ASSOCIATION BETWEEN *SODALIS GLOSSINIDIUS* AND TRYPANOSOME INFECTIONS IN TSETSE FLIES FROM THE KAFUE NATIONAL PARK ECOSYSTEM IN ZAMBIA

BY

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(Computer Number: 2019124939)

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF ZAMBIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH ANALYTICAL EPIDEMIOLOGY

THE UNIVERSITY OF ZAMBIA

LUSAKA

DECLARATION

I, SIMEGNEW ADUGNA KALLU, do hereby declare that this dissertation represents my own original work. It has been presented in accordance with the guidelines for MSc dissertation of The University of Zambia. It has not been submitted before for the award of any degree or examination in any other University.

Sign _____ Date _____

CERTIFICATE OF APPROVAL

The University of Zambia approves the dissertation submitted by **SIMEGNEW ADUGNA KALLU,** as fulfilling the partial requirements for the award of the Master of Science Degree in One Health Analytical Epidemiology of the University of Zambia.

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ABSTRACT

Tsetse flies are obligate hematophagous vectors of animal and human African trypanosomoses. They cyclically transmit pathogenic *Trypanosoma* species. Despite having a long history of chemotherapy and chemical methods of vector control, African trypanosomosis has continued causing huge economic losses. Due to their distinct reproductive biology, tsetse flies are recalcitrant to germ-line transformation. A paratransgenic approach using *Sodalis glossinidius* as a delivery system for trypanocidal components is currently of considerable interest to generate a trypanosome resistant tsetse fly. The aim of this study was to assess the association of *S. glossinidius* and trypanosome infections in a natural tsetse fly population.

Tsetse flies were caught at two sites (Chunga and Ngoma) in the Kafue National Park ecosystem, Zambia. Trapped tsetse fly species and their sex were identified morphologically using a stereomicroscope. Deoxyribonucleic acid (DNA) was extracted from the whole tsetse flies and checked for the presence of *S. glossinidius* and trypanosome DNA by polymerase chain reaction (PCR). Chi-square statistical analysis were done to check whether there was an association between the presence of *S. glossinidius* and trypanosome infections.

A total of 326 tsetse flies consisting of two species were trapped from Kafue National Park ecosystem, Zambia. These included *Glossina morsitans* that represented 82.8% (95% CI: 78.35- 86.53) and *Glossina pallidipes* that accounted for 17.2% (95% CI: 13.47-21.65) of the caught tsetse flies. Out of the total tsetse fly population, the prevalence of *S. glossinidius* was 21.8% (95% CI: 17.64-26.57) and trypanosome infection rate was 19.3% (95% CI: 15.41-23.96). The prevalence of pathogenic trypanosome species detected in this study were 6.4% , 4.6%, 4.0%, 3.7%, 3.1% and 2.5% for *T. vivax*, *T. simiae*, *T. congolense*, *T. godfreyi*, *T. simiae* Tsavo, and *T. b. brucei*, respectively. Out of 63 trypanosome infected tsetse flies, 47.6% of the flies also carried *S. glossinidius*, while the remaining flies were devoid of *S. glossinidius*. From overall data analyzed, statistically significant association was found between tsetse flies harbouring *S. glossinidius* and tsetse flies infected with trypanosomes ($p < 0.001$). The association of individual trypanosome species with presence of *S. glossinidius* indicated that statistically significant associations were found between *S. glossinidius* and *T. vivax* ($p = 0.006$), *T. simiae* ($p = 0.025$), *T. simiae* Tsavo ($p = 0.009$), and *T. godfreyi* ($p = 0.027$), but no significant association between *S. glossinidius* and *T. congolense* ($p = 0.491$), and *T. b. brucei* ($p = 0.072$).

This result supports the hypothesis that presence of *S. glossinidius* increases the susceptibility of tsetse flies to trypanosome infections and *S. glossinidius* could be a potential candidate to symbiont-mediated vector control in these tsetse species.

DEDICATION

I am dedicating this dissertation to my mother Zena Anley Kebede, my father Adugna Kallu Kassa, my wife Hibest Shewarega Sitotaw and my daughter Ablakat Simegnew Adugna.

ACKNOWLEDGEMENTS

First of all, I want to express my special gratitude to the Africa Centre of Excellence for Infectious Diseases of Humans and Animals (ACEIDHA) project for offering me fully sponsored scholarship to study at the University of Zambia, School of Veterinary Medicine. I would like to express my deepest gratitude to my supervisor Professor Martin C. Simuunza for the continuous support during my study and his patience, guidance and motivation. I am also thankful to technicians of the School of Veterinary Medicine, the University of Zambia for their full technical support during this research activity.

I would also like to thank the Ministry of Tourism and Arts, Department of National Parks and Wildlife for giving me permission to conduct this research in Kafue National Park Ecosystem. Let me extend my appreciation to the officers of Kafue National Park for making this study possible by assisting me with tsetse fly sampling in the Park.

My sincere thanks also goes to Haramaya University, College of Veterinary Medicine, for giving me a study leave to complete my Master's study outside the country.

Lastly, I would like to thank my family, especially my parents, my wife and my daughter for their belief in me, this kept my spirits and motivation high during the study period.

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LIST OF SYMBOLS AND ABBREVIATIONS

AAT	Animal African Trypanosomosis
bp	Base pair
CI	Confidence Interval
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
GMAs	Game Management Areas
HAT	Human African Trypanosomosis
ITS	Internal Transcribed Spacers
KNP	Kafue National Park
NHRA	National Health Research Authority
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
SIT	Sterile insect technique
SRA	Serum resistance associated
TAE	Tris-acetate-EDTA
UNZABREC	University of Zambia Biomedical Research Ethics Committee
WHO	World Health Organization
^{o}C	degree Celsius
µl	Microlitre

CHAPTER ONE

1.0. INTRODUCTION

1.1. Background

Tsetse flies are biological vectors of African trypanosomes which cause human African trypanosomosis (HAT) and animal African trypanosomosis (AAT) (Geiger et al., 2015). Tsetse flies (*Glossina*) occupy the 'tsetse belt' which covers an area of 10 million km^2 that is about one third of the total land of the African continent in 38 Sub-Saharan countries (Kristjanson *et al*., 1999). There are 31 different species and subspecies of tsetse flies found in Africa which can be arranged into three species groups: *morsitans* group, *palpalis* group and *fusca* group (Pollock, 1982a). In the affected areas, the density of the vector and the prevalence of trypanosome infections in the host is attributed to complex interactions between and among humans, domestic livestock, wildlife, tsetse flies, trypanosomes and different economic and ecological factors (Steverding, 2008).

Animal African trypanosomosis alternatively referred to as nagana represents a group of vector borne (*Glossina*) parasitic ailments in ruminants, camels, equines and carnivores which induces dramatic economic losses to animal producers as a result of mortality, morbidity and inefficient productiveness (Shaw et al., 2014). Within the tsetse-infested areas as an entity, trypanosomosis reduces the offtake of meat and milk by a minimum of 50% and by usually constraining farmers from the overall benefits of livestock farming including reduced efficient nutrient cycling, reduced access to animal traction, decreased revenue from milk and meat sales, reduced access to liquid capital; reduction in yields, space cultivated, and subsequently the effectivity of useful resource allocation (Swallow, 2000). The total estimated losses attributed to nagana from livestock productivity is estimated to be $4.5 billion per year (Holmes, 2013; Alsan, 2015). Different tsetse transmitted trypanosome species known to cause AAT include *T. b. brucei, T. congolense, T. simiae,* and *T. vivax* (Steverding, 2008; Adams et al., 2010; Cecchi et al., 2014).

Two trypanosome species are responsible for human African trypanosomosis (HAT), also known as sleeping sickness: *T. brucei gambiense* and *T. brucei rhodesiense* (Büscher et al., 2017). *T. b. gambiense* is found in 24 countries in West and Central Africa. This form

of the disease accounts for over 98% of reported cases of sleeping sickness, transmitted through human-tsetse contact and causes a chronic infection. *T. b. rhodesiense* is found in 13 countries in eastern and southern Africa representing less than two percent of reported cases. This parasite has a complex transmission cycle involving a wide range of wildlife and livestock reservoirs and causes an acute infection (WHO, 2020a) in humans. Even though 260 million people live in tsetse infested areas, only about 60 million are considered to be at risk of contracting sleeping sickness. The distribution of sleeping sickness areas has a focal nature and the localization of the actual areas fluctuates over the course of time (Rock et al., 2015; WHO, 2013).

Many endosymbionts have been reported in various tissues of tsetse flies, but *Wigglesworthia glossinidia*, *Sodalis glossinidius* and *Wolbachia* species are the three major bacterial species that tsetse flies harbour (O'Neill et al., 1993). *S. glossinidius* is found in the midgut, haemolymph, muscles, fat bodies, salivary glands, milk glands and reproductive system of tsetse flies and so could interact with multiple species of trypanosomes that are harbored in different tissues (Cheng and Aksoy, 1999). *S. glossinidius* lacks a clearly defined functional role within its tsetse host (Wang et al., 2013). However, it is suggested to play a role in facilitating susceptibility to trypanosome infection in tsetse by inhibiting the efficacy of the tsetse immune system (Welburn and Maudlin, 1999). N-acetyl glucosamine specific trypanocidal lectin is secreted during feeding and trypanosomes should successfully evade this lectin activity to establish in the midgut of the tsetse fly (Maudlin and Welburn, 1987). Lectin activity is influenced by *Sodalis* through the production of lectin-inhibitory sugar (Welburn et al., 1993). *S. glossinidius* has been shown to be positively associated with various species of trypanosomes, for instance *T. congolense* and *T. brucei rhodesiense* in *G. m. morsitans* (Maudlin and Welburn, 1987), *T. brucei gambiense* and *T. brucei brucei* in *G. p. gambiensis* (Geiger et al., 2007) and *T. c.* Forest, *T. brucei* s. l. and *T. b. gambiensis* in *G. p. palpalis* (Farikou et al., 2010).

In addition to sterile insect technique, the development of trypanosome refractory strains would be a much simpler and more clear-cut method to control tsetse flies (Kariithi et al., 2018; Van Den Abbeele et al., 2013). *S. glossinidius* is the first endosymbiont to be

cultured in vitro and provokes interest on its use as a potential candidate for engineering refractory arthropod disease vectors (Beard et al., 1993). As such, knowing the association between trypanosomes and *S. glossinidius* infections in tsetse flies is important in understanding the potential use of this endosymbiont in the control of trypanosomosis transmission by tsetse flies. There is little information in the knowledge of the relationship between trypanosome and *S. glossinidius* infections in tsetse flies in KNP ecosystem. Therefore, this study aimed at determining whether an association exists between trypanosomes and *Sodalis* infections in tsetse flies in KNP ecosystem.

1.2. Statement of the Problem

Within the near future, there may be no effective products forthcoming for trypanosomosis control. This is due to the increasing drug resistance (Delespaux et al., 2008) and trypanosomes' antigenic variation in the mammalian host which have hampered chemotherapy and vaccine development, respectively (Morrison et al., 2009). To design new vector control strategies, investigation of the interaction between trypanosomes and endosymbiotic microbes such as *S. glossinidius* in the tsetse flies is important and may provide new insights to this problem.

1.3. Justification of Study

Tsetse flies are found in about 37% of Zambia's land area (Mulenga et al., 2020). In spite of Zambia having had three main African trypanosomosis management programmes (aerial spraying, insecticide treated targets and trypanocidal drug use), the country has recorded several disease re-occurrences in areas where control was once undertaken (Mulenga et al., 2020). In addition to transmitting trypanosomes, tsetse have established symbiotic relationship with maternally transmitted bacteria (Wang et al., 2013). Due to their distinct reproductive biology, tsetse flies are recalcitrant to germ-line transformation. A paratransgenic approach using *Sodalis glossinidius* as a delivery system for trypanocidal components is currently of considerable interest to generate a trypanosome resistant tsetse fly (De Vooght et al., 2018). *S. glossinidius* is the only gamma proteobacterial insect symbiont to be cultured and thus amenable to genetic modification (Beard et al., 1993). Hence, understanding the interaction between trypanosomes and *Sodalis* and therefore their influence on tsetse can provide an opportunity on how to

explore such a relationship to control trypanosomosis. Therefore, this study aimed at investigating the association of trypanosomes and *S. glossinidius* infections in tsetse flies from KNP ecosystem, Zambia.

1.4. Research Questions

I. What is the prevalence of trypanosomes and *S. glossinidius* in tsetse flies in KNP ecosystem?

II. Is there an association between trypanosomes and *S. glossinidius* infections in tsetse flies in KNP ecosystem?

1.5. Study Objectives

1.5.1. General Objective

To investigate the association between trypanosomes and *S. glossinidius* infections in tsetse flies from KNP ecosystem, Zambia.

1.5.2. Specific Objectives

- To determine the prevalence and verify trypanosome species circulating in tsetse flies captured from KNP ecosystem
- To determine the prevalence of *S. glossinidius* in tsetse flies captured from KNP ecosystem
- To evaluate the association between trypanosome infections and presence of *S. glossinidius* in tsetse flies from KNP ecosystem.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Biology of Tsetse Flies

2.1.1. Taxonomy of Glossina Species

Glossina species, vectors of trypanosomes, belongs to the Kingdom Animalia, Phylum Arthropoda, Class Insecta, Order Diptera (Leak, 1998), Section Schizophora (Nirmala et al., 2001), Subsection Calyptratae (Nirmala et al., 2001), Superfamily *Hippoboscoidae* (Nirmala et al., 2001), Family *Glossinidae* (Gooding et al., 2005; Leak, 1998; Pollock, 1982a) and Genus *Glossina* (Gooding et al., 2005; Leak, 1998; Pollock, 1982a). The genus *Glossina*, the only genus in the family *Glossinidae*, which are a unique group of insects - in terms of appearance, life cycle, distribution, is of medical and veterinary importance (Gooding et al., 2005). There are 31 species and subspecies of tsetse flies, found only on the continent of Africa, south of the Sahara desert. Species range in length from approximately 6mm to 15mm, and are generally dull yellow, pale brown or dark brown in colour, with occasional black markings (Service, 2012). These 31 species and subspecies split into three subgenera based on differences in their morphological characteristics and habitat preferences: *Glossina* (*morsitans* group), *Nemorhina* (*palpalis* group) and *Austenina* (*fusca* group) (Gouteux, 1987; Krafsur, 2009; Pollock, 1982b). The *Glossina* subgenus is grouped into five species and three subspecies, the *Nemorhina* subgenus is grouped into five species and seven subspecies and the *Austenina subgenus* contains 13 species and four subspecies (Table 2.1).

Table 2. 1. Species and subspecies of tsetse flies belonging to the *morsitans*, *palpalis* and *fusca* groups.

Subgenus	Species	subspecies
Glossina		*G. m. morsitans*
(*morsitans* group)	*G. morsitans*	*G. m. submorsitans*
		G. m. centralis
	G. langipalpis	
	G. pallidipes	
	Glossina swynnertoni	
	Glossina austeni	
Nemorhina	*G. palpalis*	*G. p. palpalis*
(*palpalis* group)		*G. p. gambiensis*
	G. tachinoides	
	G. fuscipas	*G. f. fuscipes*
		G. f. martinii
		G. f. quanzensis
	G. pallicera	*G. p. pallicera*
		G. p. newsteadi
	G. caliginea	
Austenina	*G. nigrofusca*	*G. n. nigrofusca*
(*fusca* group		*G. n. hopkinsi*
	G. fusca	*G. f. fusca*
		G. f. congolensis
	G. fuscipleuris	
	G. haningtoni	
	G. schwetzi	
	G. tabaniformis	
	G. nashi	
	G. vanhoofi	
	G. medicorum	
	G. severini	
	G. brevipalpis	
	G. longipennis	
	G. frezili	

Sources: (Pollock, 1982a; WHO, 1998)

2.1.2. Life Cycle of Tsetse flies

Tsetse flies have a series of biological and demographic characteristics that make them unique among medically and veterinary important vectors (Krinsky, 2019; WHO, 2013). Their life-cycle is particularly unusual, as they do not lay eggs (Krafsur, 2009). Instead, a single larva develops within the female uterus (Krafsur, 2009). They have a very low rate of reproduction (K strategists) and differs from most insect species which produce large number of eggs (r strategists) (Leak, 1998).

Females have a pair of ovaries, a uterus and the spermathecae to store spermatozoa received from copulation with males. Most species of female tsetse usually mate once at the age of 1-3 days in their lives, except for *G. pallidipes* which mates at 7-10 days of age before or at about the time of taking the first blood meal, while males can mate several times (Pollock, 1982a). The adult female produces a single egg, which hatches to a first-stage (instar) larva in the uterus. After a period of development and moulting, a third-stage larva is deposited on the ground. This reproductive method of tsetse fly is called adenotrophic viviparity. All larval instars are retained within the uterus and are nourished by a protein and lipid rich secretion ("milk") produced by the female's accessory glands ("milk glands") (Gooding et al., 2005). Female tsetse flies produce one full-grown larva every 9-10 days, which then pupates in light or sandy soils. The main requirements for a suitable larviposition site are that it be well shaded to prevent overheating and desiccation of the puparia and that the soil be friable enough to permit larvae to burrow to a suitable depth. After being deposited, the mature third-instar larva does not feed, but quickly burrows into the friable soil and pupariates. The puparial case is thick and hard and turns black within a few hours. The pupal stage usually lasts about 20 to 80 days, depending on temperature and humidity (pupal development is not completed below 16 °C and above 36 °C) (Pollock, 1982a; WHO, 2013). The adult emerges from the puparium and forces its way to the surface using its legs and ptilinum (an eversible saclike structure in the head). The wings are quickly expanded, and the adult flies to a suitable resting area. Adults seek their first blood meal within a day of eclosion (Gooding et al., 2005).

