *G. pallidipes* (3WS, 4WS and 6SV). In addition, targeted sampling of riverine vegetation trapped three *G. brevipalpis* but it was not possible to collect sufficient numbers for further analysis. No *G. longipennis* were trapped.

	Number of flies examined per site					
	1WD	2WD	3WS	4WS	5SV	6SV
G. pallidipes	501	501	32	105	544	8
G. swynnertoni	486	485	1022	689	479	576

Table 5-3: Number of flies of each species examined from each study site

Tsetse were collected for analysis from six study sites, with two study sites in each of the following habitat types: woodland (1WD, 2WD); mixed woodland/savannah (3WS, 4WS); savannah (5SV, 6SV).

	Female	Male
G. pallidipes	1150	541
G. swynnertoni	2289	1448

Table 5-4: Number of male and female flies of each species examined

The average age of male *G. swynnertoni* by wing fray analysis was 32 days (estimated from 606 flies). The average age of male *G. pallidipes* was 43 days (estimated from 313 flies). The distribution of fly ages by site can be seen in Figure 5-2.

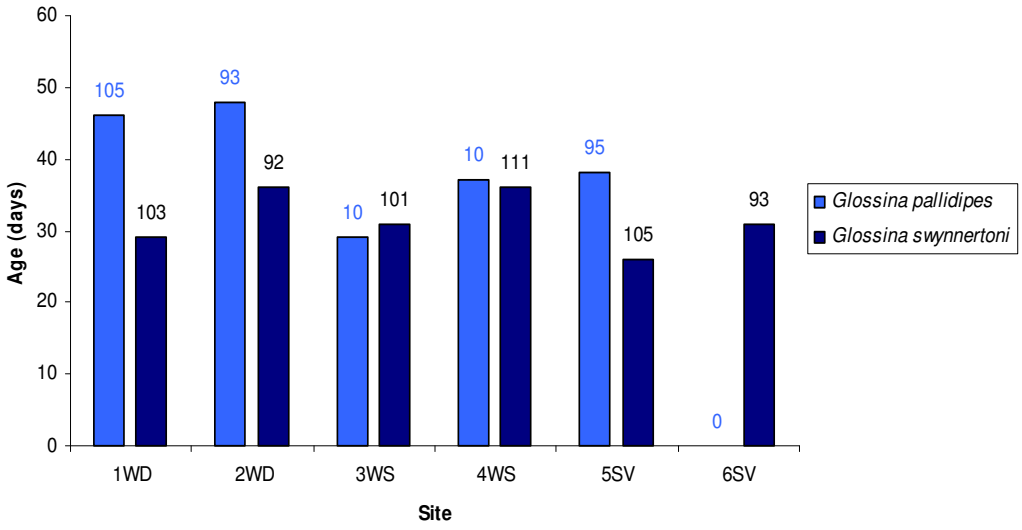


Figure 5-2: Average age of male *G. swynnertoni* and *G. pallidipes* in each study site

Age was assessed using wing fray analysis of male flies to give a population average for each tsetse species in each of the six study sites. Numbers above the bars indicate the number of flies assessed in each population.

5.4.2 Assessment of prevalence by dissection

Overall, trypanosomes were observed (in mouthparts, midgut, or both) in 3.7% of *G. pallidipes*, and 8.6% of *G. swynnertoni* examined. No salivary gland infections were seen. Using the classical trypanosome species identifications on location within the fly, the prevalence of *Duttonella, Nannomonas* and *Trypanozoon* can be seen in Table 5-5.

	G. pallidipes	***G. swynnertoni***
Duttonella	2.01 (1.4-2.8)	5.86 (5.1-6.7)
Nannomonas	1.42 (0.91-2.1)	2.09 (1.7-2.6)
Trypanozoon	0 (0-0.22)	0 (0-0.1)

Table 5-5: Prevalence of trypanosome infections identified according to location within the fly

The table shows the prevalence (%, with 95% binomial confidence intervals) of trypanosome infections in *G. swynnertoni* and *G. pallidipes*. Trypanosomes identified according to location in the tsetse fly: *Duttonella* - trypanosomes observed in mouthparts only; *Nannomonas* – mouthparts and midgut; *Trypanozoon* – salivary glands.

5.4.3 Assessment of prevalence by PCR

TBR positives in dissection-positive flies

Using PCR, *T. brucei* s.l. was detected in 22 *G. pallidipes*, out of the 63 flies in which trypanosomes were identified by microscopy, giving a proportion of 34.9% (CI 23.3-48.0). For *G. swynnertoni*, *T. brucei* s.l. was detected in 53 out of 323 flies, giving a proportion of 16.4% (CI 12.5-20.9). The location of these *T. brucei* s.l. positives within the flies can be seen in Table 5-6. Using the total number of flies dissected for each species as the denominator, the number of dissection-positive flies also testing positive for *T. brucei* s.l. by PCR out of the total number sampled for each species is 1.3% (CI 0.82-2.0) in *G. pallidipes*

and 1.4% (CI 1.0-1.8) in *G. swynnertoni*. These values are not significantly different (χ^2_1=0.047, p=0.83).

		Number of positive flies		
		MP+	**MG+**	**MP+MG+**
G. pallidipes	Microscopy	35	5	24
	PCR	7	2	MP+ only: 3 MG+ only: 6 MP+MG+: 4
G. swynnertoni	Microscopy	219	26	78
	PCR	20	12	MP+ only: 2 MG+ only: 15 MP+MG+: 4

Table 5-6: Number of flies positive on dissection which also tested positive for *T. brucei* s.l. by PCR

Flies in which trypanosomes were observed on dissection and microscopy were also analysed by PCR for *T. brucei* s.l.. The table shows the number of microscopy positives and the number of these which also tested positive by PCR, listed by part of the fly (MP+ mouthpart positive; MG+ midgut positive).

SRA/PLC multiplex PCR

All fly organs found positive by TBR PCR were analysed using the SRA multiplex PCR to differentiate *T. b. brucei* from *T. b. rhodesiense*. The SRA PCR was therefore performed on 83 mouthpart and midgut samples (from 75 flies) that had been found positive for *T. brucei* s.l.. Results are shown in Table 5-7. Only one fly was found positive using the SRA multiplex - a *G. swynnertoni* which was positive on both mouthparts and midgut (two positives in Table 5-7 are from the same fly).

	MP	MG
Analysed using SRA multiplex	40	43
PLC	4	10
SRA	1	1

Table 5-7: Number of tsetse samples testing positive for PLC and SRA

All samples testing positive by TBR PCR were also tested using PLC/SRA primers. SRA results are interpreted in conjunction with PLC results, which is included to confirm the presence of sufficient genetic material for the detection of the SRA sequence. One fly tested positive with SRA on both mouthparts and midgut (MP mouthparts; MG midgut).

TBR positives in dissection-negative flies

Seventy-eight flies in which no trypanosomes were found in any part of the fly by microscopy were also applied to FTA cards. For each fly, the mouthparts, midgut and salivary glands were each analysed by PCR. In total, *T. brucei* s.l. was identified in 17 of these flies. The location of these *T. brucei* positives can be seen in Table 5-8. Overall 21.8% (CI 13.2-32.6) of flies in which no trypanosomes were observed by microscopy tested positive for *T. brucei* s.l. by PCR. None of these samples positive for *T. brucei* s.l. revealed any PLC or SRA bands using the SRA multiplex PCR.

	Number of flies examined	MP+	MG+	SG+	MP+MG+	MP+SG+
G. swynnertoni	78	4	2	9	1	1

Table 5-8: *T. brucei* s.l. positives by PCR in dissection negative flies

All organs from seventy eight dissection negative flies were also analysed with TBR primers. The table indicated the number of flies testing positive: MP+ mouthpart positive; MG+ midgut positive; SG+ salivary gland positive.

For clarity, a summary of all microscopy and PCR results is shown in Figure 5-3 and 5-4.

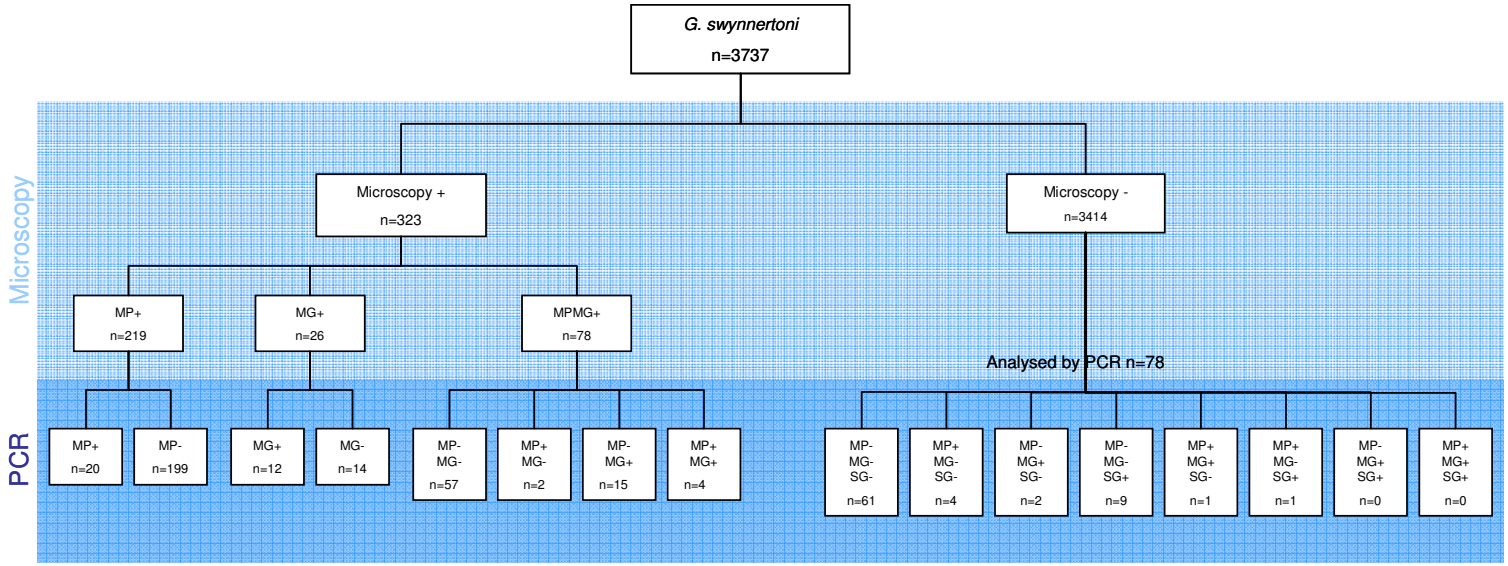


Figure 5-3: Summary of microscopy and PCR results for *G. swynnertoni*

Figure indicates (a) total number of flies analysed; (b) number of flies positive and negative by microscopy; (c) number of flies in which trypanosomes were observed listed by organ; (d) number of flies testing positive on PCR analysis with TBR primers (MP mouthparts; MG midgut; SG salivary glands).