2.1.3. Distribution and habitat of Tsetse flies

The genus *Glossina* is distributed across 38 countries of sub-Saharan Africa which occupy 10 million km^2 land area infesting more than 40% of the continent. They have adapted a wide range of habitats from central Africa humid forests to the vast, semiarid, open savannahs of Eastern Africa (Cecchi et al., 2014; Moloo, 1993). The distribution of the tsetse flies in Africa extends up to the southern edge of Sahara and Somali deserts in the North, but not beyond Kalahari and Namibian deserts in South and below 29^o S in the East (Vreysen et al., 2013). They are not found on islands East of the African continent, except for the island of Unguja in Zanzibar, where *G. austeni* was present but has been eradicated (Vreysen et al., 2000). Two subspecies *G. morsitans morsitans* and *G. fuscipes fuscipes*, were also recorded in south-western Saudi Arabia (Elsen et al., 1990).

Principally the general distribution of tsetse is determined by climate and influenced by altitude, vegetation and the presence of suitable animal hosts. The three main groups of tsetse differ in their distribution according to the availability of their preferred type of vegetation (Leak, 1998).

Almost all the species of *Palpalis* group, subgenus *Nemorhina* are found in West and Central Africa, from Lake Victoria and the Tanganyika basin to the Atlantic coast, in vegetation close to water bodies (forests, small island forests, gallery forests, "sacred" woods, river thickets, banks of lakes, mangroves). Hence species of this subgenus are also called "riverine" tsetse (Pollock, 1982a). Some species show high adaptation to manmade environmental changes and areas of intensive agricultural activities such as coffee, cocoa, mango and banana plantations and pig rearing (WHO, 1998).

Tsetse flies in the *Morsitans* group settle in open areas with savanna woodland, which are surrounded with grass, but few bushes and trees. Their distribution may be restricted by shortage of game animals on which to feed and by lack of trees. As a result of extensive deforestation of African savannas and human population pressure, some species have been forced to adapt to vegetation that they would not normally inhabit (Cecchi et al., 2008; Moloo, 1993; WHO, 2013).

Fusca groups of tsetse flies largely inhabits primary moist forests mainly in West and Central Africa (Leak, 1998; WHO, 2013). However, some species like *G. medicorum* extends to gallery forests of woodland savannahs in West Africa, *G. brevipalpis* in the rainfed thickets of East Africa, *G. longipennis* in very arid areas (thorn-bush) and sometimes in dense thickets bordering water, from southern Ethiopia and Somalia to Mozambique and the northern part of South Africa (WHO, 2013).

2.2. Biology of *Trypanosoma* Species

Trypanosomes are extracellular single-celled flagellated protozoan parasites (Radwanska et al., 2018) belonging to kingdom Protista, phylum Sarcomastigophora, class Zoomatigophora, order Kinetoplastida, family Trypanosomatidae, and genus *Trypanosoma* (Urquhart et al., 1996).

All trypanosomes are grouped in the genus *Trypanosoma* (Büscher et al., 2017; Giordani et al., 2016) and classified into stercorarian and salivarian groups based on means of transmission (O'Donoghue, 2017). The stercorarian groups are transmitted through faeces of vectors to vertebrate hosts and distributed in the Americas. They include *T. cruzi*, *T. theileri* and *T. lewisi* (WHO, 1998). The salivarian group of *trypanosomes* called so because their transmission to the vertebrate host occurs mainly via the infected saliva of blood-sucking insects. Salivarian *trypanosomes* can be subdivided into four subgenera depending on morphology and site of their cyclical development in the tsetse: *Trypanozoon*, *Duttonella*, *Nannomonas* and *Pycnomonas* (Hoare, 1964). The *Trypanozoon* subgenus contains three subspecies of trypanosomes *T. brucei brucei*, *T. b. rhodesiense* and *T. b. gambiense* which develops in the tsetse fly midgut and salivary glands; *Nannomonas* encompasses three species of animal infective trypanosomes, *T. congolense*, *T. godfreyi* and *T. simiae* that develops in the tsetse proboscis and salivary glands; while *Duttonella* subgenus contains *T. vivax* species which develops only in the tsetse fly proboscis (Table 2.2).

Table 2. 2. Tsetse transmitted *trypanosome* species/subspecies, site of cyclical development in the tsetse fly and their host ranges.

Subgenera	Species/subspecies	Site of development in tsetse	Host range
Trypanozoon	*T. brucei brucei*	Midgut and salivary gland	Domestic and wild animals
	T. b. rhodesiense		Humans, wild and domestic animals
	T. b. gambiense		
	T. congolense Kilifi		
Nannomonas	*T. congolense* Forest	Proboscis and midgut	Domestic and wild animals
	T. congolense Savannah		
	T. godfreyi		Wild and domestic suids
	T. simiae		
	T.simiae Tsavo		
Duttonella	*T. vivax*	Proboscis	Domestic and wild animals
Pycnomonas	*T. suis*	Mid gut, salivary gland and proboscis	Suids

Sources: (Rotureau and Van Den Abbeele, 2013)

Trypanozoon subgenus includes other non-tsetse transmitted species including *T. evansi* and *T. equiperdum, T. evansi* is transmitted mechanically by biting or blood sucking insects of the genus *Tabanus*, *Stomoxys*, *Atylotus* and *Lyperosia*. It has worldwide distribution except North America and Australia and causes a disease called surra with multiple host ranges including horses, mules, camels, buffalo, cattle and deer (Brun et al., 1998). *T. equiperdum* is a venereally transmitted parasite of equines and is the only pathogenic *trypanosome* that does not require an arthropod vector for its transmission. *T. equiperdum* causes a disease known as dourine which is endemic in Asia, Africa, South Eastern Europe and Central America (Constable et al., 2017).

2.3. Life Cycle and Transmission of Trypanosomes

The trypanosome transmission cycle starts when tsetse ingests blood meal from an infected mammalian host (bloodstream trypomastigotes) and then the trypanosomes undergo a cycle of development and multiplication in the tsetse digestive tract, particularly subgenus *Duttonella* in proboscis, subgenus *Nannomonas* in midgut and proboscis and subgenus *Trypanozoon* in midgut and salivary glands, until infective metacyclic trypanosomes develop (Uilenberg, 1998). In both subgenus *Nannomonas* and *Trypanozoon*, the blood stream form of trypomastigotes differentiate to procyclic trypomastigotes in the midgut and then for *Nannomonas* migrate to mouthparts while for *Trypanozoon* migrate to salivary gland (Cnops et al., 2015; Peacock et al., 2012). In the proboscis and salivary gland for each subgenus, the trypomastigotes transform into epimastigotes and then multiply and differentiated to metacyclic trypomastigotes which is an infective form that can be transmitted to another host during the tsetse blood meal feeding. The cyclical development and multiplication of *Duttonella* subgenus in the tsetse is completed in the proboscis (Ooi et al., 2016).

The cycle of development and multiplication of trypanosomes in the tsetse fly varies from one to three weeks and once infective metacyclic trypanosomes develops in the fly, it is infective throughout its life (Uilenberg, 1998). Sometimes other biting flies can transmit trypanosomes during blood meal from infected animal to a healthy animal to cause disease, if the time interval between the two meals is short enough to ensure survival of parasites in the insect mouthparts (Kruel, 2007).

T. brucei species is the only one with subspecies that can infect humans. The species is grouped into three subspecies based on host infectivity, pathogenicity and geographical occurrence (Cnops et al., 2015). HAT is caused by two subspecies with markedly different epidemiologic and geographic range: *T. b. gambiense* and *T. b. rhodesiense*. The third subspecies *T. b. brucei* is not infective to humans because it does not resist apolipoprotein A which is a serum protein (Büscher et al., 2017).

Naturally *T. b. gambiense* is transmitted by the *Palpalis* group species of tsetse flies, particularly subspecies of *G. fuscipes, G. p. gambiensis* and *G. palpalis* (Wamwiri and

Changasi, 2016), but under laboratory conditions many *Glossina* species can carry the infection (WHO, 2013). After tsetse has fed on an infected host, the trypanosomes undergo a complex process of maturation in the fly midgut and after a period of about 18 to 40 days the infective forms appear in the salivary glands of the tsetse which, thereafter remains potentially infective to any humans it bites.

T. b. rhodesiense is transmitted by tsetse flies of the *Glossina morsitans* group, such as *G. morsitans* and *G. pallidipes*, and it is a zoonosis involving a number of wildlife species, such as antelope, warthog, buffalo, and bushbuck, and domestic animals acting as reservoirs hosts (Auty et al., 2016). Rhodesiense HAT transmission, particularly in wildlife and/or livestock foci, is complex due to the involvement of multiple host and vector species within heterogeneous and often fragmented landscapes. Transmission of *T. b. rhodesiense* commonly occurs through animal-tsetse-animal cycle. Animal-tsetse-human transmission cycle occurs occasionally, but human-tsetse-human transmission cycle is very unlikely and probably only happens during outbreaks (Franco et al., 2014).

2.4. African Trypanosomosis

2.4.1. Animal African Trypanosomosis

Animal African trypanosomosis is a wasting disease of livestock caused by protozoan parasites of the genus *Trypanosoma*. The disease threatens numerous mammalian animals and some birds, reptiles and fish (Kruel, 2007). AAT affects most valuable domestic livestock including cattle, pigs, camels, goats, sheep and equines and in which the infection is caused by four main species of salivarian group of trypanosomes: *T. vivax*, *T. congolense*, *T. brucei*, and *T. simiae* (Haag et al., 1998). The epidemiology of AAT is determined mainly by the ecology of tsetse flies found only in sub Saharan Africa.

Nagana remains to be a crucial disease of livestock within the continent. Because nagana is a wasting disease, affected animals are chronically unproductive in terms of milk, meat, manure, and traction, and the mortality rate could be high, mostly in exotic and more productive animals. The disease in Africa costs livestock producers and consumers an estimated US$4.5 billion every year. It undermines environmentally sound and economically viable animal and agricultural production and become a huge constraint to

development in large areas of rural Sub-Saharan Africa (Ilemobade, 2009). The influence of AAT on a community is the result of advanced interactions between entomological, environmental, political, socio-cultural and livestock management factors (Bouyer et al., 2013). Changes in land cover and land use have a direct or indirect effect on the biodiversity of hosts and vectors, and on the various interfaces between vectors and hosts including wildlife, livestock and humans (Van den Bossche et al., 2010).

2.4.2. Human African Trypanosomosis

Human African Trypanosomosis (HAT) is a neglected tropical parasitic disease caused by haemoflagellate trypanosomes and it occurs only in sub-Saharan Africa, where there are suitable habitats for the tsetse fly vector. Although there are wider areas where suitable habitats for the tsetse fly are found, the geographic distribution of HAT is highly focal and the relationship between the parasite, the host and vector are complex (WHO, 2013). Daily activities which increase exposure of people to tsetse bite, include fishing, hunting, farming, fetching firewood and water, and working in coffee and cacao plantations, facilitates the infection and all age groups and both sexes are at risk (Büscher et al., 2017).

Gambiense HAT is caused by *T. b. gambiense*, which accounts more than 98% of the total cases (WHO, 2020a). Gambiense HAT is normally characterized by a long asymptomatic period which results in a chronic disease lasting months to years (Kennedy, 2013) and an anthroponotic disease with a minor role for animal reservoirs (Franco et al., 2014). In spite of domestic and wild animals being infected by *T. b. gambiense*, their epidemiological role is not clear yet. *T. b. gambiense* is endemic in Western and Central Africa, where the vectors infest relatively humid habitats surrounding the rivers, lake shores and wetlands (Rock et al., 2015). The highest HAT foci from Central African countries are found in Democratic Republic of the Congo, Central African Republic and Chad which constitute 86%, 5% and 2% of gHAT, respectively (Büscher et al., 2017). In West Africa three countries that report significant level of the disease are Guinea, Côte d'Ivoire and Nigeria (Simarro et al., 2010). Gambiense HAT is also reported in two East African countries: South Sudan and Uganda (WHO, 2018).

For the gambiense HAT, people living in an area of about 866,000 km^2 are estimated to be at risk. Only 1.5% of this area is at high or very high risk. The area at moderate risk is 171,000 km^2 (i.e. 20% of the total risk area). Sixty-five percent of the area within the gambiense HAT risk is in the Democratic Republic of the Congo (i.e. 566,000 km^2, or a quarter of the total country area), followed by Angola (8.5%) and Central African Republic, Congo and South Sudan (6% each) (Franco et al., 2020) .

Rhodesiense HAT is caused by *T. b. rhodesiense,* which constitute less than 2% of the total HAT reported cases. *T. b. rhodesiense* causes an acute, rapidly progressive infection in the form of epidemics in Eastern and Southern Africa. Rhodesiense HAT is commonly reported in Uganda, the United Republic of Tanzania, Malawi, Mozambique, Kenya, Rwanda, Zambia and Zimbabwe (WHO, 2020b). Approximately 71,000 km^2 are estimated to be at risk, with no area at high or very high risk, and only 10,000 km^2 at moderate risk. Zambia is the country with the largest area at risk (32,000 km^2), followed by Malawi (14,000 km^2) and Tanzania (12,000 km^2) (Franco et al., 2020). Overlapping infection of *T. b. gambiense* and *T. b. rhodesiense* happens in Uganda and possibly Tanzania and South Sudan (Gooding et al., 2005).

Symptoms of HAT occurs in two stages: early stage (stage 1) and late stage (stage 2). The early stage haemolymphatic phase of infection starts after the initial bite of an infected tsetse fly. The parasites multiply and spread throughout the bloodstream, lymphatic system and systemic organs, following which the parasites crosses the blood–brain barrier to enter the central nervous system causing the late (stage 2) encephalitic phase resulting in a wide variety of neurological symptoms (Kennedy, 2008). If untreated, or inadequately treated, HAT is almost always fatal.

2.5. Diagnosis of Trypanosomoses

Clinical signs of AAT and HAT are not specific enough to justify clinical diagnosis. As a result, laboratory testing is necessary to verify clinical suspicions. There are numerous diagnostic tests available for trypanosomoses which vary in their sensitivity and specificity, the ease with which they can be applied and their cost.

Several direct parasite detection technique based on microscopic examination can be used for the diagnosis of animal trypanosomes. The following are in the order from lowest to highest sensitivity: microscopic examination of fresh wet blood films (simplest technique); the Giemsa-stained thin blood smear, which allows identification to the subgenus level based on parasite morphology; the hematocrit concentration technique, which uses a capillary tube the buffy coat method (Desquesnes et al., 2022). In addition to direct observation of the parasites by microscopic examination, there are indirect approaches to identify trypanosome infection by explicitly demonstrating the presence of trypanosome proteins or DNA. Detection of trypanosomal DNA using PCR is the main tool for the diagnosis of trypanosome infections in humans and other mammalian hosts, as well as in tsetse flies (Desquesnes, 2017).

Several diagnostic techniques are also available to identify HAT due to T. brucei gambiense or T. b. rhodesiense in a variety of samples such as blood, lymph node juice, and cerebrospinal fluid (Bonnet et al., 2015). These include: direct examination by mini hematocrit concentration technique, Giemsa-stained thin blood smear, quantitative buffy coat or the miniature anion exchange centrifugation technique; serological antibody detection methods, such as enzyme linked immunosorbent assay, the immunofluorescence antibody test, the immune trypanolysis test and the card-agglutination trypanosomosis test for T. brucei gambiense; and molecular methods such as PCR, Loop-mediated isothermal amplification, and Real-time nucleic sequence based amplification (Mitashi et al., 2012).

2.6. Prevalence of *Trypanosomes* in Tsetse Flies

Common tsetse flies species that are distributed in Zambia includes *G. m. centralis, G. m. morsitans* and *G. pallidipes, G. fuscipes* and *G. brevipalpis* (Pollock, 1982a). The prevalence of trypanosomes in tsetse flies varies in different species of tsetse fly and also it depends on detection methods, ecological and geographic locations, availability of suitable feeding host for tsetse flies and reservoir animals for *trypanosomes* (Kasozi et al., 2021).

Using molecular techniques to investigate trypanosomes revealed a prevalence of 3.4% in the Maasai Steppe, northern Tanzania (Simwango et al., 2017a), 3.7% in Mtito Andei

Division, Makueni County, Kenya (Kulohoma et al., 2020), 10.25% in Yankari National Park and Wuya areas in Nigeria (Isaac et al., 2016), 33.8% in wildlife reserve of Santchou in the western region of Cameroon (Kamdem et al., 2020) and 58.0% at Liwonde Wild Life Reserve, Malawi (Nayupe et al., 2019).

In Zambia, prevalence of trypanosomes in natural population of *Glossina* was 11.62% in Chiawa and Chakwenga areas of Southeastern Zambia (Mekata et al., 2008), 21.20% in South Luangwa National Park (Mwandiringana et al., 2012), 23.20% in Western Zambia (Mbewe et al., 2015)and 36.40 % Luangwa valley, north-eastern Zambia (Laohasinnarong et al., 2015). A study reported higher prevalence of *trypanosomes* (43.02%) in *G. m. centralis* than (20.28%) in *G. pallidipes* from Kafue National park ecosystem and from an overall prevalence of 26.85% in these tsetse species, *T. vivax* was the most abundant with 17.45%, followed by *T. congolense* (8.73%), *T. simiae* (3.02%), *Trypanozoon* (1.68%) and *T. godfreyi* (0.34%) (Nakamura et al., 2021).

2.7. Endosymbiosis

Endosymbiosis is defined as one organism living within another resulting in a beneficial (mutualistic) or detrimental (pathogenic) outcomes for the host. It has deeply impacted the evolution of life and continues to shape the ecology of numerous species (Wernegreen, 2012). Symbiotic relationships can range from mutualistic where the host and the bacteria benefit from their relationship, to parasitic where the bacteria exploit the host resulting in a negative effect on fitness and/or reproduction;. while some relationships are commensal where the bacteria benefit without any apparent cost to the insect host (Attardo et al., 2020).

Insects that feed on unbalanced diets, particularly vertebrate blood, plant sap and woody material, commonly establish relationship with obligate mutualistic symbionts that participates on the provision of missed nutrients for the insect and/ or the degradation of food materials. Furthermore, numerous insects harbour facultative bacteria (Kikuchi, 2009).

2.8. Endosymbiosis in the Tsetse flies

The unique biology of tsetse flies contributes to the maintenance of a simple larval microbiota in comparison to other Diptera, such as mosquitoes and fruit flies, which harbour a greater complexity of bacterial taxa. One distinct feature of tsetse biology is that both sexes maintain a strictly hematophagous lifestyle, persisting solely on vertebrate blood, which limits the introduction of additional microbes through an oral/digestive route (Snyder and Rio, 2013).

Tsetse flies host at least three phylogenetically distinct and regularly associated bacterial endosymbionts both in the laboratory colonies and in wild populations namely; the obligate *Wigglesworthia glossinidia*, the facultative *Sodalis glossinidius* and the rickettsia-like *Wolbachia* species (Aksoy, 2000; Dale and Welburn, 2001; Wang *et al*., 2013). *Wigglesworthia* and *Sodalis* are found exclusively in tsetse species whilst *Wolbachia* species infects a wide range of arthropods. Endosymbionts exhibit different forms of symbiotic relationship with their insect partner; which play various functions associated with reproduction, immunity and insect fitness (Aksoy et al., 2008; Eleftherianos et al., 2013).

Wigglesworthia species maintain an obligate mutualism with tsetse flies localizing intracellularly in specialized host cells bacteriome at the anterior midgut in both sexes and also extracellularly in the female milk glands (Balmand et al., 2013). It is a gram negative rod-shaped gamma proteobacteria measured up to 8 microns in length and 1–1.4 microns in width. *Wigglesworthia* shows concordance evolution and its association with tsetse fly ancestor is predicted to be 50-80 million years old (Chen et al., 1999). The role of this endosymbiont include both nutrient provisioning, where *Wigglesworthia* supplements B vitamins lacking in the tsetse blood diet, and contributions to the maturation of host immunity which is evidenced by severely compromised immune system during adulthood when flies undergo intrauterine larval development in the absence of this bacterium (Wang et al., 2013). *Wigglesworthia glossinidia* is the only endosymbiont bacteria consistently found in all tsetse species (Attardo et al., 2020).

The second endosymbiont in the tsetse is *Wolbachia* species which is a highly diverse and widespread bacteria belonging to the order Rickettsiales subdivision alpha proteobacteria. It is transmitted transovarially through successive host generations. It's found in ovaries and testes of tsetse fly and transmitted between host generations transovarially (Cheng et al., 2000). Wolbachia has an ability to live within and manipulate cellular and reproductive processes in the host. *Wolbachia* infection is responsible for different mechanisms that facilitate female fertility which induces different host phenotypes for example cytoplasmic incompatibility, the feminization of genetically male offspring, male-killing of infected males, and parthenogenesis by infected females (Tram et al., 2003). Cytoplasmic incompatibility is the most frequently found Wolbachia induced phenotype in which sperm from Wolbachia infected males is incompatible with eggs from females that do not harbour the same Wolbachia types (Werren et al., 2008).

The third endosymbiont of tsetse fly is *Sodalis glossinidius*. *Sodalis* was first described as Rickettsia-like-organism from hemolymph of *Glossina* in 1987 (Welburn et al., 1987) and it belongs to the family *Enterobacteriaceae* within the gamma-3 subdivision of the *Proteobacteria* (Aksoy et al., 1995). Based on the 16s rDNA sequence from distant tsetse species, *S. glossinidius* do not show significant phylogenetic difference which indicates the symbiosis is more recent in its origin. The genome size of *S. glossinidius* is approximately 2 Mb. The cells are non-motile, non-spore forming, filamentous, gram-negative rods (Dale and Maudlin, 1999). It is a facultative and culturable endosymbiont in tsetse flies which can be used for genetic manipulations to produce parasite resistant tsetse flies (Kikuchi, 2009).

Sodalis is found intracellular and extracellularly in tissues throughout larval and adult tsetse flies. This bacterium shows a wide tissue tropism and colonizes various tissues of the tsetse fly including midgut, salivary glands, haemolymph, fat body, muscle, milk gland, uterus, and oviduct (Balmand et al., 2013; Cheng and Aksoy, 1999; Dale and Maudlin, 1999; O'Neill et al., 1993) and establishes stable associations. Transmission of *Sodalis glossinidius* between the tsetse flies should be predominantly vertically by milk gland secretions (Aksoy et al., 1997; Balmand et al., 2013; Cheng and Aksoy, 1999; Weiss

et al., 2006), horizontally from males to females by mating (De Vooght et al., 2015) and transovarially by haemolymph (Cheng and Aksoy, 1999).

The actual functional role of *S. glossinidius* within tsetse flies is controversial, but it is believed to influence longevity of the tsetse fly life and is involved in vectorial competence in enhancing the trypanosome susceptibility of the fly by complex biochemical mechanisms (Dale and Welburn, 2001; Farikou et al., 2010). Suggested mechanisms for enhancing the vectorial competence includes inhibition of the trypanocidal lectin secreted by the tsetse during blood meal, by N-acetylglucosamine resulting from pupae chitin hydrolysis by chitinases secreted by the fly-hosted *S. glossinidius* (Welburn and Maudlin, 1999). After emergence of the adult tsetse from pupae, there is thought to be a sufficient concentration of lectin inhibitory sugar (N-acetylglucosamine) which promotes increased trypanosome infection rates in tsetse (Dale and Welburn, 2001).

The prevalence of *S. glossinidius* in tsetse flies varies from none infected in *G. f. fuscipes* from Kenya (Lindh and Lehane, 2011) and Uganda (Alam et al., 2012) to 100% in wild fly *G. brevipalpis* species collected from Bushiri, Tanzania (Mathew, 2007). In Zambia, a study in three species of tsetse flies from Luambe National Park indicated lower infectivity rate in *G. pallidipes* with 1.4%, 17.5% in *G. m. morsitans* and higher in *G. brevipalpis* with 93.7% (Denis et al 2014), while the infectivity rate of *S. glossinidius* was estimated to be 57.7% in 85 *G. m centralis* tsetse flies caught from Kafue National Park (Gaithuma et al., 2020).

The first study conducted by Maudlin *et al*. to investigate the prevalence of *S. glossinidius* in natural population of tsetse flies and assessed the association between *S. glossinidius* presence and trypanosomes infections indicated a positive relationship in *G. p. palpalis.* The study showed that tsetse flies that were positive with *Sodalis* were six times more likely to be trypanosome positive (Maudlin et al., 1990). Thereafter, statistically significant associations between *S. glossinidius* and trypanosomes were reported in some tsetse fly species in different areas of Africa such as in *G. p. palpalis* from two HAT foci areas in Southern Cameroon (Farikou et al., 2010) and in *G. pallidipes* from Kenyan coastal forest ((Wamwiri et al., 2013). However, a study which was conducted in three

species of tsetse flies namely *G. brevipalpis*, *G. m. morsitans* and *G. pallidipes* in Luambe National Park Zambia to assess the association between presence of *S. glossinidius* and trypanosomes, did not detect statistically significant associations (Dennis et al., 2014).This may be an indication that the association between *Sodalis* and trypanosomes in tsetse flies varies with tsetse fly species and their locality.

2.9. Tsetse and Trypanosomosis Control

Developing a vaccine for trypanosomosis has been difficult, because of the ability of African trypanosomes to change their surface proteins by antigenic variation (Aksoy, 2003). Vector control and use of trypanocidal drugs are the two methods applied to control trypanosomosis by breaking their transmission cycles. In spite of the existence of effective curative and preventive trypanocides, chemotherapy and/ or chemoprophylaxis alone cannot eliminate trypanosomosis. It is not only impossible to treat all wild and domestic hosts but there is also a rising problem with drug resistance and little prospect of new products being developed for AAT or for HAT (Solano et al., 2010).

Glossina are the only real vectors of a number of species of pathogenic *trypanosomes* in sub Saharan Africa. Because of the viviparous method of reproduction, larva is retained by the female for almost all of its life, and the puparium is buried in the soil. Because of this, control of tsetse flies is only geared towards the adults (Service, 2012). Central to the control of African trypanosomosis is control of the tsetse vector, which should be very effective since transmission of trypanosomes depends on the single genus *Glossina* (Aksoy et al., 2003). Successful disruption of the transmission cycle rely on the full understanding of the interplay between tsetse flies and trypanosomes. Tsetse fly control has long been a vital choice for lowering the incidence of trypanosomosis, and many techniques have been developed over time (Torr et al., 2007). Tsetse fly control may be ecological, chemical or genetic.

2.9.1. Ecological control of tsetse flies

It consists of the modification of the habitats of tsetse flies in order to make it less suitable for the fly survival and includes bush clearing and game destruction. Bush clearing is the oldest way of reducing tsetse populations quickly, but the flies reinvade quickly when the

vegetation reappears (WHO, 2013). Habitat alteration could be more lasting than other methods, especially if the altered habitat is maintained by appropriate land use, such as cultivation. Bush clearing and destruction of tsetse hosts is no longer acceptable due to wildlife conservation and environmental concerns (Leak, 1998).

2.9.2. Chemical control of tsetse flies

After the discovery of Dichlorodiphenyltrichloroethane (DDT) and other insecticides in 1945, ground spraying became a vital method against tsetse flies and it became the option of choice for controlling trypanosomosis throughout the sub Saharan Africa for several decades (Allsopp, 2001). Different methods of chemical tsetse control have been applied including ground spraying, sequential aerial spraying, insecticide target screens or impregnated traps and insecticide treatment of hosts (Holmes, 2013). Due to unique reproductive biology of the tsetse fly and that the pupae spend their time underground, insecticides used should be active for at least the maximal pupal period or frequent application of a short acting insecticide would be necessary (Vreysen et al., 2013).

2.9.3. Genetic control of tsetse flies

Genetic control method targets to change the reproductive potential of the tsetse fly vector or its vectorial competence. Two methods are being used for genetic control: sterile insect technique and via transgenic tsetse fly symbionts. Sterile insect technique (SIT) is one method of area wide tsetse management where the tsetse fly is controlled by affecting its reproductive capacity (Vreysen, 2001). The technique relies on mass production of the tsetse flies in large production facilities, rendering males sterile by gamma irradiation or certain chemical compounds and sequential release in sustained quantities in the natural habitat of the tsetse flies to outnumber the wild male flies that compete to mate a virgin female tsetse (Vreysen et al., 2011). A mating of a sterile male with a virgin wild females will not produce offspring and females normally mate once after hatching. Eradication of *G. austeni* on Unguja Island of Zanzibar using SIT initiated interest to expand this method to large areas in tsetse infested areas of Africa (Vreysen et al., 2000).

Transgenic technology is another method of genetic control which may provide the opportunity to modify directly the genome of tsetse flies to create desirable characteristics

for existing SIT and for novel future tsetse control programmes (Aksoy et al., 2008). With this approach, rather than inserting genes directly into tsetse fly chromosome, they are inserted in to the chromosome of tsetse symbionts (De Vooght et al., 2018). This technique is still under development and has not yet been used on a large scale (Wilke and Marrelli, 2015).

2.9.4. *Chemotherapy for African trypanosomoses*

Chemotherapy is the treatment of AAT and HAT by the use of chemical drugs. Chemotherapy and chemoprophylaxis represent the mainstay of animal trypanosomosis control, ensuring animal health and production in enzootic countries. Although over 35 million doses are currently used annually in sub-Saharan Africa and they provide a major form of control for many livestock farmers, the available drugs for AAT are inadequate and the range and safety is very unsatisfactory. Only six compounds are currently licensed, namely diminazene aceturate, isometamidium chloride, suramin, homidium bromide, homidium chloride, and quinapyramine methyl sulphate (Giordani et al., 2016; Holmes, 2013; Uilenberg, 1998). Diminazene aceturate, homidium bromide and chloride and isometamidium chloride are the only commonly used drugs which have a narrow therapeutic index and drug-resistance is becoming increasingly common (Delespaux and de Koning, 2007).

Control of HAT depends on monitoring and surveillance for infection and the treatment of infected cases (Holmes, 2013). The drugs available to treat cases of HAT have limitations in effectiveness, are complicated to administer and are highly toxic (Babokhov et al., 2013). There are four main drugs available for HAT, namely suramin, pentamidine, melarsoprol and eflornithine (Pépin and Milord, 1994). Suramin is used successfully against early stage HAT mainly caused by *T. b. rhodesiense*, while pentamidine is effective against early-stage *T. b. gambiense* infection, but is less effective against *T. b. rhodesiense* infection, and is ineffective against late-stage disease (WHO, 2013). Diminazene aceturate is effective against early-stage *T. b. gambiense* and *T. b. rhodesiense* and it has also been used in combination with melarsoprol for the late-stage disease, whereas melarsoprol is effective against late-stage CNS disease caused by *T. b. gambiense* and *T. b. rhodesiense* (Babokhov et al., 2013).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study Site

Tsetse fly samples were collected from Kafue National Park (KNP) ecosystem which is situated between 14°03″S and 16°43″S and 25°13″E and 26°46″E. KNP ecosystem is the largest conservation area covering approximately 68,000 km^2 that comprises the oldest and the largest Zambian National park (22,480 square km) and the surrounding 45,406 km^2 game management areas (GMAs) which stretches over four provinces (Thapa et al., 2011) (Figure 3.1). The park is rich in animal and natural diversity and forms one of the main important terrestrial ecosystem in Africa (Mwima, 2001). The study was carried out in Chunga and Ngoma sampling locations within the KNP ecosystem and 150 km apart. The two sites were selected based on accessibility. Chunga sampling site is located approximately 150 km from Mumbwa town and it is situated along the Kafue River. The sampling points in this area are covered by thicket vegetation. Ngoma sampling site is located 26 km from Itezhi tezhi and it is situated along the Kafue River and close to Itezhi tezhi Dam. The vegetation type in Ngoma sampling points were thicket and miombo woodlands. Both areas are inside the National park (Figure 3.1).

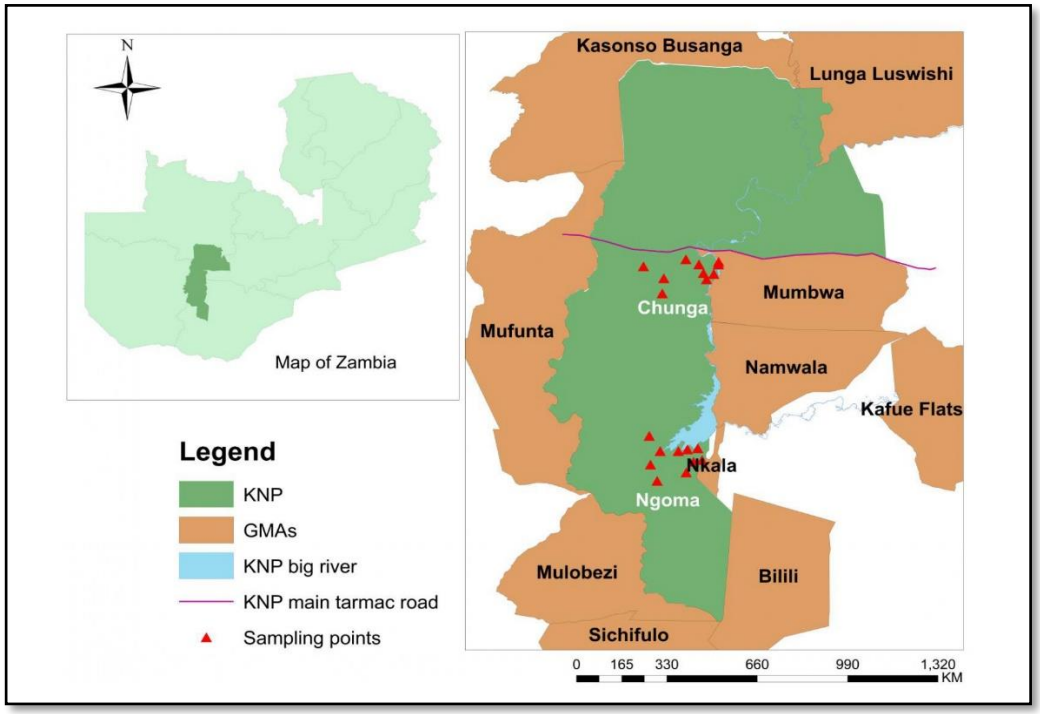


Figure 3. 1. Map of the Study Area

3.2. Study Design and Sampling

A cross-sectional study was conducted between September and December, 2021. Six Epsilon traps containing 3-n-propylphenol, octanol and 4-methylphenol in the ratio of 1:6:12 and an open 300 ml bottle containing acetone at the entrance were deployed under the tree sheds for five days in each sampling locations (Figure 3.2). All the traps were deployed between 17:00 – 18:00 hours and captured tsetse flies were collected by visiting traps in 12 hours interval daily between 06:00 and 07:00 hours and 17:00 and 18:00 hours. Mobile traps with a slow moving vehicle were also used to increase the number of trapped tsetse flies. The geographic coordinates were recorded for each trap. ArcMap in ArcGIS was used to make spatial locations of the sampling points on the map (Figure 3.1). Trapped tsetse flies were counted and grouped into teneral and non teneral flies as described by (Pollock, 1982a). Teneral flies were discarded. Non-teneral flies were stored individually in a 1.5 ml Eppendorf tubes with silica beads to dry and were transported to the University of Zambia, School of Veterinary Medicine Laboratory for tsetse fly identification and DNA extraction.