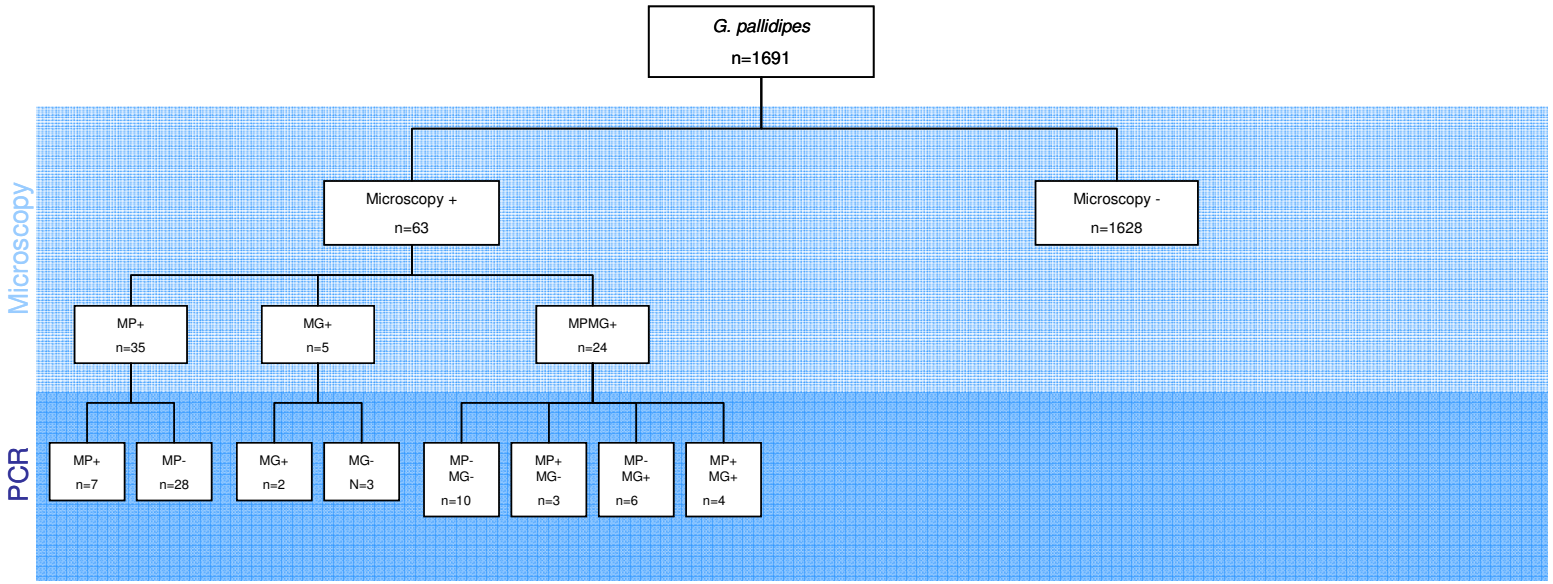


Figure 5-4: Summary of microscopy and PCR results for *G. pallidipes*

Figure indicates (a) total number of flies analysed; (b) number of flies positive and negative by microscopy; (c) number of flies in which trypanosomes were observed listed by organ; (d) number of flies testing positive on PCR analysis with TBR primers (MP mouthparts; MG midgut; SG salivary glands).

5.4.4 Assessment of risk factors using dissection and microscopy data

The effect of tsetse species, sex, study site and habitat on the proportion of flies with trypanosomes present in (a) mouthparts only, (b) mouthparts and midgut and (c) trypanosomes found in any location (including midgut only) was assessed. A summary of univariate analysis is shown in Table 5-9.

There were significant differences in the prevalence of trypanosomes between *G. pallidipes* and *G. swynnertoni* when looking at flies with trypanosomes in the mouthparts only ($p<0.001$), or with trypanosomes anywhere in the fly ($p<0.001$), but not for mouthpart and midgut infections ($p=0.065$). There were no significant differences in prevalence between male and female flies. There were significant differences in prevalence between sample sites ($p<0.02$) and habitats ($p<0.01$). The prevalence by habitat and species is shown in Figure 5-5.

Multivariate analysis was then performed. Sex was not included in the final multivariate model since no statistically significant differences were observed on univariate analysis. A mixed effects model was used to assess the differences in prevalence between habitats, with study site included as a random effect. Separate models were built for *G. pallidipes* and *G. swynnertoni* in order to investigate any differences in risk factor effects between species.

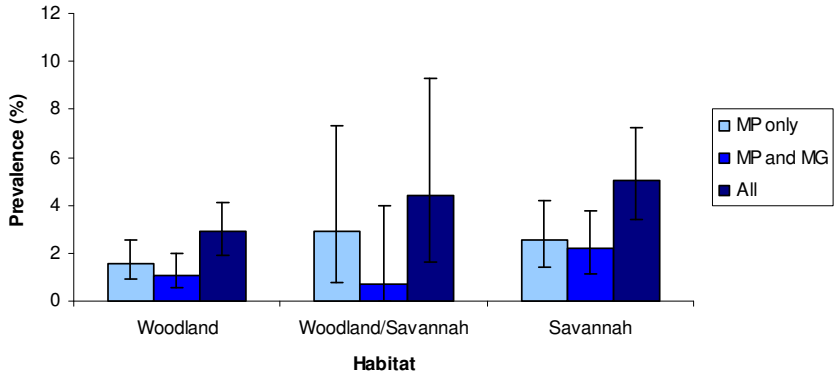
The outputs of multivariate analysis are summarised in Table 5-9. There were no statistically significant differences in prevalence of trypanosomes between habitats for *G. pallidipes*. However for *G. swynnertoni*, there were significant differences in prevalence of trypanosomes in mouthparts, mouthparts and midgut, and in flies with trypanosomes present at any location between different habitats ($p<0.049$).

	Levels	Mouthparts only		Mouthparts and midgut		Trypanosomes present	
		χ^2 and p-value	OR (CI)	χ^2 and p-value	OR (CI)	χ^2 and p-value	OR (CI)
Univariate Analysis							
Fly species	*G. pallidipes*	χ^2_1=44.9, **p<0.001**	NA	χ^2_1=3.41, p=0.065	NA	χ^2_1=46.2, **p<0.001**	NA
	G. swynnertoni		3.03 (2.1-4.4)		1.52 (0.96-2.4)		2.41 (1.8-3.2)
Sex	Female	χ^2_1=2.08, p=0.15	NA	χ^2_1=1.61, p=0.2	NA	χ^2_1=3.44, p=0.063	NA
	Male		0.82 (0.63-1.1)		0.76 (0.50-1.2)		0.81 (0.65-1.0)
Site	1WD	χ^2_5=37.6, **p<0.001**	NA	χ^2_5=13.3, **p=0.02**	NA	χ^2_5=39.0, **p<0.001**	NA
	3WS		1.89 (1.2-3.0)		1.32 (0.68-2.6)		1.62 (1.1-2.3)
	2WD		0.8 (0.46-1.4)		0.53 (0.22-1.3)		0.72 (0.47-1.1)
	5SV		1.4 (0.87-2.3)		1.76 (0.93-3.3)		1.46 (1.0-2.1)
	4WS		2.47 (1.6-3.9)		1.67 (0.85-3.3)		1.99 (1.4-2.9)
	6SV		2.35 (1.4-3.8)		1.48 (0.7-3.1)		1.83 (1.2-2.7)
Habitat	Woodland	χ^2_2=29.9, **p<0.001**	NA	χ^2_2=10.3, **p=0.01**	NA	χ^2_2=33.7, **p<0.001**	NA
	Savannah		1.94 (1.4-2.7)		2.16 (1.3-3.6)		1.86 (1.4-2.5)
	Savannah/woodland		2.38 (1.7-3.3)		1.9 (1.2-3.2)		2.06 (1.6-2.7)
Multivariate Analysis							
G. pallidipes							
Habitat	Woodland	χ^2_2=3.24, p=0.2	NA	χ^2_2=1.34, p=0.51	NA	χ^2_2=3.31, p=0.19	NA
	Savannah		1.84 (0.89-3.8)		2.21 (0.51-9.6)		1.86 (1.1-3.2)
	Savannah/woodland		1.98 (0.65-6.1)		0.69 (0.06-7.6)		1.54 (0.63-3.8)
G. swynnertoni							
Habitat	Woodland	χ^2_2=7.06, **p=0.029**	NA	χ^2_2=6.0, **p=0.049**	NA	χ^2_2=8.08, **p=0.018**	NA
	Savannah		1.64 (1.1-2.5)		2.18 (1.1-4.3)		1.60 (1.2-2.2)
	Savannah/woodland		1.67 (1.2-2.4)		1.91 (1.0-3.7)		1.54 (1.1-2.1)

Table 5-9: Summary of univariate and multivariate analysis of the effects of fly species, sex and habitat on the prevalence of trypanosomes by dissection and microscopy

The effect of fly species, sex and habitat were analysed by univariate logistic regression with binomial errors. Fly species and habitat significantly effected prevalence. A multivariate mixed effects model with study site included as a random effect showed habitat to have a significant effect on prevalence for *G. swynnertoni* but not *G. pallidipes*. Analysis was conducted separately for flies with trypanosomes present in mouthparts only, mouthparts and midguts, and trypanosomes present in any location. Table shows chi squared values (χ^2), p values and odds ratios (OR) with 95% confidence intervals (CI).

(a)



(b)

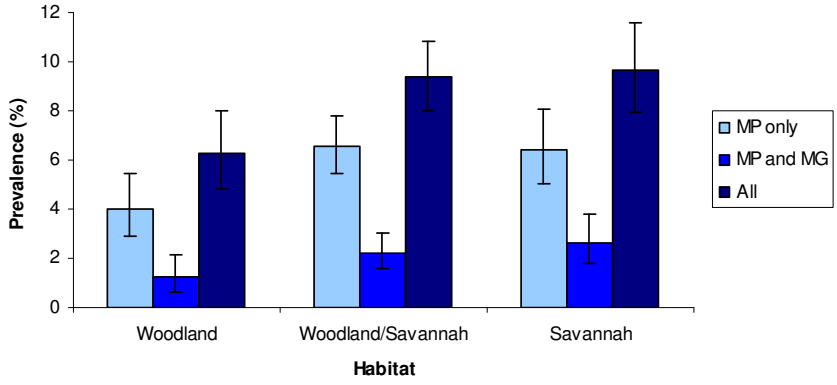


Figure 5-5: Prevalence of trypanosome infections in tsetse by microscopy in different habitats for (a) *G. pallidipes* and (b) *G. swynnertoni*

The graphs shows the proportion of flies in which trypanosomes were observed by microscopy in (a) mouthparts (MP); (b) mouthparts and midgut (MP and MG); and (c) any location in the fly (all), according to habitat, with exact binomial 95% confidence intervals.

5.4.5 Assessment of risk factors using PCR data

The effect of fly species, sex, sampling site and habitat on the proportion of flies in which trypanosomes were found by dissection, that also tested positive for *T. brucei* s.l. were assessed.

A summary of univariate analysis is shown in Table 5-10. There was a statistically significant difference in the proportion of samples testing positive by PCR between *G. pallidipes* and *G. swynnertoni* from flies positive on mouthparts and midgut (p=0.011), and flies in which trypanosomes were found in any location (p=0.002), but not from those in which trypanosomes were found in the mouthparts only. There was a statistically significant difference between male and female flies in those where trypanosomes were found in the mouthparts only (p=0.027). If analysis was performed for each fly species individually, the difference between sexes was significant for *G. swynnertoni* (p=0.013) but not for *G. pallidipes* (p=0.86). There were no statistically significant differences between sampling sites and habitats, with species combined or with separate analysis for *G. pallidipes* and *G. swynnertoni*. The proportions of flies testing positive by species and sex are shown in Figure 5-6.

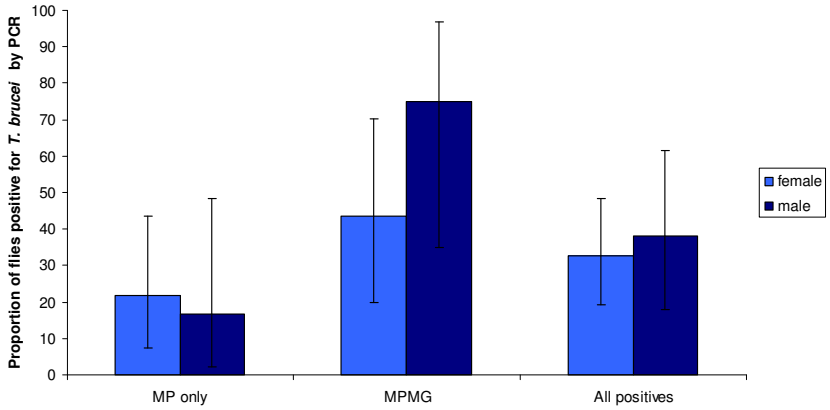
A multivariate model including fly species and sex was constructed. This did not qualitatively alter the significance of the variables, and interactions between species and sex were not statistically significant. Therefore only the simpler univariate analysis is presented here.