Figure 3. 2. Epsilon traps used to catch tsetse flies at KNP

3.3. Sex and Species Determination of Tsetse Flies

Morphological characterization were used to sort out sex and species of captured tsetse flies. Species and sex were identified using stereomicroscope based on standard published keys (Pollock, 1982a).

The species of tsetse flies were distinguished based on differences in colour of tarsal segment of front leg and colour bands on abdomen. The distinguishing feature of male and female tsetse flies was found in the posterior part of ventral abdomen. In male tsetse flies, a large rounded lump (which is called hypopygium) was easily detectable at the posterior end. In front of the hypopygium, there was a plate with strong black hairs (hectors). In females, in place of hypopygium and hectors, small genital plate were found.

3.4. Deoxyribonucleic Acid (DNA) Extraction

Each Tsetse fly was transferred to a new 2 ml microcentrifuge tube and smashed for 70 seconds at 3500 rpm in a Micro Smash^{TM} MS-100R bead cell disrupter (TOMY MEDICO. L. T. D., JAPAN) using five 3-mm diameter zirconium beads. DNA was extracted from the homogenate of each tsetse fly using QIAGEN DNeasy Blood and Tissue Kit following the manufacturer's instruction.

Briefly, 200 µl of a cell lysis buffer (Buffer ATL) was added, followed by adding 20 µl of proteinase K to each tube and mixed by vortexing. The mixture was incubated in a heat block at 56 ^{0}C for two hours and vortexing it three times in between. After briefly centrifuging the tubes to remove drops from the lid, 200 µl of Buffer AL was added and mixed thoroughly by vortexing for 15 seconds and incubated in a heating block for 10 minutes at 70 ^{0}C. Next, 200 µl of absolute ethanol was added to each mixture and vortexed for 15 seconds. Then, the mixture was pipetted and transferred into QIAamp Mini spin column in a two microliter collection tube and centrifuged at 6000 x g (8000 rpm) for one minute. Thereafter, the flow-through and collecting tubes were discarded and the QIAamp Mini spin columns were placed in a new two microliter collecting tubes. Then 500 µl of buffer AW1 was added to each spin column, centrifuged at 6000 x g (8000 rpm) for one minute, the flow-through and collecting tubes were discarded and the spin columns were placed in a new two microliter collecting tubes. Next, 500 µl of buffer AW2 was added

and centrifuged for three minutes at 20,000 x g (14,000 rpm). After the flow-through and collecting tubes were discarded, the spin columns were placed in a sterile 1.5 ml Eppendorf tubes and a final elution step was conducted by adding 60 µl buffer AE to each spin column and incubated for one minute at room temperature. Finally, spin columns with Eppendorf tubes were centrifuged at 6000 x g (8000 rpm) for one minute to collect the DNA.

The DNA was quantified using a NanoDrop^{TM} 1000 Spectrophotometer (Thermo Scientific, USA) and DNA samples were stored at – 80 ^{0}C until polymerase chain reaction (PCR) analysis.

3.5. PCR Identification of African Trypanosomes and *S. Glossinidius*

All PCR reactions were conducted using One Taq ®Quick-Load® 2X Master Mix with Standard Buffer (20 mM Tris-HCI (pH 8.9 @ 25°C), 1.8 mM $MgCl_2$, 22 mM NH_4Cl, 22 mM KCl, 0.2 mM dNTPs, 5% glycerol, 0.06% IGEPAL® CA-630, 0.05% Tween® 20, 25 units/ml OneTaq DNA Polymerase) (NEW ENGLAND BioLabs Inc., Ipswich, MA, USA). PCR reactions for both trypanosomes and *S. glossinidius* were carried out in a 10 µl reaction volume containing five µl of One Taq ®Quick-Load® 2X Master Mix, 3.2 µl of nuclease free water, 0.4 µl of forward primer, 0.4 µl of reverse primer and one µl of template DNA. Initial screening for the presence of trypanosome parasites in tsetse flies was conducted using ITS_1 CF and BR primers that target the internal transcribed spacer (Table 3.1) (Njiru et al., 2005) with PCR conditions of initial step at 94 ^{o}C for five minutes, followed by 35 cycles of 94 ^{o}C for 40 seconds, 58 ^{o}C for 40 seconds, 72 ^{o}C for 90 seconds, and final extension at 72 ^{o}C for five minutes. However, ITS_1 primer has low sensitivity against *T. vivax* species (Desquesnes et al., 2001). To solve this problem, *T. vivax* specific primers (TVIV 1 and TVIV 2) were used with the same PCR conditions except the annealing temperature, which was 60 ^{o}C.

When the ITS_1 PCR generated a PCR product of between 500 bp and 800 bp band sizes, *T. congolense* subgroup specific PCR were conducted to differentiate the subgroup Kilifi, Forest and Savannah using primers listed in Table 3.1 with PCR conditions of initial step at 94 ^{o}C for five minutes, followed by 35 cycles of 94 ^{o}C for 30 seconds, 55 ^{o}C for 30

seconds, 72 ºC for 90 seconds, and final extension step at 72 ºC for five minutes. All tsetse flies which showed band sizes between 250 and 500 bp were subject to a PCR to differentiate pathogenic trypanosome species (*T. b. rhodesiense, T. b. brucei, T. simiae, T. simiae Tsavo* and T. *godfreyi*) using species specific primers. To check the human infective trypanosome species (*T. b. rhodesiense*), the serum resistance associated (SRA) PCR using SRA284F and SRA284R primers (Radwanska et al., 2002) was done. This was done with amplification programs of initial denaturation step at 95 ºC for 15 minutes followed by 35 cycles of 94 ºC for one minute, 68 ºC for one minute, 72 ºC for one minute and a final extension step of 72 ºC for 10 minutes. The primers and annealing temperatures used for *T. vivax, T. b. brucei, T. simiae, T. simiae* Tsavo and *T. godfreyi* are also listed in Table 3.1.

The presence of *S. glossinidius* in all tsetse flies was determined using primer pairs GPO1F and GPO1R, which amplify 1,200 bp product of the extrachromosomal DNA fragment of *Sodalis* genes (O'Neill et al., 1993). The amplification program was initiated with an initial step of 94 ºC for five minutes, 35 amplification cycles with denaturation step of each cycle at 94 ºC for one minute, an annealing step at 55 ºC for one minute, and an extension step of 72 ºC for one minute followed by final extension step of 72 ºC for 10 minutes.

All PCR reactions included an appropriate positive and negative control. The PCR products were size separated by electrophoresis in 1xTAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.0) (BioConcept Ltd., Allschwil**,** Switzerland) on 1.5% agarose gel (CSL-AG100, Cleaver Scientific Ltd., Rugby, UK) stained with Ethidium bromide and visualized under UV light. Amplicon sizes were determined relative to a 100 bp DNA ladder.

Table 3. 1. PCR Primers used in this study

Organism	Target gene	Primer Name	Primer sequence (5' to 3')	Amplicon Size (bp)	Annealing temperature (°C)	Reference
***Trypanosoma* Spp.**	ITS1 rDNA	ITS1 CF	CCGGAAGTTCACCGATATTG	Variable	58	(Njiru et al., 2005)
		ITS1 BR	TTGCTGCGTTCTTCAACGAA			
***T. congolense* Kilifi**	Satellite DNA monomer	TCK 1	GTGCCCAAATTTGAAGTGAT	294	55	(Masiga et al., 1992)
		TCK 2	ACTCAAAATCGTGCACCTCG			
***T. congolense* Forest**	Satellite DNA monomer	TCF 1	GGACACGCCAGAAGGTACTT	350	55	(Masiga et al., 1992)
		TCF 2	GTTCTCGCACCAAATCCAAC			
***T. congolense* Savannah**	Satellite DNA monomer	TCS 1	CGAGAACGGGCACTTTGCGA	316	55	(Masiga et al., 1992)
		TCS 2	GGACAAAGAAATCCCGCACA			
T.b. rhodesiense	SRA gene	SRA284 F	ATAGTGACAAGATGCGTACCAACGC	284	68	(Radwanska et al., 2002)
		SRA284 R	AATGTGTTCGAGTACTTCGGTCACCT			
T. vivax		TVIV-F	CTGAGTGCTCCATGTCCCAC	142	60	(Adams et al., 2006)
		TVIV-R	CCACCAGAACACCAACCTGA			
T. brucei s. l		TBR 1	GAATATTAAACAATGCGCAG	164	58	(Masiga et al., 1992)
		TBR 2	CCATTTATTAGCTTTGTTGC			
T. simiae		TSM1	CCGGTCAAAAACGCATT	437	58	(Masiga et al., 1992)
		TSM2	AGTCGCCCGGAGTCGAT			
***T. simiae* Tsavo**		TST1	GTCCTGCCACCGAGTATGC	450	58	(Majiwa et al., 1994)
		TST2	CGAGCATGCAGGATGGCCG			
T. godfreyi		DGG1	CTGAGGCTGAACAGCGACTC	149	58	(Masiga et al., 1996)
		DGG2	GGCGTATTGGCATAGCGTAC			
S. glossinidius	GPO1	GPO1F	TGAGAGGTTCGTCAATGA	1,200	55	(O'Neill et al., 1993)
		GPO1R	ACGCTGCGTGACCATTC			

3.6. Data Management and Analysis

Data were entered into MS-excel and analyzed using R programming. The prevalence of *S. glossinidius* and trypanosomes were estimated using frequencies. The chi-square test was used to compare the prevalence of trypanosomes and *S. glossinidius* with sex, species and collection site of tsetse flies. Pearson chi-square test of association or Fisher's exact test was used to assess the association between *S. glossinidius* and trypanosome infections in tsetse flies. All the statistic were considered significant at $p \leq 0.050$.

3.7. Ethical Consideration

Ethical approval for this study was obtained from The University of Zambia Biomedical Research Ethics Committee (UNZABREC) (Ref. No: 1865-2021) and approved by the National Health Research Authority (NHRA), Zambia (Ref. No: NHRA000001/16/11/2021). All activities in the National Park were approved by the Ministry of Tourism and Arts, Department of National Parks and Wildlife, Zambia (Permit number: NPW/8/27/1).

CHAPTER FOUR

4.0. RESULTS

4.1. Distribution of Tsetse Flies

A total of 326 tsetse flies were trapped at the two sampling locations over a period of 10 days (Figure 4.1). Of the total 326 tsetse samples collected, 50 (15.3%, 95% CI: 11.83-19.85) were from Chunga sampling location and 276 (84.7%, 95% CI: 80.35-88.17) were from Ngoma sampling location of KNP. Among 326 tsetse flies collected, 270 (82.8%, 95% CI: 78.35- 86.53) were *G. morsitans* species and 56 (17.2%, 95% CI: 13.47- 21.65) were *G. pallidipes* species. Out of a total of 326 collected tsetse flies, 70.9% (95% CI: 65.7-75.5%) were male and 29.1% (95% CI: 24.5–34.3%) were female.

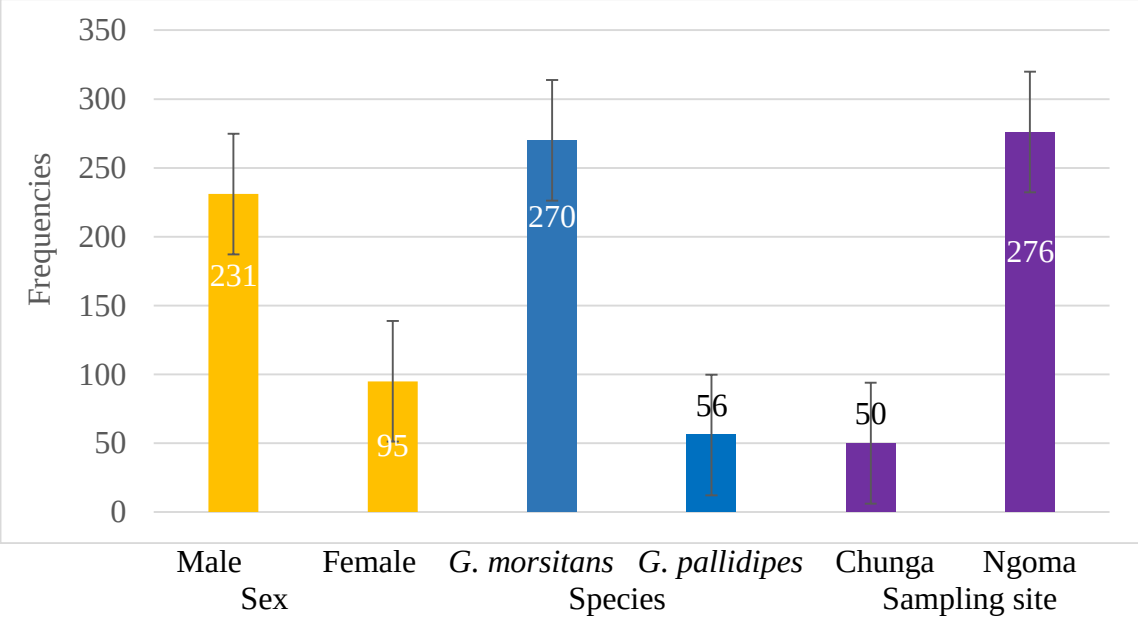


Figure 4. 1. Characteristics and distribution of captured tsetse fly

Note: Error bars corresponds to 95% confidence interval

4.2. Prevalence and Distribution of *Trypanosoma* species Identified in Tsetse flies

Of 326 tsetse fly samples subjected to PCR using general ITS_1 primers (Figure 4.2) for the presence of trypanosomes, 63 (19.3%) were found with DNA of at least one trypanosome species and 263 (80.7%) were negative indicating an overall prevalence of trypanosome infections of 19.3% (95% CI: 15.41-23.96) (Table 4.1). The prevalence of

trypanosomes in Chunga sampling location was 6.0% (*n* = 50; 95% CI: 2.06-16.22) and in Ngoma sampling location was 21.7% (*n* = 276; 95% CI: 17.28-26.98). The difference in presence of trypanosome DNA between the two sampling locations was statistically significant (X^2 = 6.73, *p* = 0.009). From 270 *G. morsitans* analyzed, trypanosome DNA was detected in 51 tsetse flies (18.9%, 95% CI: 14.67-23.98); three (6.4%) from Chunga sampling site and 48 (21.5%) from Ngoma sampling site. Out of 56 *G. pallidipes* checked for trypanosome presence, 12 (21.4%, 12.71-33.82) showed trypanosome positivity, where all positive tsetse flies were from Ngoma sampling location. No trypanosome DNA was detected in *G. pallidipes* from Chunga sampling location. From overall data across species, there was no statistically significant difference between the prevalence of trypanosome and tsetse fly species (χ^2 = 0.192; *p* = 0.661). The prevalence of trypanosomes according to sex was 20.4% (*n* = 231; 95% CI: 15.66-26.00) in male tsetse flies and 16.8% (*n* = 95; 95% CI: 10.64-25.62) in female tsetse flies. There was no statistically significant difference between male and female tsetse flies in relation to trypanosome infections (χ^2 = 0.53; *p* = 0.467).

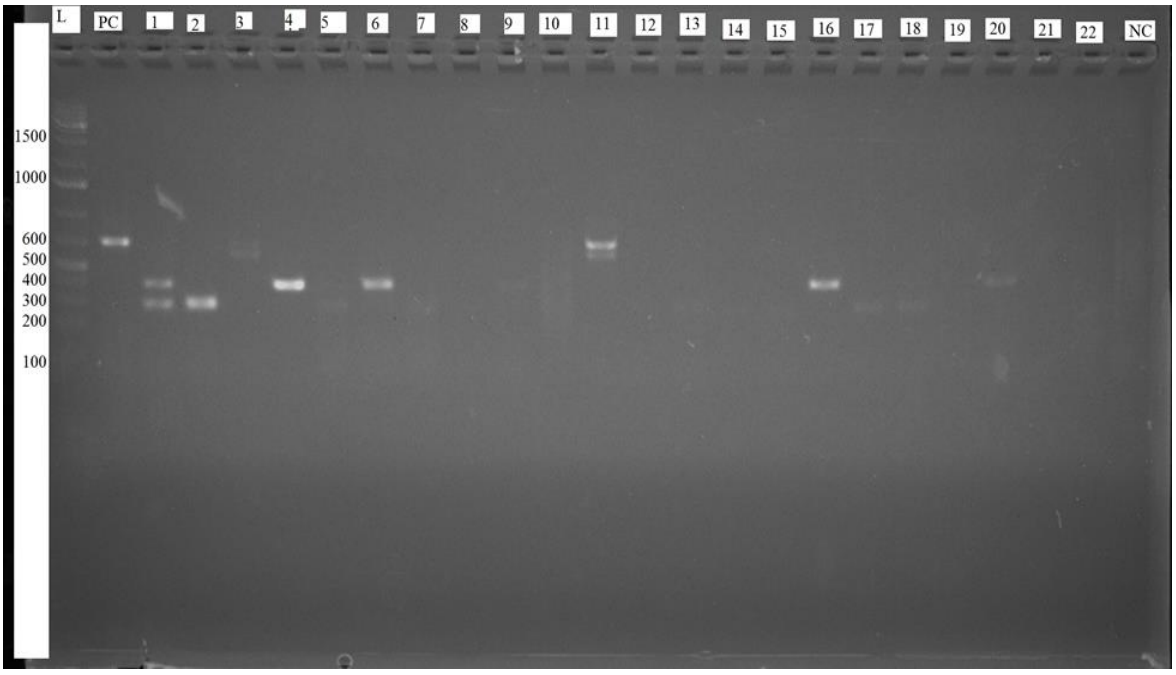


Figure 4. 2. Representative samples for ITS_1 amplification of trypanosomes
L _ 100 bp DNA Ladder; PC- Positive Control with 600 base pair amplicon size; 1,2,4,6, 11, 16, 17, 18, 20 – Positive Samples; NC – Negative Control

Table 4. 1. Prevalence of African trypanosomes in relation to sex, species and sampling site

Variable	Categories	*n*	Prevalence (%)	95% CI	*p*-value
Sampling location	Chunga	50	6.0	2.06-16.22	0.009*
	Ngoma	276	21.7	17.28-26.98	
	Overall	326	19.3	15.41-23.96	
Sex	Male	231	20.4	15.66-26.00	0.447
	Female	95	16.8	10.64-25.62	
	Overall	326	19.3	15.41-23.96	
Species	*G. morsitans*	270	18.9	14.67-23.98	0.661
	G. pallidipes	56	21.4	12.71-33.82	
	Overall	326	19.3	15.41-23.96	
G. morsitans	Male	190	20.0	14.93-26.26	0.583
	Female	80	16.3	9.75-25.84	
	Overall	270	18.9	14.67-23.98	
G. pallidipes	Male	41	22.0	12.00-36.71	1.00[a]
	Female	15	20.0	7.05-45.19	
	Overall	56	21.4	12.71-33.82	
Location	Species				
Chunga	*G. morsitans*	47	6.4	2.19-17.16	1.00[a]
	G. pallidipes	3	0.0	0.00-56.15	
	Overall	50	6.0%	2.06-16.22	
Ngoma	*G. morsitans*	223	21.5	16.64-27.38	0.859
	G. pallidipes	53	22.6	13.45-35.53	
	Overall	276	21.7	17.28-26.98	

n: sample size; 95% CI: confidence interval; a: Fisher's exact test; *: Statistically significant

Six different trypanosome species were detected in *G. morsitans* and *G. pallidipes*. *T. vivax* (6.4%, 4.25- 9.65) was the dominant trypanosome species found, followed by *T.*

simiae (4.6%, 2.81-7.45), *T. congolense* (4.0%, 2.35-6.70), *T. godfreyi* (3.7%, 2.12-6.32), *T. simiae* Tsavo (3.1%, 1.67-5.55) and *T. b. brucei* (2.5%, 1.25-4.77) (Table 4. 2).