		Mouthparts only		Mouthparts and midgut		Trypanosomes present	
	Levels	**χ^2 and p-value**	**OR**	**χ^2 and p-value**	**OR**	**χ^2 and p-value**	**OR**
Univariate Analysis							
Fly species	*G. pallidipes*	χ^2_1=3.02, p=0.082	NA	χ^2_1=6.48, **p=0.011**	NA	χ^2_1=9.69, **p=0.002**	NA
	G. swynnertoni		0.41 (0.16-1.2)		0.29 (0.11-0.76)		0.38 (0.21-0.68)
Sex	Female	χ^2_1=4.89, **p=0.027**	NA	χ^2_1=0.44, p=0.51	NA	χ^2_1=0.42, p=0.52	NA
	Male		0.33 (0.11-0.98)		1.35 (0.0.56-3.3)		0.83 (0.48-1.5)
Site	1WD	χ^2_5=4.06, p=0.54	NA	χ^2_5=4.92, p=0.43	NA	χ^2_5=7.16, p=0.21	NA
	3WS		1.92 (0.37-9.9)		0.92 (0.24-3.6)		1.25 (0.54-2.9)
	2WD		2.0 (0.31-13.1)		0.21 (0.02-2.2)		0.81 (0.27-2.5)
	5SV		2.72 (0.52-14.1)		1.1 (0.3-4.0)		1.6 (0.68-3.7)
	4WS		0.81 (0.13-5.1)		0.75 (0.18-3.1)		0.68 (0.27-1.7)
	6SV		2.06 (0.37-11.4)		0.30 (0.05-1.9)		0.63 (0.22-1.8)
Habitat	Woodland	χ^2_2=1.75, p=0.41	NA	χ^2_2=0.2, p=0.91	NA	χ^2_2=0.54, p=0.76	NA
	Savannah		1.68 (0.56-5.1)		1.19 (0.39-3.6)		1.25 (0.63-2.5)
	Savannah/woodland		0.95 (0.31-2.9)		1.28 (0.42-3.9)		1.05 (0.54-2.0)

Table 5-10: Univariate analysis of the proportion of dissection positive flies testing positive for *T. brucei* s.l. by PCR

The effect of fly species, sex and habitat were analysed by univariate logistic regression with binomial errors. Fly species and sex significantly effected prevalence. Analysis was conducted separately for flies with trypanosomes present in mouthparts only, mouthparts and midguts, and trypanosomes present in any location. Table shows chi squared values (χ^2), p values and odds ratios (OR) with 95% confidence intervals (CI).

(a)



(b)

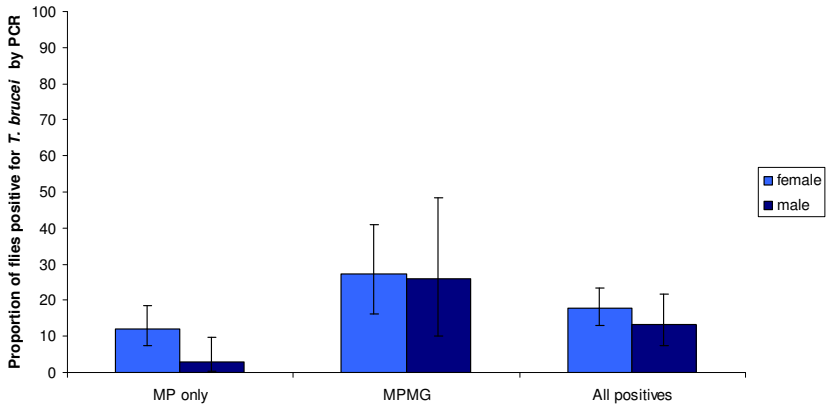


Figure 5-6: Proportion of dissection positive flies testing positive for *T. brucei* s.l. by PCR for (a) *G. pallidipes* and (b) *G. swynnertoni*

The graphs shows the proportion of flies that tested positive with TBR PCR primers, out of the in which trypanosomes were observed by microscopy in (a) mouthparts (MP); (b) mouthparts and midgut (MP and MG); and (c) any location in the fly (all), according to habitat, with exact binomial 95% confidence intervals.

Discussion

In this study 5428 tsetse were dissected and examined for the presence of trypanosomes. Over 700 tsetse organs were analysed using PCR primers which amplify *T. brucei* s.l., and samples testing positive were analysed again with PCR primers which differentiate *T. b. brucei* and *T. b. rhodesiense*.

By microscopy, no salivary gland infections were found, suggesting that the prevalence of *T. brucei* s.l. is very low. However frequent identification of *T. brucei* s.l. by PCR illustrated the difficulties in interpreting the results of microscopy and PCR. As with previous studies, classification of trypanosome species based on microscopy was at odds with PCR data.

The finding that the proportion of dissection positive flies which tested positive for *T. brucei* s.l. by PCR was much higher in *G. pallidipes* than *G. swynnertoni* is particularly significant as it suggests that *G. pallidipes* is potentially as important as *G. swynnertoni* in the transmission of HAT. This species has been mostly ignored in previous studies. Spatial heterogeneity in the prevalence of trypanosomes in *G. swynnertoni* by microscopy suggests the risk of disease transmission varies by habitat.

5.4.6 Analysis of prevalence by dissection and microscopy

Trypanosomes were classified as *Duttonella, Nannomonas* or *Trypanozoon* according to location within the tsetse and the prevalence of each group was calculated. The prevalence of *Nannomonas* in *G. swynnertoni* of 2% (CI 1.7-2.6) was identical to previous studies. The prevalence of *Duttonella* of 6% (CI 5.1-6.7) is also consistent with that found by other authors, which has ranged from 4-17% (Moloo *et al.*, 1971; Rogers & Boreham, 1973; Malele *et al.*, 2007). *G. pallidipes* have been less extensively studied but as before, the prevalence of *Duttonella* and *Nannomonas* were found to be lower than in *G. swynnertoni*. No salivary gland infections were found in either tsetse species. In line with other studies, the prevalence of salivary gland infections detectable by microscopy is very low (Moloo *et al.*, 1971; Rogers & Boreham, 1973; Moloo & Kutuza, 1974).

5.4.7 Analysis of prevalence by PCR

Overall, *T. brucei* s.l. was identified by PCR in 35% (CI 23-48) of *G. pallidipes* and 16% (CI 13-21) of *G. swynnertoni* in which trypanosomes were observed by microscopy. Other PCR based studies have wide variations in methodology, but others with similar protocols reported proportions of 15% of *G. pallidipes* in Kenya and 50% in *Glossina palpalis palpalis* in Cameroon (Morlais *et al.*, 1998; Njiru *et al.*, 2004a).

Flies with trypanosomes found in mouthparts only

Twenty percent (CI 8-37) of *G. pallidipes* and 9% (CI 6-14) of *G. swynnertoni* in which trypanosomes were observed in the mouthparts only, tested positive for *T. brucei* s.l. by PCR. These would all have been classified as *Duttonella* or *vivax*-type trypanosomes by microscopy only. The presence of *T. brucei* s.l. DNA in the mouthparts of flies where trypanosomes were observed in the mouthparts only has also been reported in other studies (Morlais *et al.*, 1998; Lefrancois *et al.*, 1999; Njiru *et al.*, 2004a). This could result from contamination from ingestion of a blood meal infected with *T. brucei* s.l.. Alternatively infections may be classified as mouthpart-only if the number of trypanosomes in the midgut (and potentially salivary glands) was low, and missed on microscopy. It has been suggested that trypanosomes may be lost from the midgut in mature *T. brucei* s.l. infections, although there is little data to support this (Peel, 1962).

Flies with trypanosomes in midgut only

Forty percent (CI 5-85) of *G. pallidipes* and 46% (CI 27-67) of *G. swynnertoni* in which trypanosomes were observed in the midgut only tested positive for *T. brucei* s.l. by PCR. By microscopy these would be assumed to be immature infections of either *Nannomonas* or *Trypanozoon* and excluded from prevalence calculations. Since PCR can detect very small numbers of trypanosomes, *T. brucei* s.l. could also detected in blood meals after feeding from a parasitaemic host, and the prevalence of *T. brucei* s.l. in tsetse midgut may be more useful as a reflection of the overall prevalence in the host population.

Flies with trypanosomes in mouthparts and midgut

Out of flies in which trypanosomes were observed by microscopy in the mouthparts and midgut, 54% (CI 32-74) of *G. pallidipes* and 27% (CI 18-38) of *G. swynnertoni* tested

positive for *T. brucei* s.l. by PCR. By microscopy, trypanosomes present in mouthparts and midgut would be classified as *Nannomonas* trypanosomes. The finding that a substantial proportion of these contain *T. brucei* s.l. in the mouthparts, midgut or both suggests that some of these in fact represent immature *T. brucei* s.l. infections. These results may also arise from mixed *Nannomonas* and *Trypanozoon* infections, or blood meal contamination with *T. brucei* s.l. as described above.

Dissection-negative flies

If the number of trypanosomes present in a fly is very small, it is more likely to be detected by PCR than by microscopy, so the detection of trypanosomal DNA in flies that appeared negative by microscopy is not surprising. Small numbers of trypanosomes, escaping detection by microscopy, may arise from early stages of infection, or from trypanosomes present in ingested blood. In experimental studies using dead trypanosomes, DNA has been found to survive in the tsetse midgut for 12 days, so even in the absence of an immature infection, *T. brucei* s.l. from several blood meals may still be detected (Raj, 2007). Similar results have been found for lymphatic filariasis, with detection of *Brugia malayi* DNA possible up to three weeks after ingesting microfilaria-positive blood, even in non-competent vector strains (Fischer *et al.*, 2007).

However, it is surprising to find dissection-negative flies with salivary glands that test positive for *T. brucei* s.l. by PCR. Whilst there is no reason to doubt the results (no evidence of contamination in sequence of results, all negative controls negative, high specificity of TBR primers (Desquesnes & Davila, 2002)) repetition may be sensible. A potential biological explanation could be the presence of mature infections where parasites have disappeared from the midgut and mouthparts, leaving a small number of trypanosomes or genetic material in the salivary glands that were not detected by microscopy.

If these are genuine positives, the most important question is whether these represent transmissible infections. One method for further investigation would be to carry out dissection as before, then divide dissection-negative salivary glands into two parts, using one part to inoculate mice and test for transmissibility, and performing PCR on the second to check for the presence of *T. brucei* s.l. DNA. Parasitaemic mice would suggest transmissible trypanosomes present in numbers too low to be detected by microscopy, whilst *T. brucei* s.l.

positives by PCR in the absence of parasitaemia in mice suggests either false positives or that the trypanosomes present are not in a transmissible form. Alternatively, further information could be obtained from the existing dataset using transmission models to test the likelihood of 13% of dissection negative flies carrying transmissible *T. brucei* s.l. infections.

5.4.8 Integrating microscopy and PCR data

These results reinforce the impression that dissection and microscopy is not a reliable technique on which to base estimates of the prevalence of transmissible infections (Otieno, 1983), highlighted by the problems of integrating microscopy and PCR data (Lehane *et al.*, 2000; Njiru *et al.*, 2004a). In the words of other authors this is "unfortunate" (Njiru *et al.*, 2004a p32) and "potentially alarming" (Lehane *et al.*, 2000 p589). However, little progress has been made as to how to interpret this information to give us useful estimates of the prevalence of transmissible infections for epidemiological studies.

T. brucei s.l., arguably the most important to assess due to its potential for human infection, presents a particular problem. The majority of studies of *T. brucei* s.l. prevalence by microscopy have calculated a prevalence of zero, even when thousands of flies have been examined. However, PCR of mouthparts, midgut and salivary glands have all revealed surprising amounts of *T. brucei* s.l. DNA, from both microscopy positive and negative flies.

It has been suggested that some infections which would be classed as immature by microscopy, may actually be transmissible. For example, inoculation of trypanosomes found in the mouthparts, from flies with trypanosomes present in the mouthparts and midgut by dissection, gave rise to *T. brucei* s.l. infections in mice, both in laboratory and field studies (Otieno, 1978; Otieno, 1983). However, rodent inoculation studies in SME using pooled tsetse showed similarly low levels of *T. brucei* s.l. infection (9 isolates out of 11 060 flies) (Moloo & Kutuza, 1974).

The question then arises as to whether it is possible to develop protocols from which the prevalence of transmissible infections could be estimated accurately. The gold standard for assessing transmissibility is rodent inoculation, but its use is limited for large scale field studies. However if the number of transmissible infections could be correlated with parameters that are easier to measure, such as prevalence of *T. brucei* s.l. in mouthparts or

midguts by PCR, this may provide a method which would be appropriate for epidemiological studies.

To some extent this approach has been taken for other pathogens, where the prevalence of immature stages is used to estimate a prevalence of transmissible infections, or directly to assess risk. For example in assessing prevalence of WNV in mosquitoes, most screening programs test the whole mosquito, detecting mosquitoes with any trace of WNV present, rather than testing the salivary glands, which would give the rate of transmissible infections (Gu *et al.*, 2008). PCR studies for *Onchocerca volvulus* on samples of *Simulium* spp. body (rather than head) give a prevalence of infected flies, but not the prevalence of transmissible infections (Rodriguez-Perez *et al.*, 1999). However, it is first necessary to be certain of the relationship between the test result and the number of transmissible infections.

Other vector-borne disease studies often use pooled samples, particularly in areas of low prevalence, relying on statistical algorithms to estimate prevalence, to allow assessment of much larger numbers of vectors (Katholi *et al.*, 1995; Martin-Sanchez *et al.*, 2006; Rodriguez-Perez *et al.*, 2006). With a few exceptions (for example Njiru *et al.*, 2004a analysed pooled negative flies), pooled samples have not been widely used for studies on tsetse. Given the extremely low prevalence of *T. b. rhodesiense*, pooled analysis may be useful, but obviously still requires interpretation of what is actually represented by PCR positive flies.

Current techniques are still valuable for assessing spatiotemporal trends, or for looking at presence and absence of a pathogen, for example in a particular vector species or location, although it should be borne in mind that detection of a trypanosome species by PCR in a potential vector is not necessarily evidence of cyclical transmission. However, currently almost every author takes a different approach to sample collection and analysis. There is clearly a need to develop consistent protocols to generate data that allows epidemiological comparisons.

5.4.9 Risk factors for trypanosome infection

The effects of tsetse species, sex and habitat were assessed on (a) the prevalence of trypanosomes in tsetse by dissection and microscopy, and (b) the proportion of dissection-positive flies that tested positive for *T. brucei* s.l. by PCR.

Tsetse Species

The prevalence of flies with trypanosomes observed in the mouthparts only, and with trypanosomes found anywhere in the fly differed significantly between *G. swynnertoni* and *G. pallidipes*. The difference in prevalence for mouthpart and midgut infections also approached statistical significance. If trypanosomes were assigned to species groups according to their location in the fly, the prevalence of *T. vivax* was 3.0 times higher (CI 2.1-4.4) and the prevalence of *T. congolense* 1.5 times higher (CI 0.96-2.4) in *G. swynnertoni* compared to *G. pallidipes*.

This trend has been reported in other studies in Serengeti, although the majority have focussed on *G. swynnertoni*. This could arise either from differing exposure to trypanosomes i.e. from different host feeding patterns, or from differences in the proportion of trypanosomes that survive to immature or mature infections, i.e. differences in vector competence (the innate ability of tsetse to be refractory to trypanosome infections).

Traditionally *G. swynnertoni* are considered to feed predominantly on suids, and *G. pallidipes* predominantly on bovids (Weitz, 1963). In SME *G. swynnertoni* are adaptable, but feed mostly on warthog and buffalo (Moloo *et al.*, 1971; Rogers & Boreham, 1973). The feeding habits of *G. pallidipes* in SME have not been studied. In other areas they feed on buffalo, bushbuck and warthog (Clausen *et al.*, 1998). This would suggest that the feeding habits of the two species may not be sufficiently different to explain the difference in trypanosome prevalence. However, since these studies on host feeding preferences have been conducted in different ecosystems, and have not considered the relative densities of available hosts, they are unlikely to provide sufficient detail and accuracy to draw conclusions about feeding preferences and trypanosome prevalence. Further work on the host feeding preferences of *G. pallidipes* and *G. swynnertoni* in SME would help to answer this question.

It is interesting to note that the proportion of dissection positive flies that then tested positive for *T. brucei* s.l. by PCR is 2.7 times higher (CI 1.5-4.8) for *G. pallidipes* than for *G. swynnertoni*. This suggests that although *G. swynnertoni* has a higher prevalence of trypanosomes overall, the proportion of these contributed by *T. brucei* s.l. is higher in *G. pallidipes*. Calculating the number of dissection positive flies that also test positive for *T. brucei* s.l. by PCR out of the total number sampled for each species, there is no difference between *G. pallidipes* (1.3% CI 0.8-2.0) and *G. swynnertoni* (1.4% CI 1.0-1.8).

This has important implications. *G. pallidipes* has been somewhat ignored by previous studies in SME because lower trap catches and lower prevalence by dissection have suggested it is less important as a vector of *T. brucei* s.l. than *G. swynnertoni*. However, both of these reasons may be unjustified. Whilst time constraints precluded counting trap catches in this study, it was observed that in some locations the number of *G. pallidipes* was very high – in some cases over 500 flies per trap per day. The extremely low prevalence of salivary gland infections found previously has precluded statistical assessment of *G. pallidipes* as a vector of *T. brucei* s.l., and in the absence of any other information, the assumption has been made that the prevalence of trypanosomes in any location in the tsetse by microscopy is an indicator of the prevalence of *T. brucei* s.l.. It seems this may not be the case. Whether the prevalence of *T. brucei* s.l. by PCR in dissection-positive flies, as presented here, can be used as an indicator for the prevalence of transmissible *T. b. rhodesiense* is so far unclear, but further work to explore the vector role of *G. pallidipes* is important.

Analysis of the role of *G. brevipalpis* and *G. longipennis* was not possible since no *G. longipennis*, and very small numbers of *G. brevipalpis* were caught, even when targeting sampling to areas of suitable riverine habitat. *G. longipennis* has only been reported once in SME (Mlengeya *et al.*, 2003). Since *G. longipennis* and *G. brevipalpis* are morphologically similar and can be hard to differentiate, and the distribution of *G. longipennis* is not usually predicted to extend this far west in Tanzania (Rogers & Robinson, 2004), further confirmation of the presence of *G. longipennis* in SME may be necessary. *G. brevipalpis* cannot be excluded as a vector of *T. brucei* s.l.. However, the refractoriness of *G. brevipalpis* to *T. brucei* s.l. infection in laboratory studies (Moloo & Kutuza, 1988), combined with its very limited distribution in SNP, and its disinclination to feed from man (Clausen *et al.*, 1998), suggest it is unlikely to play an important role.

Sex

No statistically significant differences were found in the prevalence of trypanosome infections by dissection and microscopy between male and female flies. However there was a statistically significant difference in the proportion of mouthpart dissection-positive flies that then tested positive for *T. brucei* s.l. by PCR between male and female *G. swynnertoni*. The proportion of flies testing positive was 4.9 (CI 1.1-22) times higher in female flies compared to male flies. Other authors have found sex to have a significant effect on prevalence but have not published details of this ratio (Lehane *et al.*, 2000). Maturation of *T. brucei* s.l. infections under laboratory conditions is affected by the sex of the tsetse, with male flies maturing more *T. brucei* s.l. infections than females (Dale *et al.*, 1995). However carrying a salivary gland infection brings a fitness cost and mortality in male infected flies may therefore be higher (Maudlin *et al.*, 1998). A potential explanation for the higher rate of *T. brucei* detection in female flies is that since *T. brucei* s.l. infections in male flies are more likely to mature, and potentially lead to mortality, *T. brucei* s.l. would be more commonly detected in female flies, where it is more likely to remain as an immature infection and not affect fly survival. However, the fitness cost of *T. brucei* s.l. may not manifest in male flies in the field since parasite-induced mortality is not observed until flies are aged over approximately 50 days, which is unusual in wild populations (Maudlin *et al.*, 1998).

Habitat

By multivariate analysis, statistically significant differences in the prevalence of trypanosomes were observed for *G. swynnertoni* between the three habitat types sampled. The prevalence of *G. swynnertoni* with trypanosomes present in any location was 1.6 times higher (CI 1.2-2.2) in savannah, and 1.5 times higher (CI 1.1-2.1) in mixed savannah and woodland, when compared to woodland. There was no significant difference in the prevalence of trypanosomes in *G. pallidipes* between habitats.

This is the first reporting of statistically significant differences in trypanosome prevalence between difference habitats in the same ecosystem. There are a number of factors which may vary between habitats that could provide potential explanations for this variation. Firstly, trypanosome prevalence in tsetse is affected by population age, with larger numbers of flies carrying trypanosomes in older populations. Variation in trypanosome prevalence between habitats could result from differences in age structure of the tsetse populations. However the

data collected in this study on the average age of tsetse in each habitat does not support this. The average age of the tsetse population from woodland is consistent with the populations in savannah and mixed savannah and woodland; the savannah population, with the highest prevalence, in fact had the lowest average age.

A second potential explanation is that the differences in prevalence arise from variation in the species composition and density of wildlife hosts between habitats, leading to differences in host feeding patterns. It is interesting that differences were observed for *G. swynnertoni* but not for *G. pallidipes*. This may simply be a function of sample size – both the number of *G. pallidipes* examined and the proportion with trypanosomes were lower, decreasing the likelihood of a significant result. However, it is possible that species differences could account for this result. The feeding habits of *G. swynnertoni* are known to be adaptable, so the normal diet of a *G. swynnertoni* population may vary between habitats. Whilst the feeding habits of *G. pallidipes* in SME are less clear, a fly species with less catholic tastes may seek out the same species in any habitat, leading to a more homogeneous prevalence. As discussed above, no recent studies have been carried out in SME on feeding habits of either species so blood meal analysis results reported in the literature have been used. Assessment of host feeding preferences using PCR based methods recently described would be valuable. Blood meal samples were collected from each study site as part of this study, but analysis was outwith the scope of this thesis.

Thirdly, differences in prevalence in the wildlife host population between different habitats could lead to variation in prevalence in the tsetse population. The statistically significant relationships between the density of *G. pallidipes* and *G. swynnertoni* and the prevalence of trypanosomes in lions and hyaenas (Chapter 4) suggest that prevalence is spatially heterogeneous, at least in some species. However differentiating the effects of trypanosome prevalence in hosts from the effects of host species composition is not possible without more detailed knowledge of tsetse feeding habits.

No salivary gland infections were found by microscopy in this study. If the prevalence of trypanosomes overall by dissection and microscopy is an indicator for the prevalence of *T. brucei* s.l. and ultimately *T. b. rhodesiense*, the spatial heterogeneity in prevalence also suggests heterogeneity in risk of transmission to man. Any attempt to generate maps estimating risk of disease transmission in SME would have to take this into account.

5.4.10 Study design and methodology

This chapter describes a cross-sectional study performed during the dry season. The annual migration of wildebeest and zebra, and to some extent Thomson's gazelle and eland, causes large seasonal changes in the diversity and density of hosts available. Seasonal variations occur in both the density and activity (Challier, 1982) and trypanosome infection rates (Woolhouse *et al.*, 1994; Msangi *et al.*, 1998) of tsetse. A longitudinal study was outwith the scope of this project; however it would be valuable to build on this study to assess temporal trends.

A stratified design allowed sampling over a range of habitats, to give an accurate estimate of prevalence overall, whilst allowing assessment of the effect of habitat on prevalence. However, due to the necessity of transporting flies to the laboratory at Seronera twice daily, sample sites were selected within 40km of Seronera, and within 1km of a road. In addition, flies could not be sampled from dense woodland or grassland. Therefore firm conclusions can only be drawn about open woodland, savannah and mixed woodland/savannah habitats in this central area of SNP. However, there is no obvious reason to believe that these habitats are not representative of other similar parts of the SNP and extrapolation of these results is probably justifiable.

This study was randomised as far as possible; study sites were randomly selected, and as far as possible all viable non-teneral flies caught were examined, in order to prevent non-random selection of flies for examination out of the total caught. All traps introduce some bias into the sample. Traps such as the epsilon trap are known to catch more female flies, older flies, and flies at later stages of the hunger cycle. However, recording the sex of each fly examined, and assessing average age in each study site allowed the potentially confounding effects of these variables to be considered in analysis.

PCR analysis in this study only targeted *T. brucei* s.l.. Although *T. brucei* s.l. is of most interest due to its potential for human infection, identification of the other trypanosome species would have been useful. Approximately 200 samples of tsetse organs in this study were also analysed using primers based on the ribosomal internal transcribed spacer regions, used in Chapters 3 and 4 to identify the trypanosomes circulating in wildlife. However it appears that the ITS primers described by Cox *et al* (2005) are not appropriate for identifying

trypanosomes in tsetse. The resulting agarose gels contained a very high number of bands, which were often at unexpected sizes. This may be due to the inconsistency between expected and obtained sequence lengths discussed in Chapter 3. It is also possible that the primers do not work in the presence of the large amount of trypanosomal DNA likely to be present in these samples. It may be possible to optimise these primers for use on samples from tsetse. Alternatively the TRYP ITS primers appear to be more successful in identifying trypanosomes in tsetse (Adams *et al.*, 2006).