T. congolense and *T. simiae* infection rates were higher in *G. morsitans* (4.4% and 4.8%, respectively) than *G. pallidipes* (1.8% and 3.6%, respectively). *T. simiae* Tsavo was identified in 3.7% of *G. morsitans* tsetse flies, but not in *G. pallidipes.* The prevalence of *T. vivax* was 6.3% in *G. morsitans* and 7.1% in *G. pallidipes*. *T. b. brucei* and *T. godfreyi* were identified more frequently in *G. pallidipes* (5.4% for each) than *G. morsitans* (1.9% and 3.3%, respectively). There were no significant difference in the prevalence of each trypanosome species detected between species (Table 4.2).

Table 4. 2. Prevalence of trypanosome species according to tsetse fly species

	Tsetse fly species		Total (95% CI)
	G. morsitans (95% CI)	*G. pallidipes*(95% CI)	
n	270	56	326
T. congolense	4.4% (2.56-7.61)	1.8% (0.32-9.45)	4.0% (2.35-6.70)
		$p = 0.705$	
T. vivax	6.3% (3.97-9.85)	7.1% (2.81-16.98)	6.4% (4.25-9.65)
		$p = 0.768$	
T. b. brucei	1.9% (0.79-4.26)	5.4% (1.84-14.61)	2.5% (1.25-4.77)
		$p = 0.142$	
T. simiae	4.8% (2.84-8.06)	3.6% (0.98-12.12)	4.6% (2.81-7.45)
		$p = 1.000$	
T. godfreyi	3.3% (1.76-6.21)	5.4% (1.84-14.61)	3.7% (2.12-6.32)
		$p = 0.440$	
T. simiae Tsavo	3.7% (2.02-6.68)	0.0% (0.0-6.42)	3.1% (1.67-5.55)
		$p = 0.221$	

n = number of tsetse fly samples, CI = Confidence interval, *p* = p-value

The prevalence of *T. vivax, T. congolense*, *T. simiae, T. godfreyi, T. simiae* Tsavo, and *T. b. brucei* in male tsetse flies were 6.5%, 4.8%, 4.3%, 3.9%, 3.0%, and 2.6%, respectively and in female tsetse flies were 6.3%, 2.1%, 5.3%, 3.2%, 3.2%, and 2.1%, respectively (Table 4.3). No statistically significant difference were found in prevalence of each trypanosome species detected between the sexes of the tsetse flies (Table 4.3).

Table 4. 3. Prevalence of trypanosome species according sex

	Tsetse fly sex		
	Male (95% CI)	Female (95% CI)	Total (95% CI)
n	231	95	326
T. congolense	4.8% (2.68-8.32)	2.1% (0.58-7.35)	4.0% (2.35-6.70)
		$p = 0.360$	
T. vivax	6.5% (3.97-10.44)	6.3% (2.93-13.10)	6.4% (4.25- 9.65)
		$p = 0.003$	
T. b. brucei	2.6% (1.20-5.55)	2.1% (0.58-7.35)	2.5% (1.25-4.77)
		$p = 1.000$	
T. simiae	4.3% (2.37-7.78)	5.3% (2.27-11.73)	4.6% (2.81-7.45)
		$p = 0.773$	
T. godfreyi	3.9% (2.06-7.24)	3.2% (1.08-8.88)	3.7% (2.12-6.32)
		$p = 1.000$	
***T. simiae* Tsavo**	3.0% (1.48-6.12)	3.2% (1.08-8.88)	3.1% (1.67-5.55)
		$p = 1.000$	

n: number of tsetse fly samples, CI: Confidence interval, *p*: p-value

The prevalence of each trypanosome species detected in Chunga sampling site were 2.0% except *T. simiae* Tsavo which was not detected in this sampling site, while the prevalence in Ngoma sampling site were 7.3%, 5.1%, 4.4%, 4.0%, 3.6%, and 2.5% for *T. vivax*, *T. simiae*, *T. congolense*, *T. godfreyi*, *T. simiae* Tsavo, and *T. b. brucei*, respectively (Table 4.4). Despite the prevalence of each trypanosome species being higher in Ngoma than Chunga sampling site, no statistical significant difference were found (Table 4.4).

Table 4. 4. Prevalence of trypanosome species according to tsetse fly sampling site

	Tsetse fly sampling site		
	Chunga (95% CI)	Ngoma (95% CI)	**Total (95% CI)**
n	50	276	326
T. congolense	2.0% (0.35-10.50)	4.4% (2.50-7.44)	4.0% (2.35-6.70)
		$p = 0.700$	
T. vivax	2.0% (0.35-10.50)	7.3% (4.74-10.93)	6.4% (4.25- 9.65)
		$p = 0.220$	
T. b. brucei	2.0% (0.35-10.50)	2.5% (1.23-5.14)	2.5% (1.25-4.77)
		$p = 1.000$	
T. simiae	2.0% (0.35-10.50)	5.1% (3.05-8.33)	4.6% (2.81-7.45)
		$p = 0.483$	
T. godfreyi	2.0% (0.35-10.50)	4.0% (2.24-6.99)	3.7% (2.12-6.32)
		$p = 0.700$	
***T. simiae* Tsavo**	0.0% (0.0-7.13)	3.6% (1.98-6.54)	3.1% (1.67-5.55)
		$p = 0.371$	

n: number of tsetse fly samples, CI: Confidence interval, *p*: p-value

Among thirteen tsetse flies which were positive for *T. congolense,* eight tsetse flies had *T. congolense* Kilifi subgroup (Figure 4.3), two with *T. congolense* Forest subgroup, and one with *T. congolense* Savannah. Two tsetse flies had mixed *T. congolense* subgroups. Of these one had mixed infection of *T. congolense* Kilifi and *T. congolense* Forest. The other had *T. congolense* Forest mixed with *T. congolense* Savannah.

The human infective *T. b. rhodesiense* was not detected in both species of tsetse flies.

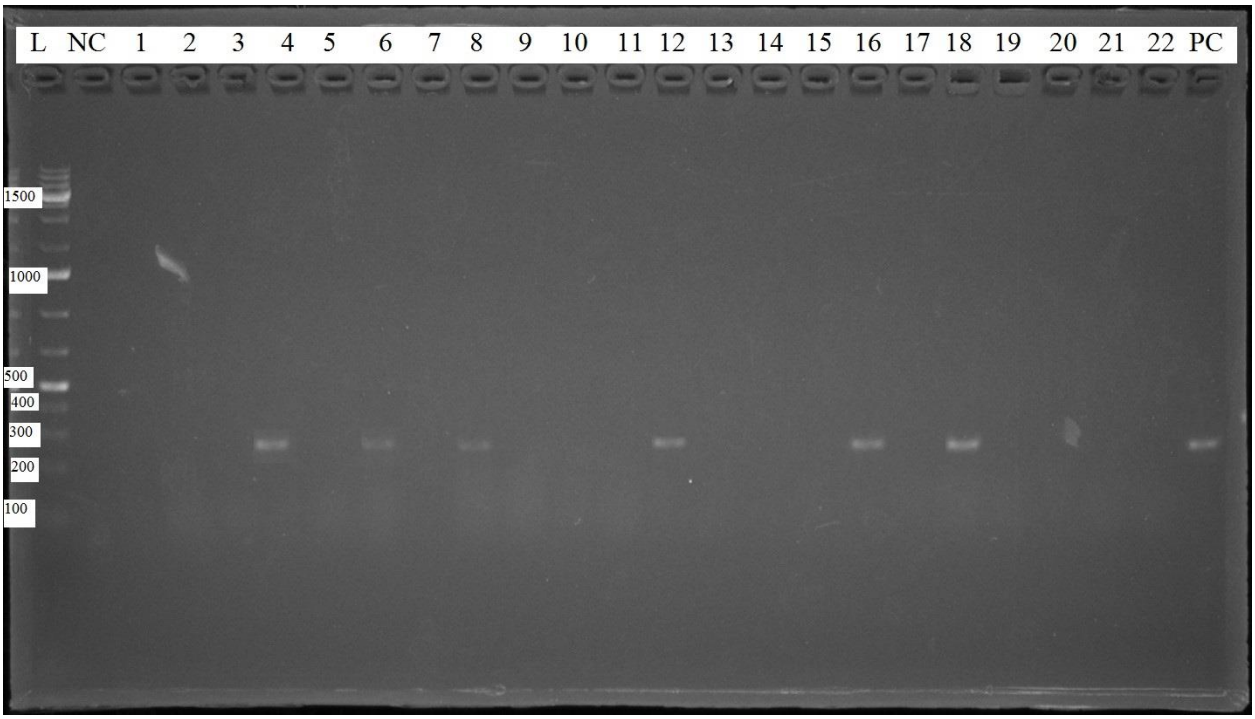


Figure 4. 3. Satellite DNA monomer amplifications using TCK 1 and TCK 2 primers

L – 100 bp DNA ladder; NC – Negative Control; PC – Positive Control; 4, 6, 8, 12, 16, 18 – *T. congolense* subgroup Kilifi positive samples.

According to type of infections (single or multiple), most tsetse flies were infected with single trypanosome species (49/63, 77.8%), followed by tsetse flies infected by two *trypanosoma* species (12/63, 19.0%) and tsetse flies that had three *trypanosoma* species (2/63, 3.2%) (Figure 4.4). Multiple infections with two trypanosomes species included three tsetse flies that had a mixture of *T. simiae* and *T. godfreyi*, three tsetse flies that had *T. simiae* and *T. simiae* Tsavo, two tsetse flies infected with *T. congolense* and *T. simiae*, one tsetse fly with *T. congolense* and *T. vivax*, one tsetse fly contained *T. congolense* mixed with *T. b. brucei*, one tsetse fly with *T. b. brucei and T. vivax* and one tsetse fly with *T. simiae* Tsavo and *T. godfreyi*. Triple infections were found in two tsetse flies which had *T. simiae*/*T. godfreyi*/*T. simiae* Tsavo and *T. simiae*/*T. congolense*/*T. simiae* Tsavo.

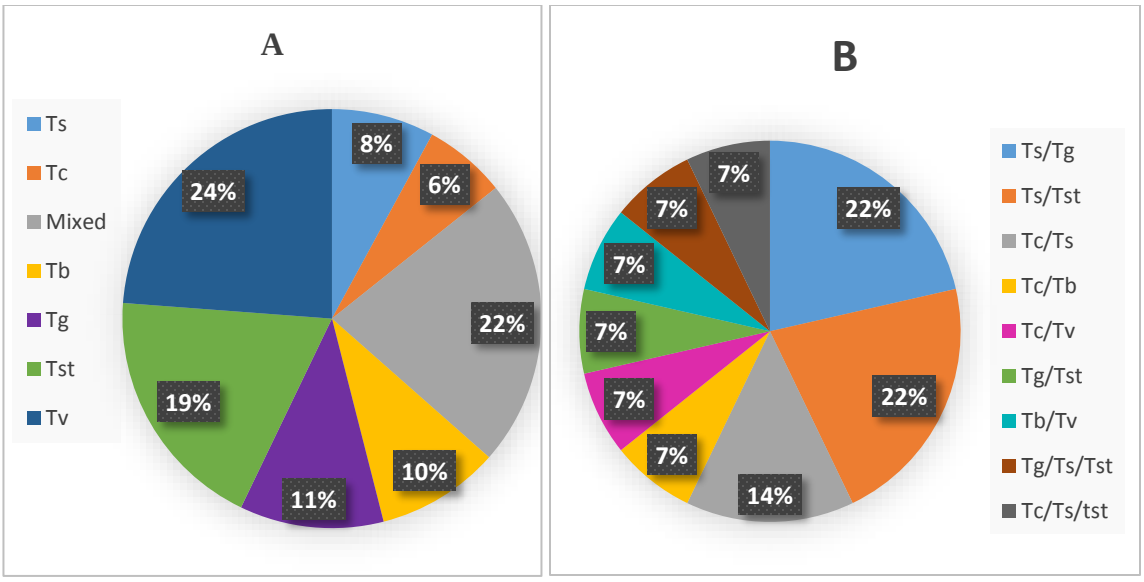


Figure 4. 4. Distribution of single (A) and multiple (B) infections of *trypanosome* in tsetse flies

Tc _ *T. congolense*, Tb _ *T. b. brucei*, Tg _ *T. godfreyi*, *Ts* _ *T. simiae*, *Tst* _ *T. simiae Tsavo*, and *Tv* _ *T. vivax.*

4.3. Prevalence of *S. glossinidius* in Tsetse flies

Among 326 tsetse flies checked for the presence of *S. glossinidius* using GPO1 primers (Figure 4.6), 71 were positive with an overall prevalence of 21.8% (95% CI: 17.64-26.57). Prevalence of *S. glossinidius* was higher in female tsetse flies (24.2%; 95% CI: 16.71, 33.72) than in male ones (20.8%; 95% CI: 16.05, 26.47). In relation to tsetse fly species, *S. glossinidius* positivity was slightly higher in *G. morsitans* (22.2%; 95% CI: 17.67, 27.55) than in *G. pallidipes* (19.6%; 95% CI: 11.34, 31.84). Tsetse flies from Chunga (26.0%: 95% CI: 15.87, 39.55) had a slightly higher prevalence of *S. glossinidius* than those from Ngoma (21.0%; 95% CI: 16.62, 26.20) (Figure 4.5). There was no significant difference on prevalence of *S. glossinidius* between tsetse fly species (p =0.670), sex (p = 0.495), and sampling site (p = 0.549) (Figure 4.5).

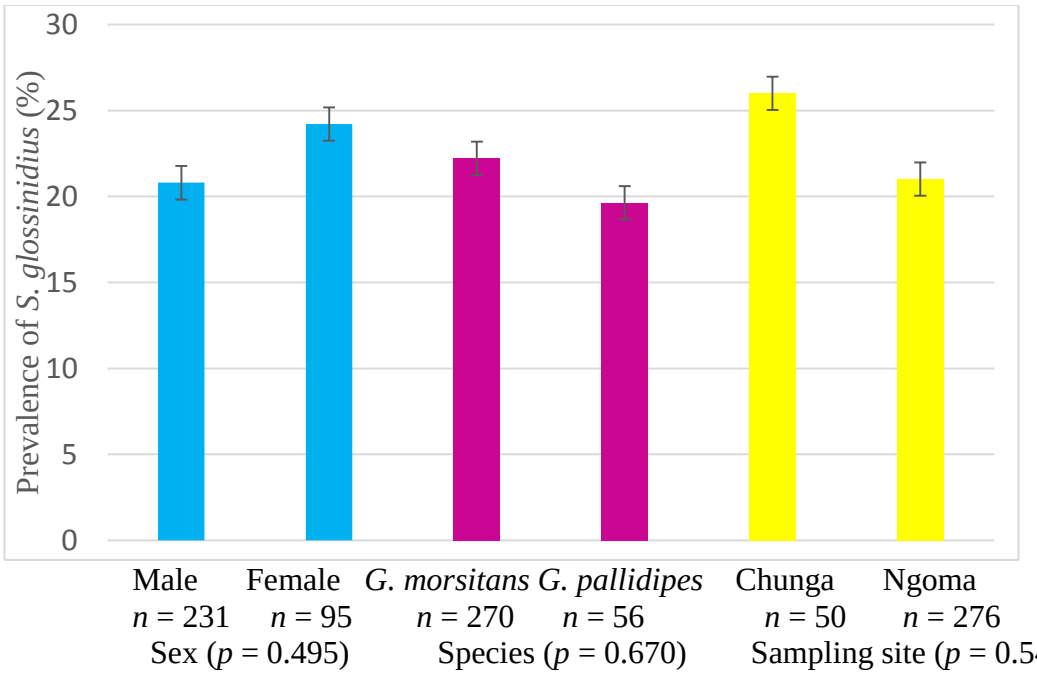


Figure 4. 5. Prevalence of *S. glossinidius* in tsetse flies

n = number of tsetse flies analyzed in each population, *p* = p- values

S. glossinidius prevalence in *G. morsitans* species from Chunga sampling site (27.66%, 95% CI: 16.94-41.76) was higher than Ngoma sampling site (21.08%, 95% CI: 16.24-26.90), but the difference was not statistical significant (*p* = 0. 324) (Table 4.5). The prevalence of *S. glossinidius* in *G. pallidipes* from Ngoma was 20.75% (95% CI: 12.00-33.46), but none of the *G. pallidipes* were infected by *S. glossinidius* from Chunga. Based on the species and sex of tsetse flies, a higher positivity of *S. glossinidius* was found in male *G. morsitans* (25.26%, 95% CI: 19.62-31.89**)** than female *G. morsitans* **(**22.5%, 95% CI: 14.73-32.79); whereas the prevalence was higher in female *G. pallidipes* (33.33%, 95% CI: 15.18-58.29) than male *G. pallidipes* (14.63%, 95% CI: 6.88-28.44**).** There was no statistically significant difference in prevalence between male *G. morsitans* and female *G. morsitans* species (*p* = 0.943) and between male *G. pallidipes* and female *G. pallidipes* (*p* = 0.142) (Table 4.5).