5.5 Conclusions

This study highlights the problems, raised by other authors, in interpreting the results of microscopy and PCR to give meaningful estimates of the prevalence of transmissible trypanosome infections. This is a serious concern which questions long term assumptions about the development of trypanosomes in tsetse and the meaning of microscopy findings. The ability to generate data to use in studies on transmission and disease ecology is likely to be limited until this is resolved.

Although *G. pallidipes* plays an important role in HAT transmission in other areas, its potential importance in *T. brucei* s.l. transmission in SME has not been previously recognised. The spatial heterogeneity observed in the prevalence of trypanosome infections in tsetse may arise for several reasons, which deserve further research. This heterogeneity would have to be taken into account if an attempt was made to develop the maps of predicted tsetse density (Chapter 2) into maps of disease risk.

Chapter 6: Discussion

Unravelling the complexities of a disease with multiple wildlife hosts and multiple tsetse vector species is not trivial. After over a century of anecdotal evidence, field observations, experimental studies and conjecture, the role of wildlife in the transmission of trypanosomes is still unclear. However, recently frameworks used in the studies of other vector-borne diseases with wildlife reservoirs showed that not only is it possible to understand transmission, but that spatiotemporal predictions of human disease risk and targeted control are realistic aims, even in such complex systems. With the challenge of these ideas, the promise showed by new diagnostic tests, and renewed concern about human African trypanosomiasis (HAT) in Serengeti due to cases in tourists, a new look at this system was due.

The questions regarding trypanosome transmission in a complex ecosystem were not all going to be answered in one thesis. The general aims of this thesis were therefore to critically review the existing literature, and to establish base line values for prevalence of trypanosomes in host and vector populations. Accordingly, Chapter 1 reviewed the frameworks used to study transmission of other vector-borne pathogens with wildlife reservoirs, and background information on trypanosomiasis, and Chapter 2 provided information on the Serengeti Mara ecosystem (SME), and summarised trypanosome research specific to Tanzania and the SME. Chapter 3 assessed the use of ITS PCR in wildlife. Chapter 4 explored this data further by analysis of risk factors associated with carrying trypanosome infections in wildlife at a species and individual level. Chapter 5 set out to assess the prevalence of trypanosomes in *Glossina swynnertoni* and *Glossina pallidipes*, the main tsetse species in the SME, and explored the difficulties of obtaining meaningful estimates of the prevalence of transmissible infections.

6.1 Detecting trypanosomes in wildlife and tsetse populations

The use of ITS primers for the first time on blood samples collected from wildlife identified a range of trypanosome species, providing information on host range and trypanosome diversity. The potential identification of *Trypanosoma godfreyi* and *Trypanosoma simiae* Tsavo in warthog is particularly exciting as this is the first report of these species in wildlife hosts. The range and diversity of trypanosomes found in wildlife in this study is likely to be

reflected in the tsetse population. *Trypanosoma congolense, Trypanosoma brucei brucei*, *Trypanosoma brucei rhodesiense, Trypanosoma simiae, T. simiae* Tsavo and *T. godfreyi* have been identified before in tsetse in SME (Adams *et al.*, 2006). The sample size in the study by Adams *et al* was small (n=700) however, given the low prevalence of trypanosomes in the vector population, and further work to establish precise prevalence estimates is necessary. In this study only the prevalences of *Trypanosoma brucei* sensu lato and *T. b. rhodesiense* were assessed in tsetse. However the parallel sample sets collected from wildlife and tsetse in this study provide a huge opportunity for further work. Midguts from engorged flies, also collected during this study, provide material for blood meal analysis. Analysis of trypanosome prevalence in wildlife and tsetse in conjunction with the feeding preferences of tsetse provides the opportunity for identification and quantification of the transmission of each trypanosome species. Given the spatial heterogeneity in the prevalence of trypanosomes in *G. swynnertoni*, it would be particularly interesting to examine the relationship between wildlife species density and diversity, and the prevalence of different trypanosome species in tsetse.

However, this study identified a major hurdle which will have to be overcome before any attempts at quantifying transmission are possible. Current techniques do not allow the prevalence of transmissible trypanosome infections in tsetse to be estimated. Several authors have highlighted the inadequacies of differentiating trypanosome species on the basis of location within the fly, a technique accepted for many years. Development of molecular techniques such as PCR confirmed the low specificity of the dissection and microscopy method. Whilst PCR has been useful in identifying trypanosomes circulating in tsetse populations, little consideration has been given to how to interpret PCR data for use in epidemiological studies.

The main difficulty is how to correlate positive results by PCR with the prevalence of mature, and therefore transmissible, infections. For example, a positive PCR result for *T. brucei* s.l. in a midgut sample indicates that trypanosomal DNA has been detected in the fly. This could be due to the present of a mature infection (if trypanosomes were also present in the salivary glands), an immature infection, the presence of *T. brucei* s.l. in a recent blood meal, or simply remnants of trypanosomal DNA from previous blood meals or infections. Other studies have not addressed this question; PCR results are often presented in such a way that an unwary reader could interpret them as the prevalence of transmissible infections. If

there is a consistent relationship between the number of flies testing positive for *T. brucei* s.l. by PCR and the number of mature *T. brucei* s.l. infections, and this ratio is quantified, PCR provides a useful indicator of prevalence by which smaller sample sets can yield useful results (compared to the many thousands of flies which must be analysed by dissection). However, evidence that the proportion of trypanosomes which are able to mature is influenced by factors such as temperature may make this unlikely.

Until this is resolved, interpretation of PCR data from tsetse must be done with care, and studies aiming to generate epidemiological parameters should give careful consideration to the meaning of data generated.

6.2 Wildlife as reservoirs of HAT

Proving the existence of a disease reservoir is always difficult. It is hard to conceive a method by which this could definitively be done for HAT. However, some conclusions can be drawn through consideration of existing evidence.

The potential candidates for reservoirs of HAT in the SME are wildlife, livestock and man. It has already been established that for HAT caused by *T. b. rhodesiense*, with its short duration of infection, and therefore infectiousness, man does not act as an important reservoir for tsetse, except perhaps in an epidemic situation (Welburn *et al.*, 2006). In SME, the hypothesis that the sporadic occurrence of HAT results from spillover into wildlife from infected cattle reservoirs in the surrounding areas does deserve consideration. However there are several pieces of evidence that do not support this. Firstly, anecdotal evidence (reviewed in Chapter 1) describes the continuing occurrence of HAT in areas cleared of humans and livestock. Secondly the geographical distribution of wildlife samples testing positive for *T. brucei* s.l. and *T. brucei rhodesiense* does not correlate with increased transmission towards the borders of the protected areas, where cattle would occur (Figure 4-3). This is true even when migratory species and carnivores (which could eat species which have migrated away from the borders) are excluded. Therefore wildlife are certainly the most likely candidates for trypanosome reservoirs in SME.

The role of livestock in this disease system remains unclear. *T. b. rhodesiense* has been identified at low prevalence (1%, Kaare *et al.*, 2007) in cattle herds around the protected areas. The question of direction of pathogen movement is always a difficult one to address. Given the importance of cattle as reservoirs of HAT in other areas, it would not be surprising if both wildlife and cattle populations were able to maintain HAT transmission independently.

6.3 The importance of individual wildlife species as reservoirs of HAT

Over the last century, studies have identified *T. brucei* s.l. by microscopy in a wide range of wildlife species. The use of molecular techniques in this study identified *T. brucei* s.l. in cheetah for the first time. In general the results obtained in this study were consistent with those reported previously, providing useful validation of older, microscopy-based studies.

It is intriguing that the prevalence of *T. brucei rhodesiense* was so high in warthogs in a previous study compared to this one, despite the consistencies in study site and sample analysis. The potential hypothesis of long term variation in prevalence which correlates with human incidence highlights the need for longitudinal studies of trypanosome prevalence in wildlife. Longitudinal studies are difficult to conduct due to the logistical and financial difficulties of sampling sufficient numbers of animals over a sufficient period of time. However, single cross-sectional studies are unable to incorporate features which are potentially important in natural ecosystems, such as seasonal variation, and the need for longitudinal studies may be unavoidable in understanding transmission dynamics (Hazel *et al.*, 2000).

Whilst the prevalence of *T. brucei* s.l. in warthogs was not significantly higher than in other species, warthogs contained the highest diversity of trypanosomes, with *T. congolense, T. simiae*, *T. simiae Tsavo* and *T. godfreyi* (pending confirmation) also identified. *T. vivax* has also been identified in warthogs (Kaare *et al.*, 2007). *T. godfreyi, T. simiae* and *T. simiae* Tsavo frequently group together in mixed infections in tsetse (Lehane *et al.*, 2000). This is unsurprising given that all these species were detected in warthogs in this study, and that warthogs are popular food sources for both *G. swynnertoni* and *G. pallidipes* (Moloo *et al.*, 1971; Rogers & Boreham, 1973; Clausen *et al.*, 1998). Whether or not warthogs are

ultimately important in transmission of *T. b. rhodesiense*, they are certainly an interesting species for further studies on the diversity, classification and interactions of trypanosomes.

Whilst a high prevalence of *T. brucei* s.l. and *T. congolense* in lions has been reported before, this was the first study to include a reasonable number of samples from spotted hyaena (n=78). The prevalence in hyaena was also high, supporting the hypothesis that carnivores may become infected through alternative routes of transmission. Despite high prevalence of trypanosome infections lion and hyaena are not commonly fed on by any of the species of tsetse present in SME (<0.3% of feeds, Clausen *et al.*, 1998). Lions carrying trypanosome infections are found on the grassland plains, where no tsetse can persist (Figure 4-3). This adds to a body of evidence, reviewed in Chapter 1, which supports the theory that carnivores can become infected via consumption of infected prey.

In Chapter 4, significant positive relationships were found between tsetse density and trypanosome prevalence in Felidae and Hyaenidae. Although perhaps initially appearing to contradict the theory of infection via consumption, if the prevalence of trypanosome infections in prey species increases with tsetse density, exposure via consumption will also increase. Whilst no statistically significant relationship was found between prevalence of trypanosome infections in other species and tsetse density, this may have been more of a reflection of changing species composition in areas of higher tsetse density. This could be investigated further by assessing how prevalence varies with tsetse species in individual prey species, and incorporating lion feeding habits. In areas where there are no tsetse, such as the grassland plains, prey species such as wildebeest and Thomson's gazelle still carry trypanosome infections. These animals also spend time in the woodlands, where they are exposed to tsetse, on their annual migration cycles. The relative importance of infection via tsetse compared to infection via consumption of infected prey is not known. Although the prevalence of trypanosomes is high in lion and hyaena, they are unlikely to play an important role in transmission since they are rarely fed on by tsetse.

6.4 Where next with wildlife reservoirs?

In other multihost, vector-borne disease systems, different host species vary widely in their importance in pathogen transmission. This ability can be measured using the parameters of realised reservoir competence (the probability that a vector feeding on an individual of a

given species becomes infected), or reservoir potential (the average number of infected vectors produced by an individual of a given species) (reviewed in Chapter 1). Reservoir potential is the product of realised reservoir competence and the number of vectors fed by an individual of a given species.

Realized reservoir competence can also be assessed as a product of the probability the individual host will be infected, or infection prevalence, and the probability that if the host is infected it will be able to transmit the infection to a feeding vector, or infectivity. These parameters, and factors that may affect them, are summarised in Figure 6-1, adapted from a framework used for investigation of Lyme Disease (Brunner *et al.*, 2008).