Table 4. 5. *S. glossinidius* prevalence in *G. morsitans* and *G. pallidipes* based on sex and sampling site

Species	Location	*n*			Prevalence (95% CI)			*p*-value
		M	F	Total	M	F	Overall	
G. morsitans	Chunga	22	25	47	27.3% (13.15-48.15)	28.0% (14.28-47.58)	27.7% (16.94-41.76)	
	Ngoma	168	55	223	21.4% (15.90-28.24)	20.0% (11.55-32.36)	21.1% (16.24-26.90)	0. 324
	Total	190	80	270	25.3% (19.62-31.89)	22.5% (14.73-32.79)	22.2% (17.67-27.55)	
					$p = 0.943$			
G. pallidipes	Chunga	2	1	3	0.0%	0.0%	0.0%	
	Ngoma	39	14	53	15.4%, 7.25-29.73	35.7% (16.34-61.24)	20.8 (12.00-33.46)	1.000
	Total	41	15	56	14.6%, 6.88-28.44	33.3% (15.18-58.29)	19.6% (11.34-31.84)	
					$p = 0.142$			

n: number of tsetse flies sampled and tested for *S. glossinidius*, F: Female tsetse flies, M: male tsetse flies, CI: Confidence interval, *p*: p-value

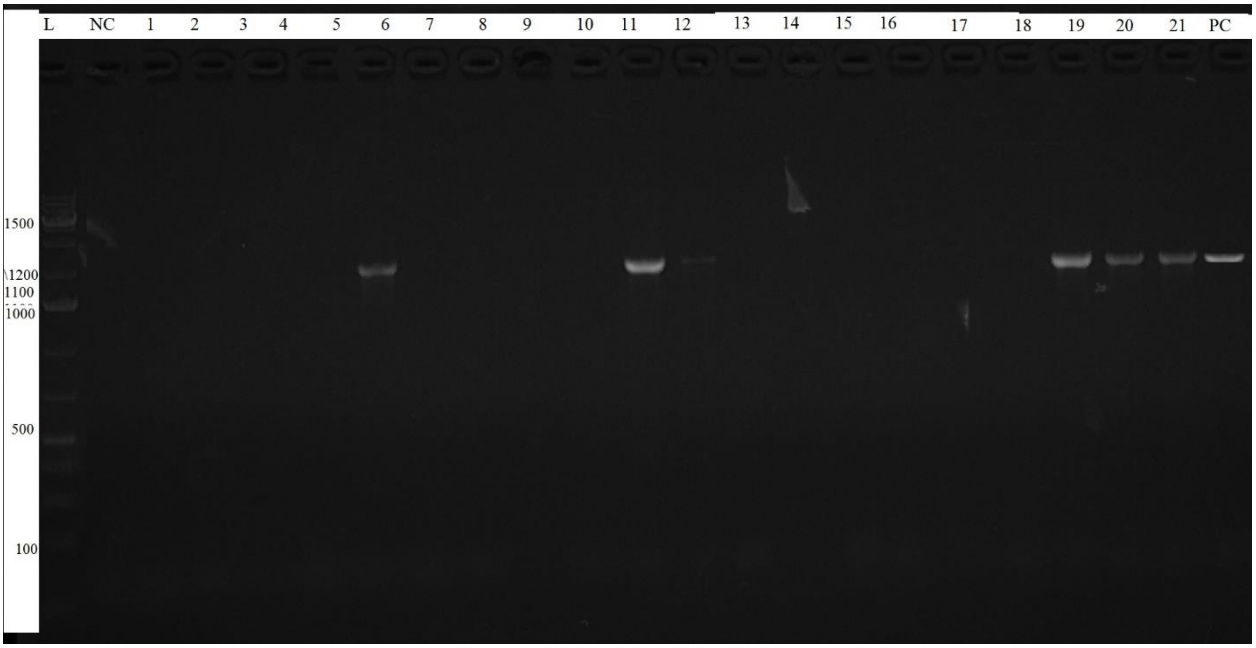


Figure 4. 6. Representative DNA amplification using GPO1 primers

L – 100bp DNA Ladder; NC- Negative control; PC – Positive control; 6, 11, 12, 19, 20, 21 positive samples

4.4. Association between *S. glossinidius* and prevalence of African trypanosomes

Among the 326 tsetse flies analyzed, 21.8% had *S. glossinidius*, whereas 19.3% harboured at least one trypanosome species. The number of tsetse flies infected by *S. glossinidius* (71) was higher than the number of tsetse flies infected by trypanosomes (63). Out of 63 trypanosome infected flies, 47.6% of the flies also carried *S. glossinidius*, while the remaining flies were devoid of *S. glossinidius*. The analysis done from overall dataset indicated that there was significant association between tsetse flies harbouring *S. glossinidius* and tsetse flies infected with trypanosome species (X^2 = 30.61, $p < 0.001$). The association varied between sampling sites, with tsetse flies from Ngoma showed statistically significant association (X^2 = 30.39, $p < 0.001$) between trypanosomes and the bacteria, while tsetse flies from Chunga showed no statistically significant association (p = 0.162). In *G. morsitans*, twenty-five out of sixty tsetse flies with *S. glossinidius* were infected with trypanosomes, with statistically significant association (X^2 = 26.12, p <0.001). From eleven *S. glossinidius* positive *G. pallidipes* tsetse flies, five had trypanosomes, with statistical significant association (p = 0.045). Statistically significant

association were also observed between *S. glossinidius* and trypanosome prevalence in male tsetse flies (X^2 = 28.42, $p < 0.001$), but no significant association was found between the presence of *S. glossinidius* and trypanosome prevalence in female tsetse flies (p = 0.058) (Table 4.6).

Table 4. 6. Association between *S. glossinidius* and *trypanosome* according to tsetse fly species, sex and sampling site

	Overall		***G. morsitans***		***G. pallidipes***		**Male**		**Female**		**Chunga**		**Ngoma**	
	T+	T-	T+	T-	T+	T-	T+	T-	T+	T-	T+	T-	T+	T-
S+	30	41	25	35	5	6	23	26	7	16	2	11	28	30
S-	33	222	26	184	7	38	24	159	9	63	1	36	32	190
	X^2 = 30.61, $p < 0.001$		X^2 = 26.12, $p < 0.001$		p = 0.045		X^2 = 28.42, $p < 0.001$		p = 0.058		p = 0.162		X^2 = 30.39, $P < 0.001$	

T+: Trypanosome positive, T-: Trypanosome negative, S+: *Sodalis* positive, S-: *Sodalis* negative, *p:* p-value

Of the ten tsetse flies infected with *T. simiae* Tsavo, six (60.0%) were also infected by *S. glossinidius*. From 15 tsetse flies infected with *T. simiae*, seven (46.7%) were also infected with *S. glossinidius*. Among 21 tsetse flies infected by *T. vivax*, ten (47.6%) were co-infected with *S. glossinidius*. Among 13 tsetse flies infected by *T. congolense*, co-infection with *S. glossinidius* was 30.8%, while for *T. b. brucei* and *T. godfreyi* co-infection rate were 50.0% (Table 4.4). The association of individual trypanosome species with presence of *S. glossinidius* indicated that statistically significant associations were found between *S. glossinidius* and *T. vivax* (p = 0.006), *T. simiae* (p = 0.025), *T. simiae* Tsavo (p = 0.009), and *T. godfreyi* (p = 0.027), but no significant association between *S. glossinidius* and *T. congolense* (p = 0.491), and *T. b. brucei* (p = 0.072) (Table 4.4).

Table 4. 7. Association between *S. glossinidius* and *trypanosoma* species detected in tsetse flies

	T. congolense		***T. vivax***		***T. b. brucei***		***T. simiae***		***T. s.* Tsavo**		***T. godfreyi***	
	Tc+	Tc-	Tv+	Tv-	Tbb+	Tbb-	Ts+	Ts-	Tst+	Tst-	Tg+	Tg-
S+	4	67	10	61	4	67	7	64	6	65	6	66
S-	9	247	11	244	4	251	8	247	4	251	6	249
	$p = 0.491$		$p = 0.006$		$p = 0.072$		$p = 0.025$		$p = 0.009$		$p = 0.027$	

Tc+: *T. congolense* positive, Tc-: *T. congolense* negative; Tv+: *T. vivax* positive, Tv-: *T.vivax* negative; Tbb+: *T. b. brucei* positive, Tbb-: *T. b. brucei* negative; Ts+: *T. simiae* positive, Ts-: *T. simiae* negative; Tst+: *T. simiae* Tsavo positive, Tst-: *T. simiae* Tsavo negative; Tg+: *T. godfreyi* positive, Tg-: *T. godfreyi* negative; *p:* p-value; OR: Odds Ratio; CI: Confidence Interval

CHAPTER FIVE

5.0. DISCUSSION

5.1. Prevalence of trypanosome species

In this study, six species of trypanosomes were detected in *G. morsitans* and *G. pallidipes* tsetse flies from the KNP. The overall prevalence of trypanosomes in these tsetse flies was estimated to be 19.3%. This result was comparable with the 23.2% prevalence reported in Western Zambia (Mbewe et al., 2015), but it was lower than other studies in Zambia such as the 43.86% in Luambe National Park (Dennis et al., 2014), 36.4% in the Luangwa valley, north-eastern Zambia (Laohasinnarong et al., 2015), and 26.85% in Kafue Ecosystem (Nakamura et al., 2021). This result was in line with the 17.4% prevalence reported from Ghana (Nakayima et al., 2012). The prevalence of trypanosomes in this work was lower than the prevalence of trypanosomes reported by different studies in National Parks and Wildlife Reserve areas from outside of Zambia which includes: 85.1% Nkhotakota Wildlife Reserve, Malawi (Musaya et al., 2017), 58% in Liwonde Wild Life Reserve, Malawi (Nayupe et al., 2019), 42.1% in the Shimba Hills National Reserve and Nguruman regions in Kenya (Channumsin et al., 2018), 39.3% in the wildlife reserve of Santchou in the western region of Cameroon (Kamdem et al., 2020), 38% in Nech Sar National Park in Ethiopia (Rodrigues et al., 2019),. The result was higher compared to other studies such as 0.8% in Jomoro district of the western region of Ghana (Apaatah et al., 2020), 2.40% in the Maasai Mara National Reserve, a wildlife-human-livestock interface (Makhulu et al., 2021), 3.4% in Maasai Steppe, northern Tanzania (Simwango et al., 2017b), 6.31% in the sleeping sickness focus of Zimbabwe in Hurungwe District (Shereni et al., 2016), 10.7% in the sleeping sickness endemic focus of northwestern Uganda (Opiro et al., 2021), and 11.4% in tsetse flies collected from Kenyan coastal forests and South Africa (Wamwiri et al., 2013).

In this study, the prevalence of trypanosome infections was higher in male than female tsetse flies. This result is in disagreement with that from other studies where they reported higher prevalence of trypanosomes in females than male tsetse flies (Gaithuma et al., 2019; Isaac et al., 2016). It is however in agreement with experimental studies in the same

tsetse species (Moloo et al., 1992) in which males had higher trypanosome infectivity than female tsetse flies.

In the present study, most pathogenic AAT causing trypanosome species were detected. *T. vivax* was the most dominant, which is in line with a study which reported a high prevalence of *T. vivax* in tsetse flies and cattle in the same study area (Nakamura et al., 2021). The result is also in agreement with other studies in Zambia (Mbewe et al., 2015) and other parts of Africa (Makhulu et al., 2021; Simwango et al., 2017a). Other trypanosome species detected were *T. simiae, T. congolense, T. godfreyi*, *T. simiae* Tsavo, and *T. b. brucei*. The higher prevalence of *T. vivax* in tsetse flies compared with other trypanosome species may be due to the differences in development cycles; where the entire developmental stages of *T. vivax* is only in proboscis of the tsetse flies unlike *T. congolense*, *T. simiae, T. godfreyi*, and *T. simiae* Tsavo which completes the developmental stage in proboscis and midgut and *T. b. brucei* in midgut and salivary gland of the tsetse flies where they can be affected by low pH, protease activity and lectins in the midgut (Dyer et al., 2013; Rotureau and Van Den Abbeele, 2013).

Despite a recent HAT case in adult male (Squarre et al., 2016) being reported and presence of human infective *T. b. rhodesiense* in vervet monkey, sable antelope and buffalo (Squarre et al., 2020) and in cattle (Nakamura et al., 2021) from KNP ecosystem were recorded, the human infective trypanosome species was not detected in this study. Although *T. b. rhodesiense* was not detected in the current study, the presence of the most competent tsetse fly vectors of *T. b. rhodesiense* (*G. morsitans* and *G. pallidipes*) in KNP and surrounding GMAs and high prevalence of *T. b. rhodesiense* in wildlife and cattle in the area, coordinated surveillance and diagnosis in the KNP ecosystem is required.

5.2. Prevalence of *S. glossinidius*

The overall prevalence of *S. glossinidius* estimated from this study is lower than the 31.3% that was reported from southwest Nigeria (Odeniran et al., 2019) and the 34.0% that was reported from the Shimba Hills and Nguruman regions in Kenya (Channumsin et al., 2018). However, the current results are higher than 6.6% in the Maasai Mara National Reserve, a wildlife-human-livestock interface (Makhulu et al., 2021) and 15.9%

prevalence reported in the Shimba Hills National Reserve, a wildlife-human- livestock interface on Kenya's south coast (Wamwiri et al., 2013).

The result showed no statistical significant difference in the prevalence of *S. glossinidius* in tsetse fly species. In *G. morsitans*, the prevalence of *S. glossinidius* was higher than the 17.5% reported in Luambe National Park, Zambia (Dennis et al., 2014) and 15.9% obtained from Western Zambia (Mbewe et al., 2015). However, this value is lower than the prevalence of 29.6% that reported from Zimbabwe (Mathew, 2007) and 28.6% reported from Adamawa region of Cameroon (Kame-Ngasse et al., 2018). In this study, the prevalence of *S. glossinidius* in *G. pallidipes* was higher than 1.4%, 6.5%, 15.9%, and 16% recorded in Luambe National Park, Zambia (Dennis et al., 2014), Maasai Mara National Reserve, Kenya (Makhulu et al., 2021), Shimba Hills National Reserve, Kenya (Wamwiri et al., 2013) and tsetse flies collected from Zimbabwe (Mathew, 2007), respectively. This result was however lower than the 83.3% in *G. pallidipes* collected from Tanzania (Mathew, 2007). These differences may be linked to environmental and ecological variations between sampling areas which can highly affect the biology of tsetse flies and the presence of different *S. glossinidius* genotypes (Farikou et al., 2011).

In this study, there was no significant difference in prevalence of *S. glossinidius* between male and female tsetse flies. This finding is in agreement with those of other studies that reported similar results (Dennis et al., 2014; Mathew, 2007). Data analyzed for individual tsetse species also indicated no significant difference in the prevalence of *S. glossinidius* between sexes of *G. morsitans* and *G. pallidipes*.

5.3. Association between *S. glossinidius* and trypanosome infections in tsetse flies

From the overall data analyzed, the co-infection rate between *S. glossinidius* and trypanosomes in tsetse flies in this study were lower than 37% reported from "Faro and Déo" division of the Adamawa region of Cameroon (Kame-Ngasse et al., 2018) and 32.2% in two historical human African trypanosomiasis foci in Cameroon (Farikou et al., 2010), but higher than 2% co-infection rate in Kenyan coastal forests (Wamwiri et al., 2013).

In the current study, significant association was found between the presence of *S. glossinidius* and presence of trypanosomes in tsetse flies. This maybe an indication that presence of *S. glossinidius* favors trypanosome infections in tsetse flies. This is in agreement with other studies from Cameroon (Farikou et al., 2010), Zambia (Mbewe et al., 2015) and Kenya (Makhulu et al., 2021), where significant association were reported between *S. glossinidius* and trypanosome infections in different tsetse fly species. There however was variation in degree of association between tsetse fly species, sex and sampling locations.