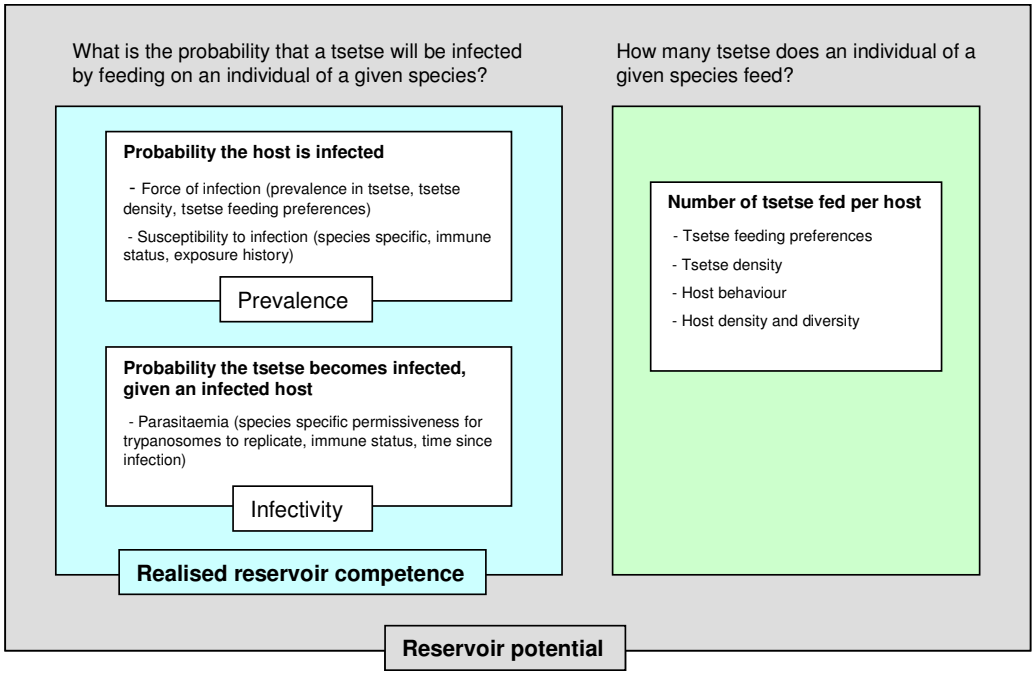


Figure 6-1: The relationships between components of reservoir potential

Reservoir potential is determined by the number of tsetse fed per host, and the realised reservoir competence, which is a product of prevalence and infectivity of host species (adapted for trypanosomiasis from Brunner *et al*., 2008).

The parameters of prevalence and number of tsetse fed per host can be estimated from data collected during this study and from literature values. How important is the parameter of infectivity? Brunner *et al* (2008) found prevalence and infectivity to be strongly positively

correlated for hosts of Lyme disease. It could be argued that the behaviour of PCR analysis and tsetse are similar - the likelihood of detecting trypanosomes by PCR or of a feeding tsetse becoming infected, is a function of the length and degree of parasitaemia in the host, particularly at the generally low levels of parasitaemia common in wildlife. Is it possible to simply use PCR prevalence as an indicator of realized reservoir competence? This assumes there are no factors which determine whether a tsetse becomes infected or not, once it is feeding on a host, other than parasitaemia.

If this is the case, the product of this measure of realized reservoir competence and the number or proportion of vectors which feed on a given species gives an indication of reservoir potential. This would give a measure of the importance of each wildlife species as reservoirs and would be a valuable exercise. However, it is complicated by the existence of two vector species, present at different densities and with different feeding preferences.

6.5 Application to other areas

Whilst wildlife does not appear to play an important role in HAT transmission in Uganda, in other areas where wildlife is present at higher densities the situation may be similar to SME. Investigating the consistency of the results obtained in this study across different ecosystems would be particularly interesting. SME has an unusually high density and diversity of wildlife, with the added complexity of annual migration cycles. However, research in protected areas in Zambia suggests that at least some features are consistent, such as particularly high prevalence of *T. brucei* s.l. and *T. congolense* in lions (N. Anderson, pers. comms.). A recent case control study in Western Tanzania suggested that seeing wildlife (lions, elephants, hyaenas and monitor lizard) was a risk factor for HAT cases (Matemba, 2008). This was only significant on univariate analysis, becoming non-significant in a multivariate model. However further investigations confirming wildlife as a risk factor for infection would suggest that wildlife play an important role in HAT transmission in other areas. Over 40% of Tanzania is comprised of protected areas (World Resources Institute, 2007), where the highest densities of wildlife are found. If wildlife does provide a source of infection for HAT, people working in, or living close to, other protected areas where tsetse are present should be a priority for disease surveillance.

6.6 Implications for disease management

Human African trypanosomiasis remains a serious concern in Serengeti National Park. The fear is predominantly related to the threat to the tourist industry of a perceived disease risk to tourists. The public health burden of the disease outside the protected area remains unclear. No cases feature in the national level HAT records, but the likelihood of correct HAT diagnosis at a district hospital around SNP is low. However, it is also possible that low incidence outside SNP reflects land use change to arable farming, with little vegetation remaining for tsetse. As Mr William Ngowo of Maswa GR stated of the neighbouring area, inhabitated by Sukuma people: "Sukuma and trees can't live together". The protected areas, with their high density of tsetse, may reflect the highest risk area for HAT transmission. Two HAT cases reportedly occurring last year support this: both were in men entering the protected area illegally to hunt wildlife.

Identification of unusually high prevalence of *T. b. rhodesiense* in warthogs in SME in a previous study had suggested that options for control strategies targeted at warthogs should be explored. However, this study did not confirm the importance of warthogs, and development of these control strategies cannot now be justified. Control strategies aimed at the wide range of species in which *T. brucei* s.l. has been identified are impossible, and unless future research proves that a particular species is vital in transmission, control will have to continue focusing on the tsetse vector.

Spatial heterogeneity in distribution (Chapter 2) and prevalence (Chapter 5) of trypanosome infections in tsetse show that the risk of disease transmission to man is not uniform. Tsetse challenge is determined by the density of tsetse, prevalence of *T. b. rhodesiense* and the proportion of tsetse feeding on man. Neither *G. swynnertoni* nor *G. pallidipes* feed preferentially on man, so feeds on man are likely to be opportunistic, or occur if wildlife hosts are unavailable (for example if there is movement of animals away from an area due to migration or drought).

Tsetse challenge is therefore likely to predominantly reflect density and prevalence, and a measure of overall disease risk could be calculated by incorporating tsetse challenge for both *G. swynnertoni* and *G. pallidipes*. Production of a map to illustrate the heterogeneity in disease risk is likely to be of use, particularly with respect to focusing tsetse control

operations from limited resources. Validation of risk maps would, however, be almost impossible, due to the small number of cases, and the difficulty of determining where people became infected.

In summary, this thesis has begun the process of understanding a disease which is intertwined with every aspect of Serengeti – wildlife, tsetse, vegetation, climate, poverty, tourism. Hopefully future research can build on the work in this thesis to further the understanding of the transmission dynamics of HAT in this complex ecosystem.

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Appendix 1: Larger Mammal Species of the Serengeti-Mara region

(Mduma & Hopcraft, 2008)

Order Primates	
Papio anubis	Olive baboon
Colobus guereza	Black and white colobus
Erythrocebus patas baumstarki	Ikoma patas monkey
Cercopithecus aethiops	Vervet monkey
Galago crassicaudatus	Greater galago
Galago senegalensis	Bushbaby, lesser
Order Pholidota	
Manis temmicki	Ground pangolin
Order Lagomorpha	
Lepus capensis	Cape hare
Lepus crawshayi	Crawshay's hare
Pronolagus rupestris	Red rock hare
Order Rodentia	
Hystrix crisata	North African crested porcupine
Hystrix africae australis	Cape crested porcupine
Pedetes capensis	Spring hare
Order Carnivora	
Panthera leo	Lion
Panthera pardus	Leopard
Acinonyx jubatus	Cheetah
Leptailurus serval	Serval
Caracal caracal	Caracal
Felis sylvestris	African Wildcat
Canis aureus	Golden jackal
Canis mesomelas	Black-backed jackal
Canis adustus	Side-striped jackal
Lycaon pictus	African wild dog
Octocyon megalotis	Bat-eared fox
Ictonyx striata	Zorilla
Poecilogale albinucha	African striped weasel
Melivora capensis	African honey badger
Viverra civetta	African civet
Nandinia binotata	Palm civet
Genetta genetta	Common genet
Herpestes ichneumon	Egyptian mongoose
Herpestes sanguineus	Slender mongoose

Helogale undulata	Dwarf mongoose
Herpestes paludinosus	Marsh mongoose
Mungos mungo	Banded mongoose
Ichneumia albicaudata	White-tailed mongoose
Proteles cristatus	Aardwolf
Crocuta crocuta	Spotted hyaena
Hyaena hyaena	Striped hyaena
Lutra maculicollis	Spotted-necked otter
Aonyx capensis	Cape clawless otter
Order Tubulidentata	
Orycteropus afer	Aardvark
Order Proboscidea	
Loxodonta africana	African elephant
Order Hyracoidea	
Dendrohyrax arboreus	Tree hyrax
Heterohyrax brucei	Bush hyrax
Procavia capensis	Rock hyrax
Order Perissodactyla	
Equus burchelli	Burchell's zebra
Diceros bicornis	Black rhinoceros
Order Artiodactyla	
Potamochoerus porcus	Bushpig
Phacochoerus africanus	Warthog
Hylochoerus meinertzhageni	Giant forest hog
Hippopotamus amphibius	Hippopotamus
Giraffa camelopardalis	Giraffe
Sylvicarpa grimmia	Grey duiker
Raphicerus campestris	Steinbuck
Ourebia ourebi	Oribi
Oreotragus oreotragus	Klipspringer
Madoqua kirkii	Kirk's dikdik
Redunca redunca	Bohor reedbuck
Redunca fulvorufula	Mountain reedbuck
Kobus defassa	Defassa waterbuck
Aepyceros melampus	Impala
Gazella thomsoni	Thomson's gazelle
Gazella granti	Grant's gazelle
Hippotragus equinus	Roan antelope
Damaliscus korrigum	Topi
Alcephalus buselaphus	Coke's hartebeest
Connochaetes taurinus	Wildebeest

Tragelaphus scriptus	Bushbuck
Tragelaphus strepsiceros	Greater kudu
Tragelaphus imberbis	Lesser kudu
Taurotragus oryx	Eland
Oryx beisa	Orvx
Syncerus caffer	African buffalo

Appendix 2: Health management of horses under high challenge from trypanosomiasis: A case study from Serengeti, Tanzania

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Veterinary Parasitology 154 (2008) 233–241
veterinary parasitology
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Health management of horses under high challenge from trypanosomes: A case study from Serengeti, Tanzania

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Received 5 September 2007; received in revised form 23 February 2008; accepted 27 February 2008

Abstract

Horses kept for recreational riding purposes by a wildlife tourism company in a heavily tsetse fly-infested region of north-western Tanzania were systematically monitored to investigate the occurrence, presentation and management of tsetse-transmitted trypanosomosis. During a 23-month period, 18 clinical cases were diagnosed (*Trypanosoma brucei* or *Trypanosoma congolense* were identified) and treated and trypanosomes were implicated of involvement in four deaths. Pyrexia consistently aided early detection (17 cases). Ataxia, weight loss and anaemia were seen in chronic cases and conferred a poor prognosis. Delaying treatment by more than 2 days from the onset of clinical signs led to prolonged disease course and more severe anaemia. Early detection, prompt treatment, thorough post-treatment health monitoring and rigorous prophylactic measures helped keep clinical cases to manageable levels, but re-infection remained a constant, insidious threat.

Keywords: Horse; Tanzania; *Trypanosoma* spp

1. Introduction

Tsetse-transmitted trypanosomes, protozoan blood-borne parasites which cause potentially fatal disease in horses, are a major constraint to horse keeping in Africa, with the more trypanosome tolerant donkeys used where draught power is required (Barrowman, 1990). However, many aspects of trypanosomosis in horses remain poorly understood, and previous studies have only focussed on the management of single outbreaks. With the growth of horse safaris within the expanding wildlife tourism industry, management of horses under trypanosome challenge is likely to become increasingly important.

Horses are susceptible to infection with *Trypanosoma brucei*, *T. congolense* and *T. vivax* (Stephen, 1970). During an outbreak of trypanosomosis in Kenya in 1990 all three trypanosome species were isolated. *T. congolense* was considered the main species responsible for clinical signs. Both *T. congolense* and *T. vivax* were identified as sole pathogens in sick horses. *T. brucei* was only present in mixed infections and was considered incidental (Kihurani et al., 1994). The implication of *T. brucei* as the sole pathogen in an outbreak in Zambia in 1970 (Awan and Johnston, 1979) reveals some uncertainty regarding its role in causing disease. However, with the advent of molecular

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doi:10.1016/j.vetpar.2008.02.034

technologies, tools now exist to differentiate species with a high degree of accuracy compared to morphological examination often previously relied on.

Clinical signs recognised in association with trypanosomosis in horses include ataxia, paralysis and ventral oedema. Pyrexia, anaemia, anorexia, jaundice, tachycardia and keratoconjunctivitis may also be reported (Kihurani et al., 1994; Stephen, 1970; Taylor and Authie, 2004). The frequency of these clinical signs and their value as diagnostic or prognostic indicators has not previously been evaluated, although it has been suggested that differences in disease presentation exist between trypanosome species.

Maintenance of horse populations in tsetse-infested areas relies on chemotherapy for treatment and prophylaxis. Most trypanocidal drugs have been developed for use in cattle or camels. Treatment regimens in horses have been extrapolated from these species, but are associated with uncertain efficacy and frequent side effects.

Diminazene aceturate is widely used in cattle at a dose rate of 3.5–7 mg/kg bodyweight. When used at 3.5 mg/kg bodyweight in horses, side effects are common: of seven horses treated for *T. brucei* infection in Zambia, all became anorexic, and three developed ataxia (Awan and Johnston, 1979), and in treatment of *T. evansi* in Thailand four out of eight horses developed moderate to severe side effects including oedema, hypersalivation, recumbancy, restlessness and dsypneoa, which was fatal in one case (Tuntasuvan et al., 2003). Diminazine is rapidly excreted and considered to have no prophylactic effect.

Isometamidium chloride is used in horses at a recommended dose of 0.5 mg/kg as both treatment and prophylaxis. The period of prophylactic cover is variable but in general lasts for 2–4 months in cattle. Severe local reaction at the injection site is commonly reported (Kinabo and Bogan, 1988).

Quinapyramine is no longer used in cattle but is produced for the treatment of *T. evansi* in camels. In addition to quinapyramine sulphate, Triquin® (Wocklhardt Europe Ltd., Ireland) contains quinapyramine chloride which forms a depot at the injection site and provides prophylactic cover in camels for approximately 3 months. Quinapyramine has been used to treat *T. brucei* and *T. evansi* infections in horses. Side effects of hypersalivation, restlessness and colic are described; the proportion of horses affected is not reported (Awan and Johnston, 1979; Leach and Roberts, 1981; Maqbool et al., 1996).

Melarsomine is used for treatment of *T. evansi* infections in camels and horses. Efficacy is also reported against *T. brucei* in horses (Raynaud et al., 1989). Melarsomine has no prophylactic activity.

We describe 2 years of health monitoring in a well-managed stable of horses that are kept for recreational riding purposes by a wildlife tourism company operating in a heavily tsetse-infested region of north-western Tanzania. Documentation of natural infections gave the opportunity for detailed study into the presentation and management of equine trypanosomosis. This case study evaluates (i) the clinical picture seen in natural infections of horses; (ii) a range of diagnostic methods used; and (iii) the success of treatment and prophylactic regimens attempted.

2. Materials and methods

2.1. Study area

The study population of horses is owned by Singita-Grumeti Reserves Ltd., and kept adjacent to Grumeti Game Reserve, a wildlife protected area which borders the north-west of Serengeti National Park in northern Tanzania. The property has an area of about 2500 ha which is dominated by acacia wooded grassland and has very high densities of *Glossina swynnertoni* and *G. pallidipes*. Tsetse populations vary seasonally, increasing after the start of the rainy season and declining during the dry season (Challier, 1982). Sixteen horses (predominately thoroughbred) are kept by the tourist lodge and ridden in the surrounding area for game viewing. Horses are periodically bought and sold to maintain a constant population in the stable. All introductions come from areas outside the tsetse fly belt and are thus naïve to trypanosome infection.

2.2. Health monitoring and diagnostic procedures

Systematic health monitoring was carried out between January 2005 and November 2006, following cases of undiagnosed and recurrent illness. A diagnostic protocol was established for rapid identification of trypanosome infections and medical records were kept for all horses which detailed clinical signs during disease episodes. Any change from normal demeanour or appetite was noted. Rectal temperatures of all horses were routinely taken twice daily and those above 38.5 °C were then taken repeatedly throughout the day. Pyrexia was recorded if rectal temperature exceeded 39 °C. Anaemia or jaundice was initially assessed subjectively by examination of buccal and scleral mucous membranes and the limbs, sheath and ventral areas were examined for oedema. Tachycardia and

cardiac arrhythmia were frequently detected on auscultation carried out once pyrexia was noticed, and as such were often recorded as presenting signs.

Jugular venipuncture was performed on all horses showing pyrexia or other clinical signs consistent with trypanosome infection, and the blood collected in 10 ml heparinized tubes. Fresh blood films were examined microscopically on site. Blood samples were then centrifuged and a Giemsa-stained buffy coat smear examined microscopically. Following a negative result, the above tests were repeated daily, during periods of pyrexia if observed. Anaemia was assessed by measuring packed cell volume (PCV) by centrifugation of blood samples in micro-haematocrit capillary tubes, and carried out both routinely in healthy horses to establish the normal PCV of each horse, and during episodes of disease. Anaemia was recorded if the PCV fell below 30%.

2.3. Molecular analysis

Samples of heparinized blood from microscopy-positive horses were applied to Whatman Classic FTA Cards for characterisation of trypanosome species using polymerase chain reaction (PCR) in the United Kingdom. Cards were sealed in foil pouches with desiccant and maintained at room temperature. Two 2 mm punches per sample were analysed using a PCR which detects differences in length of the internal transcribed spacer (ITS) regions of the trypanosome ribosomal genes, enabling differentiation of the main African trypanosome species (Cox et al., 2005). The wash protocol, primer sequences and PCR conditions were as detailed by Cox et al. (2005).

Samples showing band sizes specific for *T. brucei* sensu lato were screened for the presence of the human-serum-resistance-associated (SRA) gene to differentiate *T. brucei brucei* from *T. b. rhodesiense* (pathogenic to humans), following the protocol described by Picozzi et al. (2005).

2.4. Treatment regimen

Of the four trypanocides described above, two were used in this study. Melarsomine was unavailable in this area during the study period. Diminazine was not used due to concerns over side effects and lack of prophylactic cover. Quinapyramine is regarded as the most effective treatment for *T. brucei* in horses (Fenger, 2004) and was therefore chosen for treatment (Triquin®, Wockhardt Europe Ltd., Ireland). The severe side effects caused by quinapyramine meant that isometamidium (Trypamidium-Samorin®, Merial, France) was preferred for routine prophylaxis. However, isometamidium is more difficult to administer, therefore quinapyramine was used in fractious horses.

When trypanosomes were found on microscopic examination of fresh blood film or buffy coat, parenteral treatment was immediately administered using quinapyramine (0.025 ml of suspension per kg bodyweight subcutaneously, when one bottle of Triquin® (1.5 g quinapyramine sulphate, 1 g quinapyramine chloride) was made up with 15 ml sterile water). Flunixin meglumine (Finadyne®, Norbrook, UK) was used as supporting treatment for alleviation of pyrexia. A vitamin/mineral supplement (Mirablud®, Bayer, South Africa) was given for 10 days to counter anaemia. Recovering horses were rested for at least 10 days, or approximately 1 week per day of pyrexia. Buffy coat samples were examined daily after treatment until parasites disappeared and PCV was monitored regularly during recovery.

2.5. Prevention

Quinapyramine (Triquin®, Wockhardt Europe Ltd., Ireland) (in 8 horses) and isometamidium (Trypamidium-Samorin®, Merial, France) (in 12 horses) were administered as prophylaxis. Horses were only covered by chemoprophylaxis for part of the study period due to unavailability of drugs at the start of the study.

A combination of physical and chemical measures was put in place to reduce tsetse fly density near the stable complex where horses spent the majority of their time. Acetone-baited tsetse control targets (Vale et al., 1988) treated with deltamethrin (Glossinex®, Ecomark Ltd., Zimbabwe) were deployed in the grazing paddocks and around the stables. Commercial equine insect repellents containing a synthetic pyrethroid (Ultrashield®, Absorbine, USA) were applied topically. Nighttime usage of stable lighting was minimised to reduce the attraction of tsetse flies and other insects, and thick vegetation was cut back around the buildings.

2.6. Statistical analysis

A positive case was defined as parasites being found by microscopy, in combination with at least one clinical sign consistent with trypanosomosis. Incidence was calculated as the number of new cases per horse-year at risk and incidence rate ratio used to compare periods when horses were covered by chemoprophylaxis to periods they were not. For further analysis, only the first case recorded for each horse was considered. At the

beginning of the study period, difficulties were experienced obtaining drugs for treatment and treatment was delayed in some cases. Cases treated within 2 days of the first recorded clinical signs were regarded as treated promptly. Cases in which treatment was administered after more than 2 days were regarded as having delayed treatment. After assessment using the Anderson–Darling test for normality, a two-sample *T*-test was used to compare the PCV in cases where treatment was delayed to cases treated promptly. Fishers exact tests were used to identify associations between clinical signs and case outcome, and between treatment delay and resolution of clinical signs. Statistical analysis was conducted in R 2.4.1 (The R Foundation for Statistical Computing, http://www.r-project.org).

3. Results

Records were examined for 24 horses, present for all or part of the 23-month study period. Eighteen cases of trypanosome infection occurred in 14 horses.

3.1. Trypanosome species identification

Blood samples were collected onto FTA card from six of the eighteen cases, and were positive for *T. brucei* s.l. (three cases), *T. congolense* (one case) and *T. brucei* s.l. and *T. congolense* together (one case) by PCR. One sample was negative. Further analysis of the four *T. brucei* s.l. positive samples ruled out the human infective sub-species, *T. b. rhodesiense*.

3.2. Clinical signs and treatment

The clinical signs observed in each case were varied (Fig. 1). Depression, pyrexia and jaundice were the most common clinical signs (Fig. 1). At presentation, pyrexia was the most common clinical sign, either alone or in combination with other signs; only in one case was pyrexia not observed (Fig. 2).

Most horses responded quickly to treatment with quinapyramine, with pyrexia and oedema disappearing within 1 or 2 days (Fig. 3). In cases treated promptly (within 2 days of clinical signs first being observed) clinical signs were statistically significantly more likely to resolve within 5 days after treatment (Fishers Exact test, *P*-value 0.029). The distribution of case outcomes can be seen in Table 1.

Whilst cases that resolved quickly generally showed pyrexia, jaundice, depression and anorexia (Fig. 3), long-standing cases after delayed treatment were characterised by ataxia, anaemia, weight loss and behavioural changes (Fig. 4). Pyrexia and jaundice were not observed in the later phases of chronic cases. Ataxia, weight loss and severe anaemia (PCV < 20%) were all statistically significant risk factors in case fatality (Fishers Exact test, *P*-values <0.01, 0.011, 0.015, respectively) (Table 1).

PCV (normal range 30–46% (Knottenbelt, 2005)) was reduced in every case of trypanosomosis (Fig. 5). Anaemia was severe when treatment was delayed, reaching as low as 12% in two cases. PCV was statistically significantly lower when treatment was delayed by more than 2 days (mean PCV 18.1%,

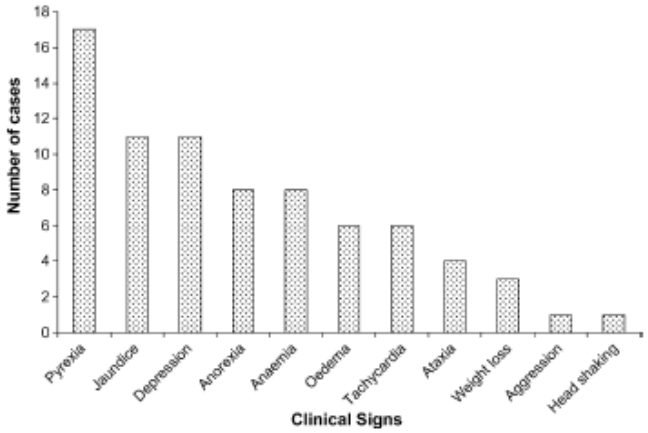

Fig. 1. Relative frequency of clinical signs observed during cases of trypanosomosis in horses ($n = 18$).