There were no large difference between the proportion of co-infected tsetse flies with *Sodalis* and trypanosomes in *G. morsitans* and *G. pallidipes* (9.26% and 8.93% respectively) and the association test result was the same between the two species of tsetse flies. Significant associations were found between *Sodalis* and trypanosome infections in *G. morsitans* and *G. pallidipes* tsetse fly species. This findings are in line with other studies conducted in *G. m. centralis* (Mbewe et al., 2015), *G. pallidipes* (Wamwiri et al., 2013), *G. pallidipes* and *G. swynnertoni* (Makhulu et al., 2021) and *G. p. palpalis* (Farikou et al., 2010) where all reported a significant association between *S. glossinidius* and trypanosome infections in the respective tsetse fly species. This result supported the hypothesis that presence of *S. glossinidius* increases susceptibility and establishment of trypanosome infections in *G. morsitans* and *G. pallidipes* tsetse flies. But the current result is in contrast with a study in *G. morsitans* and *G. pallidipes* of tsetse flies in Luambe National Park, Zambia (Dennis et al., 2014), where no significant associations between *S. glossinidius* and trypanosome infections were found. This difference may be (in addition to presence/absence of *S. glossinidius*) due to a difference in *S. glossinidius* genotype which may affect the association between *S. glossinidius* and trypanosome infections as described by Geiger and his colleagues (Geiger et al., 2005). Based on sex of tsetse flies, twenty three male tsetse flies and only seven female tsetse flies were co-infected with *S. glossinidius* and trypanosomes. There was significant association between *S. glossinidius* and trypanosome infections in male tsetse flies, but no significant association was observed between the endosymbiont and trypanosome infections in female tsetse flies. These differences may be due to small number of female tsetse flies collected and checked

for the endosymbiont and trypanosome infections. Separate analysis of data for each sampling site indicates there is a difference in statistical association between the endosymbiont and trypanosome infections, where statistically significant association was observed in Ngoma sampling site, but not in Chunga sampling site. This difference is maybe due to low trypanosome infection rate and small number of tsetse flies captured in Chunga sampling site.

The association between *S. glossinidius* and each trypanosome species infection were also examined. The result of this analysis clearly indicated that significant associations were found between *S. glossinidius* and *T. simiae, T. vivax, T. simiae* Tsavo and *T. godfreyi*. But, there was no significant association between *S. glossinidius* and *T. congolense* and *T. b. brucei*, which may have been due to the low number of co-infected tsetse flies (four for each).

CHAPTER SIX

6.0. CONCLUSION AND RECOMMENDATION

6.1. Conclusions

This study revealed the presence of *S. glossinidius* in *G. morsitans* and *G. pallidipes* tsetse flies from KNP. The study showed that the prevalence of *S. glossinidius* did not vary significantly with species, sex and sampling site of tsetse flies. *G. morsitans* and *G. pallidipes* also carries diverse trypanosome species in KNP ecosystem which indicates the circulation of pathogenic trypanosome species in the study area. The result also shows that some tsetse flies were infected by both the endosymbiont and trypanosome, while others were infected by the endosymbiont or trypanosome only, or no infection at all. The association between *S. glossinidius* and trypanosome infections seems to vary according to tsetse fly sex and trypanosome species. *T. simiae, T. simiae* Tsavo, *T. vivax* and *T. godfreyi* were significantly associated with *S. glossinidius*. According to this study, *S. glossinidius* should not be strictly necessary for *G. morsitans* and *G. pallidipes* tsetse flies to become infected with trypanosomes, but its presence would strongly favor the infection. It is therefore possible that *S. glossinidius* could be a potential target for genetic transformation to control vectors of trypanosomes.

6.2. Recommendations

Pathogenic trypanosome species circulating in KNP ecosystem indicates a need for continuous surveillance and diagnosis of AAT. In addition to those factors assessed in this study, investigation of the host-feeding pattern of *G. morsitans* and *G. pallidipes* in KNP ecosystem would also be interesting. For a better understanding of the association between presence of *S. glossinidius* and trypanosome infections in *G. morsitans* and *G. pallidipes*, further research on genetic comparison between *S. glossinidius* detected in tsetse flies co-infected with trypanosomes and *S. glossinidius* detected without trypanosome infections is recommended.

7.0. REFERENCES

Adams, E.R., Malele, I.I., Msangi, A.R., Gibson, W.C., 2006. Trypanosome identification in wild tsetse populations in Tanzania using generic primers to amplify the ribosomal RNA ITS-1 region. *Acta Tropica* 100, 103–109. https://doi.org/10.1016/j.actatropica.2006.10.002

Adams, Hamilton, P.B., Gibson, W.C., 2010. African trypanosomes: celebrating diversity. *Trends in parasitology* 26. https://doi.org/10.1016/j.pt.2010.03.003

Aksoy, S., 2003. Control of tsetse flies and trypanosomes using molecular genetics. *Veterinary Parasitology* 115, 125–145. https://doi.org/10.1016/s0304-4017(03)00203-6

Aksoy, S., 2000. Tsetse--A haven for microorganisms. *Parasitology Today* 16, 114–118. https://doi.org/10.1016/s0169-4758 (99)01606-3

Aksoy, S., Chen, X., Hypsa, V., 1997. Phylogeny and potential transmission routes of midgut-associated endosymbionts of tsetse (Diptera:*Glossinidae*). *Insect Molecular Biology* 6, 183–190. https://doi.org/10.1111/j.1365-2583.1997.tb00086.x

Aksoy, S., Gibson, W.C., Lehane, M.J., 2003. Interactions between tsetse and trypanosomes with implications for the control of trypanosomiasis. *Advances in Parasitology* 53, 1–83. https://doi.org/10.1016/s0065-308x(03)53002-0

Aksoy, S., Pourhosseini, A.A., Chow, A., 1995. Mycetome endosymbionts of tsetse flies constitute a distinct lineage related to Enterobacteriaceae. *Insect Molecular Biology* 4. https://doi.org/10.1111/j.1365-2583.1995.tb00003.x

Aksoy, S., Weiss, B., Attardo, G., 2008. Paratransgenesis applied for control of tsetse transmitted sleeping sickness. *Advances in Experimental Medicine and Biology* 627, 35–48. https://doi.org/10.1007/978-0-387-78225-6_3

Alam, U., Hyseni, C., Symula, R.E., Brelsfoard, C., Wu, Y., Kruglov, O., Wang, J., Echodu, R., Alioni, V., Okedi, L.M., Caccone, A., Aksoy, S., 2012. Implications of microfauna-host interactions for trypanosome transmission dynamics in *Glossina fuscipes fuscipes* in Uganda. Applied and Environmental Microbiology 78, 4627–4637. https://doi.org/10.1128/AEM.00806-12

Allsopp, R., 2001. Options for vector control against trypanosomiasis in Africa. *Trends in Parasitology* 17, 15–19. https://doi.org/10.1016/S1471-4922(00)01828-6

Alsan, M., 2015. The Effect of the TseTse Fly on African Development. *American Economic Review* 105, 382–410. https://doi.org/10.1257/aer.20130604

Apaatah, F., Osae, M., Nwaefuna, E., Aboagye-Antwi, F., Egyir-Yawson, A., Bimi, L., 2020. Trypanosome prevalence in pigs and tsetse flies from selected areas of Jomoro district of the western region of Ghana. *Veterinary Parasitology: Regional Studies and Reports* 21, 100444. https://doi.org/10.1016/j.vprsr.2020.100444

Attardo, G.M., Scolari, F., Malacrida, A., 2020. Bacterial Symbionts of Tsetse Flies: Relationships and Functional Interactions between Tsetse Flies and Their Symbionts. *Results and problems in cell differentiation* 69, 497–536. https://doi.org/10.1007/978-3-030-51849-3_19

Auty, H., Morrison, L.J., Torr, S.J., Lord, J., 2016. Transmission dynamics of Rhodesian sleeping sickness at the interface of wildlife and Livestock areas. *Trends in Parasitology*. http://dx.doi.org/10.1016/j.pt.2016.05.003

Babokhov, P., Sanyaolu, A.O., Oyibo, W.A., Fagbenro-Beyioku, A.F., Iriemenam, N.C., 2013. A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis. *Pathogens* and *Global Health* 107, 242–252. https://doi.org/10.1179/2047773213Y.0000000105

Balmand, S., Lohs, C., Aksoy, S., Heddi, A., 2013. Tissue distribution and transmission routes for the tsetse fly endosymbionts. *Journal of Invertebrate Pathology* 112 Suppl, S116-122. https://doi.org/10.1016/j.jip.2012.04.002

Beard, C.B., O'Neill, S.L., Mason, P., Mandelco, L., Woese, C.R., Tesh, R.B., Richards, F.F., Aksoy, S., 1993. Genetic transformation and phylogeny of bacterial symbionts from tsetse. *Insect Molecular Biology* 1, 123–131. https://doi.org/10.1111/j.1365-2583.1993.tb00113.x

Bonnet, J., Boudot, C., Courtioux, B., 2015. Overview of the diagnostic methods used in the field for human African trypanosomiasis: What could change in the next years? *Biomed Research International* 2015, 583262. https://doi.org/10.1155/2015/583262

Bouyer, J., Bouyer, F., Donadeu, M., Rowan, T., Napier, G., 2013. Community- and farmer-based management of animal African trypanosomosis in cattle. *Trends in Parasitology* 29, 519–522. https://doi.org/10.1016/j.pt.2013.08.003

Brun, R., Hecker, H., Lun, Z.-R., 1998. *Trypanosoma evansi* and *T. equiperdum*: distribution, biology, treatment and phylogenetic relationship (a review). *Veterinary Parasitology* 79, 95–107. https://doi.org/10.1016/S0304-4017(98)00146-0

Büscher, P., Cecchi, G., Jamonneau, V., Priotto, G., 2017. Human African trypanosomiasis. *The Lancet* 390, 2397–2409. https://doi.org/10.1016/S0140-6736 (17)31510-6

Cecchi, G., Mattioli, R.C., Slingenbergh, J., de la Rocque, S., 2008. Land cover and tsetse fly distributions in sub-Saharan Africa. *Medical and Veterinary Entomology* 22, 364–373. https://doi.org/10.1111/j.1365-2915.2008.00747.x

Cecchi, G., Paone, M., Feldmann, U., Vreysen, M.J.B., Diall, O., Mattioli, R.C., 2014. Assembling a geospatial database of tsetse-transmitted animal trypanosomosis for Africa. *Parasites and Vectors* 7, 39. https://doi.org/10.1186/1756-3305-7-39

Channumsin, M., Ciosi, M., Masiga, D., Turner, C.M.R., Mable, B.K., 2018. *Sodalis glossinidius* presence in wild tsetse is only associated with presence of trypanosomes in complex interactions with other tsetse-specific factors. *BMC Microbiology* 18, 163. https://doi.org/10.1186/s12866-018-1285-6

Chen, X., Li, S., Aksoy, S., Lord, J., 1999. Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. *Journal* of *Molecular Evolution* 48, 49–58. https://doi.org/10.1007/pl00006444

Cheng, Q., Aksoy, S., 1999. Tissue tropism, transmission and expression of foreign genes in vivo in midgut symbionts of tsetse flies. *Insect Molecular Biology* 8, 125–132. https://doi.org/10.1046/j.1365-2583.1999.810125.x

Cheng, Q., Ruel, T.D., Zhou, W., Moloo, S.K., Majiwa, P., O'Neill, S.I., Aksoy, S., 2000. Tissue distribution and prevalence of *Wolbachia* infections in tsetse flies, *Glossina* spp. *Medical and Veterinary Entomology* 14. https://doi.org/10.1046/j.1365-2915.2000.00202.x

Cnops, J., Magez, S., De Trez, C., 2015. Escape mechanisms of African trypanosomes: why trypanosomosis is keeping us awake. *Parasitology* 142, 417–427. https://doi.org/10.1017/S0031182014001838

Constable, P., Hinchcliff, K.W., Done, S., Gruenberg, W., 2017. Veterinary Medicine - 11th Edition, 11th ed. Saunders Ltd, vet.

Dale, C., Maudlin, I., 1999. *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. *International Journal of Systematic Bacteriology* 49, 267–275. https://doi.org/10.1099/00207713-49-1-267

Dale, C., Welburn, S.C., 2001. The endosymbionts of tsetse flies: manipulating host-parasite interactions. *International Journal of Parasitology* 31, 628–631. https://doi.org/10.1016/s0020-7519 (01)00151-5

De Vooght, L., Caljon, G., Van Hees, J., Van Den Abbeele, J., 2015. Paternal Transmission of a Secondary Symbiont during Mating in the Viviparous Tsetse Fly. *Molecular Biology and Evolution* 32, 1977–1980. https://doi.org/10.1093/molbev/msv077

De Vooght, L., Van Keer, S., Van Den Abbeele, J., 2018. Towards improving tsetse fly paratransgenesis: stable colonization of *Glossina morsitans morsitans* with genetically modified *Sodalis*. *BMC Microbiology* 18. https://doi.org/10.1186/s12866-018-1282-9

Delespaux, V., de Koning, H.P., 2007. Drugs and drug resistance in African trypanosomiasis. *Drug Resistance Updates* 10, 30–50. https://doi.org/10.1016/j.drup.2007.02.004

Delespaux, V., Geysen, D., Van den Bossche, P., Geerts, S., 2008. Molecular tools for the rapid detection of drug resistance in animal trypanosomes. *Trends in Parasitology* 24, 236–242. https://doi.org/10.1016/j.pt.2008.02.006

Dennis, J.W., Durkin, S.M., Horsley Downie, J.E., Hamill, L.C., Anderson, N.E., MacLeod, E.T., 2014. *Sodalis glossinidius* prevalence and trypanosome presence in tsetse from Luambe National Park, Zambia. *Parasites and Vectors* 7, 378. https://doi.org/10.1186/1756-3305-7-378

Desquesnes, M., Gonzatti, M., Sazmand, A., Thévenon, S., Bossard, G., Boulangé, A., Gimonneau, G., Truc, P., Herder, S., Ravel, S., Sereno, D., Jamonneau, V., Jittapalapong, S., Jacquiet, P., Solano, P., Berthier, D., 2022. A review on the diagnosis of animal trypanosomoses. *Parasites and Vectors* 15, 64. https://doi.org/10.1186/s13071-022-05190-1

Desquesnes, M., 2017. Compendium of diagnostic protocols of the OIE reference laboratory for animal trypanosomoses of African origin. Organization for Animal Health (OIE), Montpellier.

Desquesnes, M., McLaughlin, G., Zoungrana, A., Dávila, A.M., 2001. Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. *International Journal of Parasitology* 31, 610–614. https://doi.org/10.1016/s0020-7519(01)00161-8

Dyer, N.A., Rose, C., Ejeh, N.O., Acosta-Serrano, A., 2013. Flying tryps: survival and maturation of trypanosomes in tsetse flies. *Trends in Parasitology* 29, 188–196. https://doi.org/10.1016/j.pt.2013.02.003

Eleftherianos, I., Atri, J., Accetta, J., Castillo, J., 2013. Endosymbiotic bacteria in insects: guardians of the immune system? *Frontiers in Physiology* 4.

Elsen, P., Amoudi, M.A., Leclercq, M., 1990. First record of *Glossina fuscipes fuscipes* Newstead, 1910 and *Glossina morsitans submorsitans* Newstead,

1910 in southwestern Saudi Arabia. *Annales de la Société belge de médecine tropicale* 70, 281–287.

Farikou, O., Njiokou, F., Cuny, G., Geiger, A., 2011. Microsatellite genotyping reveals diversity within populations of *Sodalis glossinidius*, the secondary symbiont of tsetse flies. *Veterinary Microbiology* 150, 207–210. https://doi.org/10.1016/j.vetmic.2011.01.021

Farikou, O., Njiokou, F., Mbida Mbida, J.A., Njitchouang, G.R., Djeunga, H.N., Asonganyi, T., Simarro, P.P., Cuny, G., Geiger, A., 2010. Tripartite interactions between tsetse flies, *Sodalis glossinidius* and trypanosomes--an epidemiological approach in two historical human African trypanosomiasis foci in Cameroon. *Infection, Genetics and Evolution* 10, 115–121. https://doi.org/10.1016/j.meegid.2009.10.008

Franco, J.R., Cecchi, G., Priotto, G., Paone, M., Diarra, A., Grout, L., Simarro, P.P., Zhao, W., Argaw, D., 2020. Monitoring the elimination of human African trypanosomiasis at continental and country level: Update to 2018. WHO.

Franco, J.R., Simarro, P.P., Diarra, A., Jannin, J.G., 2014. Epidemiology of human African trypanosomiasis. *Clinical Epidemiology* 257–275.

Gaithuma, A., Yamagishi, J., Hayashida, K., Kawai, N., Namangala, B., Sugimoto, C., 2020. Blood meal sources and bacterial microbiome diversity in wild-caught tsetse flies. *Scientific Reports* 10, 5005. https://doi.org/10.1038/s41598-020-61817-2

Gaithuma, A.K., Yamagishi, J., Martinelli, A., Hayashida, K., Kawai, N., Marsela, M., Sugimoto, C., 2019. A single test approach for accurate and sensitive detection and taxonomic characterization of Trypanosomes by comprehensive analysis of internal transcribed spacer 1 amplicons. *PLoS Neglected Tropical Diseases* 13, e0006842. https://doi.org/10.1371/journal.pntd.0006842

Geiger, A., Ponton, F., Simo, G., 2015. Adult blood-feeding tsetse flies, trypanosomes, microbiota and the fluctuating environment in sub-Saharan Africa. *International Society for Microbial Ecology Journal* 9, 1496–1507. https://doi.org/10.1038/ismej.2014.236

Geiger, A., Ravel, S., Frutos, R., Cuny, G., 2005. *Sodalis glossinidius* (Enterobacteriaceae) and vectorial competence of *Glossina palpalis gambiensis* and *Glossina morsitans morsitans* for *Trypanosoma congolense* savannah type. *Current Microbiology* 51, 35–40. https://doi.org/10.1007/s00284-005-4525-6

Geiger, A., Ravel, S., Mateille, T., Janelle, J., Patrel, D., Cuny, G., Frutos, R., 2007. Vector competence of *Glossina palpalis gambiensis* for *Trypanosoma brucei*

s.l. and genetic diversity of the symbiont *Sodalis glossinidius*. *Molecular Biology and Evolution* 24, 102–109. https://doi.org/10.1093/molbev/msl135

Giordani, F., Morrison, L.J., Rowan, T.G., DE Koning, H.P., Barrett, M.P., 2016. The animal trypanosomiases and their chemotherapy: a review. *Parasitology* 143, 1862–1889. https://doi.org/10.1017/S0031182016001268

Gooding, R.H., Haines, L.R., Pearson, T.W., 2005. Tsetse Flies, the *Glossinidae*, and Transmission of African Trypanosomes, in: Marquardt W. C. Biology of Disease Vectors. ELSEVIER Academic Press, pp. 153–164.