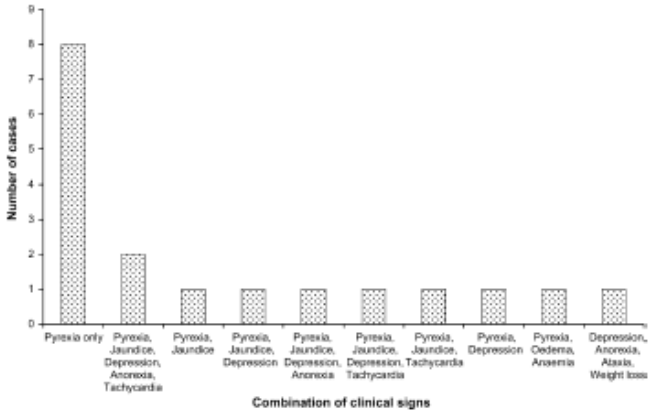

Fig. 2. Combinations of clinical signs seen *at presentation* in cases of trypanosomosis in horses ($n = 18$).

Fig. 5b, calculated from means of lowest PCV observed for each case during a 1-month period post-presentation) compared to cases treated within 2 days of presentation (mean PCV 29.1%, Fig. 5a) (two-sample T-test, d.f. = 11, $T = 3.71$, $P < 0.01$).

Thirteen cases were treated successfully, requiring no further treatment. In one case (case 17 in Fig. 4) the horse exhibited signs of mild head shaking which resolved after a second treatment 2 months after the initial episode. Four horses died despite treatment. These cases occurred at the beginning of the study period when treatment was delayed due to difficulties in obtaining drugs for treatment. One of these horses (case 18 in Fig. 4) is the only case that did not show pyrexia at presentation (Fig. 2) and showed a pattern of clinical signs consistent with chronic infection (anaemia, weight loss and ataxia). This horse is likely to have become infected before the start of the study period.

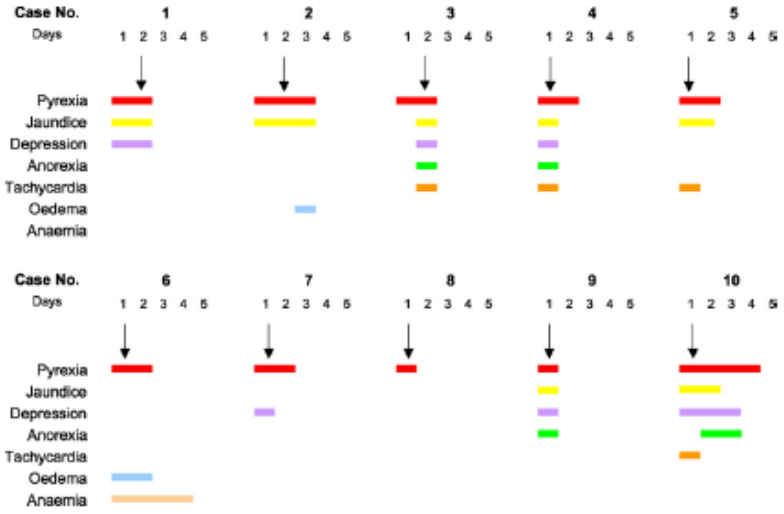

Fig. 3. Pattern of clinical signs seen in cases of trypanosomosis in horses where treatment was administered within 2 days of the onset of clinical signs. Arrow represents time of treatment.

Table 1
Distribution of the explanatory variables and outcomes used in statistical analysis

Resolution of clinical signs after treatment	Resolved in <5 days	Resolved in >5 days
Treatment <2 days after presentation		
Yes	6	1
No	1	5

Case outcome	Recovery	Fatality
Ataxia		
No	10	0
Yes	0	4
Weight loss		
No	10	1
Yes	0	3
Severe anaemia		
No	8	0
Yes	2	4

Post-mortem examination of the horse showed severe lymphoplasmacytic meningo-encephalomyelitis consistent with the presence of trypanosomes in the central nervous system.

Adverse effects were seen in all of the 15 horses where quinapyramine was used for treatment or prophylaxis. These effects were: colic, sweating and restlessness lasting 0.5–3 h, localised non-painful swelling at the injection site persisting for several months in some cases. No adverse effects were seen in the 12 horses where isometamidium was used.

Assuming a period of prophylactic cover of 3 months for both quinapyramine and isometamidium, the incidence rate whilst covered by prophylaxis was 0.39 cases per horse-year at risk, compared to 0.98 when not covered. The incidence rate ratio is 2.50 (95% CI 0.84–7.36).

4. Discussion

4.1. Diagnosis

The systematic diagnostic procedure used in this study helped to identify trypanosome infections as early as possible. Clinical signs previously described for trypanosomosis in horses, namely ventral and limb oedema, ataxia and paralysis (Taylor and Authie, 2004), were only observed late in the course of disease in our study. Kerato-conjunctivitis was not observed during the study. Pyrexia, jaundice and depression were the most prevalent, although less specific, early clinical signs. Pyrexia was a consistent feature of acute cases. Twice daily temperature monitoring is therefore a valuable and easily implemented tool for early case

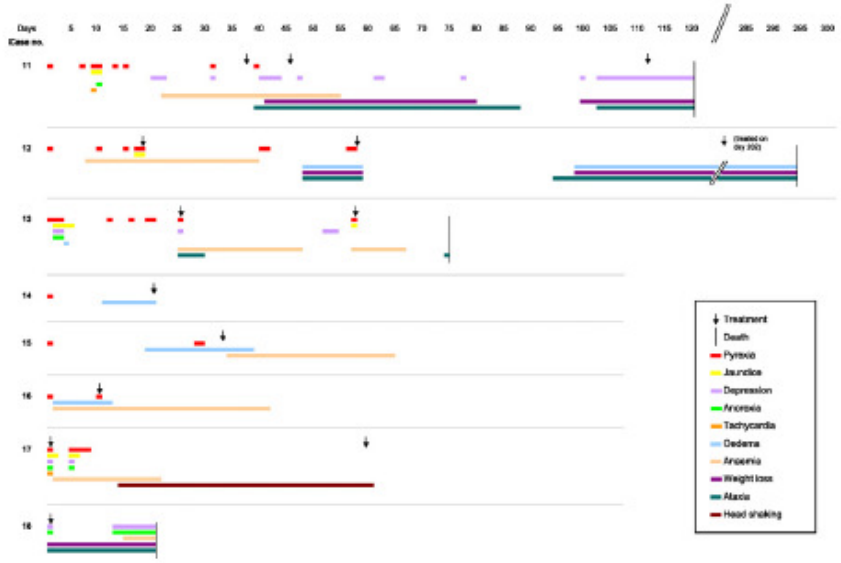

Fig. 4. Pattern of clinical signs seen in each case of trypanosomosis in horses where treatment was delayed for more than 10 days. Two anomalous cases are included (case 17 treated promptly but required second treatment; case 18 likely to have been infected before start of study period).

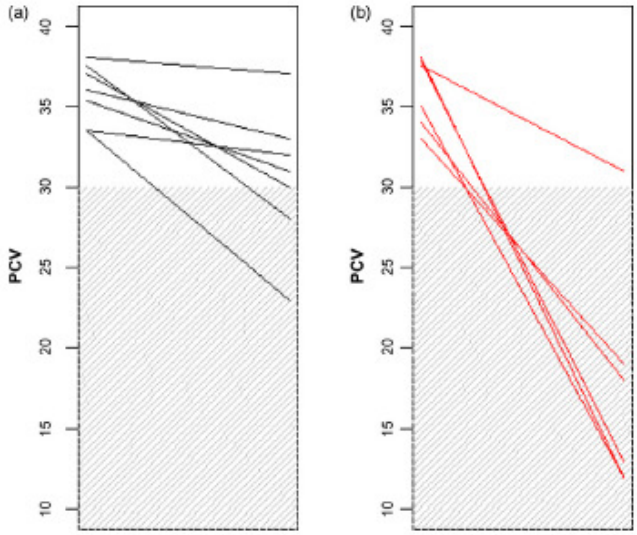

Fig. 5. Change in PCV during cases of trypanosomosis in horses. (a) Where treatment was administered <2 days after clinical signs were first observed ($n = 7$) and (b) where treatment was administered >2 days after clinical signs observed ($n = 6$). Lines join two points (i) initial PCV is the normal level for each individual horse when healthy and (ii) is the lowest PCV reached during a 1-month period post-presentation. Shaded area represents anaemia.

indication. Repeated examination of blood smears is essential in pyrexic cases to confirm trypanosomosis and eliminate other causes of pyrexia such as equine babesiosis (*Babesia caballi*) and theileriosis (*Theileria equi*). Anaemia, a clinical sign frequently utilised for diagnosis of trypanosomosis in cattle (Murray and Dexter, 1988), was uncommon in acute infections. Therefore monitoring PCV to identify cases of trypanosomosis may only be reliable as an aid in detecting more chronic infections. Diagnosis of chronic trypanosomosis becomes more difficult as parasitaemia detectable by microscopy is rarely seen (Van den Bossche et al., 2005), and is therefore more reliant on the clinical signs observed. Identification of a chronic case showing signs that include ataxia, anaemia and weight loss must confer a poor prognosis.

Molecular diagnostics showed that in contrast to previous findings (Kihurani et al., 1994), *T. congolense* and *T. b. brucei* are both able to cause disease as sole pathogens in horses. These trypanosome species are prevalent in the adjacent Serengeti ecosystem in a range of wildlife species (Kaare et al., 2007). Analysis of further case records to determine individual clinical presentations for different trypanosome species would be valuable.

4.2. Treatment

The susceptibility of horses to trypanosome infections makes prompt treatment essential. Delaying treatment increases both the course of disease and the severity of clinical signs. Quinapyramine is an effective treatment in acute infections, with severe but transient side effects. Use of quinapyramine in chronic cases, whilst sometimes leading to a short-term improvement in condition, did not usually prevent progression of disease in the longer term. The neurological signs such as ataxia, head shaking and behavioural changes observed in these cases indicate that trypanosomes have invaded the central nervous system. Quinapyramine, isometamidium and diminazene are unable to cross the blood brain barrier. However, melarsomine is able to penetrate the cerebrospinal fluid (Raynaud et al., 1989) and it is hoped this may provide a future treatment option for cases in which neurological signs are present.

4.3. Prophylaxis

Maintenance of insecticide-treated tsetse control targets and use of pour on or spray formations is costly

and cannot prevent horses from some contact with tsetse. Tsetse challenge varies considerably both spatially and temporally. Maintenance of a rigorous prophylactic protocol is necessary, and particularly important at times of high tsetse challenge. Cases are likely to be minimised by combining physical tsetse control measures with strategic chemoprophylaxis. In this location resistant trypanosome populations are unlikely to arise due to the presence of a large and diverse wildlife population which forms the source of infection for the horses. However quinapyramine has been implicated in the development of multiple drug resistance and the use of sanative pairs of trypanocides may be prudent. Further work is needed to quantify the protective effect of chemoprophylaxis and to determine the relative efficacy of quinapyramine and isometamidium.

5. Conclusion

We conclude that health management of horses under high challenge from trypanosomes requires considerable management skill and resources. The clinical presentation of trypanosomosis in horses differs from that in cattle and early diagnosis and prompt treatment are essential to prevent progression to chronic illness with a poor prognosis. This study has highlighted that with a combination of prompt diagnosis and treatment, strategic use of prophylactics and implementation of measures to reduce fly exposure, cases may be reduced to manageable levels. However, infection remains a constant threat and demands continual vigilance. Being a susceptible exotic species, it appears that domestic horses can only be maintained under conditions of high trypanosome challenge if health and stable management are of the highest standard.

Acknowledgements

We gratefully acknowledge the material and financial contributions given to this study on a pro-rata basis by the partner organisations: Singita-Grumeti Reserves Ltd., the University of Edinburgh and the Messerli Foundation of Switzerland that provides full funding for the Tanzania Wildlife Research Institute (TAWIRI) Wildlife Veterinary Programme. We are grateful to Rob Jackson for his invaluable advice on treatment protocols, Vijay Varma for his commitment to the project and ongoing veterinary care of the horses at Singita-Grumeti Reserves Ltd., and Maulidi Mdaki for sample processing and initial diagnostic work in the TAWIRI Veterinary Laboratory. We thank Sarah Cleaveland, Darren Shaw, Crawford Revie and two anonymous referees for helpful comments on the manuscript.

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