Gouteux, J.P., 1987. A new *Glossina* from the Congo: *Glossina* (Austenina) *frezili* sp. nov. (Diptera: *Glossinidae*)]. *Tropical medicine and parasitology* 38, 97–100.

Haag, J., O'hUigin, C., Overath, P., 1998. The molecular phylogeny of trypanosomes: evidence for an early divergence of the Salivaria. *Molecular and Biochemical Parasitology* 91, 37–49. https://doi.org/10.1016/s0166-6851 (97)00185-0

Hoare, C.A., 1964. Morphological and taxonomic studies on mammalian trypanosomes. X. Revision of the systematics. *Journal of Protozoology* 11, 200–207. https://doi.org/10.1111/j.1550-7408.1964.tb01741.x

Holmes, P., 2013. Tsetse-transmitted trypanosomes--their biology, disease impact and control. *Journal of Invertebrate Pathology* 112 Suppl, S11-14. https://doi.org/10.1016/j.jip.2012.07.014

Ilemobade, A.A., 2009. Tsetse and trypanosomosis in Africa: the challenges, the opportunities. *Onderstepoort Journal of Veterinary Research* 76, 35–40. https://doi.org/10.4102/ojvr.v76i1.59

Isaac, C., Ciosi, M., Hamilton, A., Scullion, K.M., Dede, P., Igbinosa, I.B., Nmorsi, O.P.G., Masiga, D., Turner, C.M.R., 2016. Molecular identification of different trypanosome species and subspecies in tsetse flies of northern Nigeria. *Parasites and Vectors* 9, 301. https://doi.org/10.1186/s13071-016-1585-3

Kamdem, C.N., Tiofack, A.A.Z., Mewamba, E.M., Ofon, E.A., Gomseu, E.B.D., Simo, G., 2020. Molecular identification of different trypanosome species in tsetse flies caught in the wildlife reserve of Santchou in the western region of Cameroon. *Parasitology Research* 119, 805–813. https://doi.org/10.1007/s00436-020-06606-6

Kame-Ngasse, G.I., Njiokou, F., Melachio-Tanekou, T.T., Farikou, O., Simo, G., Geiger, A., 2018. Prevalence of symbionts and trypanosome infections in tsetse flies of two villages of the "Faro and Déo" division of the Adamawa

region of Cameroon. *BMC Microbiology* 18, 159. https://doi.org/10.1186/s12866-018-1286-5

Kariithi, H.M., Meki, I.K., Schneider, D.I., De Vooght, L., Khamis, F.M., Geiger, A., Demirbaş-Uzel, G., Vlak, J.M., iNCE, I.A., Kelm, S., Njiokou, F., Wamwiri, F.N., Malele, I.I., Weiss, B.L., Abd-Alla, A.M.M., 2018. Enhancing vector refractoriness to trypanosome infection: achievements, challenges and perspectives. *BMC Microbiology* 18, 179. https://doi.org/10.1186/s12866-018-1280-y

Kasozi, K.I., Zirintunda, G., Ssempijja, F., Buyinza, B., Alzahrani, K.J., Matama, K., Nakimbugwe, H.N., Alkazmi, L., Onanyang, D., Bogere, P., Ochieng, J.J., Islam, S., Matovu, W., Nalumenya, D.P., Batiha, G.E.-S., Osuwat, L.O., Abdelhamid, M., Shen, T., Omadang, L., Welburn, S.C., 2021. Epidemiology of Trypanosomiasis in Wildlife—Implications for Humans at the Wildlife Interface in Africa. *Frontiers in Veterinary Science* 8, 621699. https://doi.org/10.3389/fvets.2021.621699

Kennedy, P.G., 2013. Clinical features, diagnosis, and treatment of human African trypanosomiasis (sleeping sickness). *Lancet Neurology* 12, 186–194. https://doi.org/10.1016/S1474-4422 (12)70296-X

Kikuchi, Y., 2009. Endosymbiotic bacteria in insects: their diversity and culturability. *Microbes and Environment* 24, 195–204. https://doi.org/10.1264/jsme2.me09140s

Krafsur, E.S., 2009. Tsetse flies: Genetics, evolution, and role as vectors. *Infection, Genetics and Evolution* 9, 124–141. https://doi.org/10.1016/j.meegid.2008.09.010

Krinsky, W.L., 2019. Tsetse Flies (*Glossinidae*)., in: Mullen G. R. and Derdun R. A. Medical and Veterinary Entomology. Academic Press/ELSEVIER, Amsterdam, The Netherlands, pp. 369–382.

Kristjanson, P.M., Swallow, B.M., Rowlands, G.J., Kruska, R.L., de Leeuw, P.N., 1999. Measuring the costs of African animal trypanosomosis, the potential benefits of control and returns to research. *Agricultural Systems* 59, 79–98. https://doi.org/10.1016/S0308-521X (98)00086-9

Kruel, D., 2007. Trypanosomiasis (Deadly disease and Epidemics). Chelsea House Publishers, New York, NY 10001.

Kulohoma, B.W., Wamwenje, S.A.O., Wangwe, I.I., Masila, N., Mirieri, C.K., Wambua, L., 2020. Prevalence of trypanosomes associated with drug resistance in Shimba Hills, Kwale County, Kenya. *BMC Research Notes* 13, 234. https://doi.org/10.1186/s13104-020-05077-3

Laohasinnarong, D., Goto, Yasuyuki, Goto, Yasuhuki, Asada, M., Nakao, R., Hayashida, K., Kajino, K., Kawazu, S., Sugimoto, C., Inoue, N., Namangala, B., 2015. Studies of trypanosomiasis in the Luangwa valley, north-eastern Zambia. *Parasites and Vectors* 8, 497. https://doi.org/10.1186/s13071-015-1112-y

Leak, S.G.A., 1998. Tsetse biology and ecology: their role in the epidemiology and control of trypanosomosis. CABI Publishing, New York, USA.

Lindh, J.M., Lehane, M.J., 2011. The tsetse fly *Glossina fuscipes fuscipes* (Diptera: *Glossina*) harbours a surprising diversity of bacteria other than symbionts. *Antonie Van Leeuwenhoek* 99, 711–720. https://doi.org/10.1007/s10482-010-9546-x

Majiwa, P.A., Thatthi, R., Moloo, S.K., Nyeko, J.H., Otieno, L.H., Maloo, S., 1994. Detection of trypanosome infections in the saliva of tsetse flies and buffy-coat samples from antigenaemic but aparasitaemic cattle. *Parasitology* 108, 313–322. https://doi.org/10.1017/s0031182000076150

Makhulu, E.E., Villinger, J., Adunga, V.O., Jeneby, M.M., Kimathi, E.M., Mararo, E., Oundo, J.W., Musa, A.A., Wambua, L., 2021. Tsetse blood-meal sources, endosymbionts and trypanosome-associations in the Maasai Mara National Reserve, a wildlife-human-livestock interface. *PLoS Neglected Tropical Diseases* 15, e0008267. https://doi.org/10.1371/journal.pntd.0008267

Masiga, D.K., McNamara, J.J., Laveissière, C., Truc, P., Gibson, W.C., 1996. A high prevalence of mixed trypanosome infections in tsetse flies in Sinfra, Côte d'Ivoire, detected by DNA amplification. *Parasitology* 112, 75–80. https://doi.org/10.1017/s0031182000065094

Masiga, D.K., Smyth, A.J., Hayes, P., Bromidge, T.J., Gibson, W.C., 1992. Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *International Journal for Parasitology* 22, 909–918. https://doi.org/10.1016/0020-7519(92)90047-o

Mathew, C.Z., 2007. Biological and molecular aspects of *Sodalis glossinidius*. The University of Edinburgh, College of Medicine and Veterinary Medicine, Edinburgh.

Maudlin, I., Welburn, S.C., 1987. Lectin mediated establishment of midgut infections of *Trypanosoma ongolense* and *Trypanosoma brucei* in *Glossina morsitans*. *Tropical medicine and parasitology* 38, 167–70.

Maudlin, I., Welburn, S.C., Mehlitz, D., 1990. The relationship between rickettsia-like-organisms and trypanosome infections in natural populations of tsetse in Liberia. *Tropical Medicine and Parasitology* 41, 265–267.

Mbewe, N.J., Mweempwa, C., Guya, S., Wamwiri, F.N., 2015. Microbiome frequency and their association with trypanosome infection in male *Glossina morsitans centralis* of Western Zambia. *Veterinary Parasitology* 211, 93–98. https://doi.org/10.1016/j.vetpar.2015.04.027

Mekata, H., Konnai, S., Simuunza, M., Chembensofu, M., Kano, R., Witola, W.H., Tembo, M.E., Chitambo, H., Inoue, N., Onuma, M., Ohashi, K., 2008. Prevalence and source of trypanosome infections in field-captured vector flies (*Glossina pallidipes*) in southeastern Zambia. *Journal of Veterinary Medical Science* 70, 923–928. https://doi.org/10.1292/jvms.70.923

Mitashi, P., Hasker, E., Lejon, V., Kande, V., Muyembe, J.-J., Lutumba, P., Boelaert, M., 2012. Human African trypanosomiasis diagnosis in first-line health services of endemic Countries, a systematic review. *PLOS Neglected Tropical Diseases* 6, e1919. https://doi.org/10.1371/journal.pntd.0001919

Moloo, S.K., 1993. The distribution of *Glossina* species in Africa and their natural hosts. *International Journal of Tropical Insect Science* 14, 511–527. https://doi.org/10.1017/S1742758400014211

Moloo, S.K., Sabwa, C.L., Kabata, J.M., 1992. Vector competence of *Glossina pallidipes* and *G. morsitans centralis* for *Trypanosoma vivax*, *T. congolense* and *T. b. brucei*. *Acta Tropica* 51, 271–280. https://doi.org/10.1016/0001-706x(92)90045-y

Morrison, L.J., Marcello, L., McCulloch, R., 2009. Antigenic variation in the African trypanosome: molecular mechanisms and phenotypic complexity. *Cell Microbiol*ogy 11, 1724–1734. https://doi.org/10.1111/j.1462-5822.2009.01383.x

Mulenga, G.M., Henning, L., Chilongo, K., Mubamba, C., Namangala, B., Gummow, B., 2020. Insights into the Control and Management of Human and Bovine African Trypanosomiasis in Zambia between 2009 and 2019-A Review. *Tropical Medicine and Infectious Disease* 5. https://doi.org/10.3390/tropicalmed5030115

Musaya, J., Chisi, J., Senga, E., Nambala, P., Maganga, E., Matovu, E., Enyaru, J., 2017. Polymerase chain reaction identification of *Trypanosoma brucei rhodesiense* in wild tsetse flies from Nkhotakota Wildlife Reserve, Malawi. *Malawi Medical Journal* 29.

Mwandiringana, E., Gori, E., Nyengerai, T., Chidzwondo, F., 2012. Polymerase chain reaction (PCR) detection of mixed trypanosome infection and blood meal origin in field-captured tsetse flies from Zambia. *African Journal of Biotechnology* 11, 14490–14497. https://doi.org/10.4314/ajb.v11i79

Mwima, H., 2001. A Brief History of Kafue National Park, Zambia. Koedoe : *African Protected Area Conservation and Science* 44. https://doi.org/10.4102/koedoe.v44i1.186

Nakamura, Y., Hayashida, K., Delesalle, V., Qiu, Y., Omori, R., Simuunza, M., Sugimoto, C., Namangala, B., Yamagishi, J., 2021. Genetic Diversity of African Trypanosomes in Tsetse Flies and Cattle from the Kafue Ecosystem. *Frontiers in Veterinary Science* 8, 599815. https://doi.org/10.3389/fvets.2021.599815

Nakayima, J., Nakao, R., Alhassan, A., Mahama, C., Afakye, K., Sugimoto, C., 2012. Molecular epidemiological studies on animal trypanosomiases in Ghana. *Parasites and Vectors* 5, 217. https://doi.org/10.1186/1756-3305-5-217

Nayupe, S.F., Simwela, N.V., Kamanga, P.M., Chisi, J.E., Senga, E., Musaya, J., Maganga, E., 2019. The use of molecular technology to investigate trypanosome infections in tsetse flies at Liwonde Wild Life Reserve. *Malawi Medical Journal* 31, 233–237. https://doi.org/10.4314/mmj.v31i4.3

Nirmala, X., Hypša, V., Žurovec, M., 2001. Molecular phylogeny of Calyptratae (Diptera: Brachycera): the evolution of 18S and 16S ribosomal rDNAs in higher dipterans and their use in phylogenetic inference. *Insect Molecular Biology* 10, 475–485. https://doi.org/10.1046/j.0962-1075.2001.00286.x

Njiru, Z.K., Constantine, C.C., Guya, S., Crowther, J., Kiragu, J.M., Thompson, R.C.A., Dávila, A.M.R., 2005. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. *Parasitology Research* 95, 186–192. https://doi.org/10.1007/s00436-004-1267-5

Odeniran, P.O., Macleod, E.T., Ademola, I.O., Welburn, S.C., 2019. Endosymbionts interaction with trypanosomes in *Palpalis* group of *Glossina* captured in southwest Nigeria. *Parasitology International* 70, 64–69. https://doi.org/10.1016/j.parint.2019.01.011

O'Donoghue, P., 2017. Haemoprotozoa: Making biological sense of molecular phylogenies. *International Journal of Parasitology: Parasites and Wildlife* 6, 241–256. https://doi.org/10.1016/j.ijppaw.2017.08.007

O'Neill, S.L., Gooding, R.H., Aksoy, S., 1993. Phylogenetically distant symbiotic microorganisms reside in *Glossina* midgut and ovary tissues. *Medical and Veterinary Entomology* 7, 377–383. https://doi.org/10.1111/j.1365-2915.1993.tb00709.x

Ooi, C.-P., Schuster, S., Cren-Travaillé, C., Bertiaux, E., Cosson, A., Goyard, S., Perrot, S., Rotureau, B., 2016. The Cyclical Development of *Trypanosoma vivax* in the Tsetse Fly Involves an Asymmetric Division. *Frontiers in*

Cellular and Infection Microbiology 6. https://doi.org/10.3389/fcimb.2016.00115

Opiro, R., Opoke, R., Angwech, H., Nakafu, E., Oloya, F.A., Openy, G., Njahira, M., Macharia, M., Echodu, R., Malinga, G.M., Opiyo, E.A., 2021. Apparent density, trypanosome infection rates and host preference of tsetse flies in the sleeping sickness endemic focus of northwestern Uganda. *BMC Veterinary Research* 17, 365. https://doi.org/10.1186/s12917-021-03071-w

Peacock, L., Cook, S., Ferris, V., Bailey, M., Gibson, W., 2012. The life cycle of *Trypanosoma* (*Nannomonas*) *congolense* in the tsetse fly. *Parasites and Vectors* 5, 109. https://doi.org/10.1186/1756-3305-5-109

Pépin, J., Milord, F., 1994. The treatment of human African trypanosomiasis. *Advances in Parasitology* 33, 1–47. https://doi.org/10.1016/s0065-308x(08)60410-8

Pollock, J.N., 1992. Training Manual for Tsetse Control Personnel: Tsetse biology, systematics and distribution; techniques. FAO, Rome.

Pollock, J.N., 1982. Training Manual for Tsetse Control Personnel: Control methods and side effects. FAO, Rome.

Radwanska, M., Chamekh, M., Vanhamme, L., Claes, F., Magez, S., Magnus, E., de Baetselier, P., Büscher, P., Pays, E., 2002. The serum resistance-associated gene as a diagnostic tool for the detection of *Trypanosoma brucei rhodesiense*. *American Journal of Tropical Medicine and Hygiene* 67, 684–690. https://doi.org/10.4269/ajtmh.2002.67.684

Radwanska, M., Vereecke, N., Deleeuw, V., Pinto, J., Magez, S., 2018. Salivarian Trypanosomosis: A Review of Parasites Involved, Their Global Distribution and Their Interaction With the Innate and Adaptive Mammalian Host Immune System. *Frontiers in Immunology* 9. https://doi.org/10.3389/fimmu.2018.02253

Rock, K.S., Stone, C.M., Hastings, I.M., Keeling, M.J., Torr, S.J., Chitnis, N., 2015. Mathematical models of human African trypanosomiasis epidemiology. *Advances in Parasitology* 87: 53-133. https://doi.org/doi: 10.1016/bs.apar.2014.12.003.

Rodrigues, C.M.F., Garcia, H.A., Sheferaw, D., Rodrigues, A.C., Pereira, C.L., Camargo, E.P., Teixeira, M.M.G., 2019. Genetic diversity of trypanosomes pathogenic to livestock in tsetse flies from the Nech Sar National Park in Ethiopia: A concern for tsetse suppressed area in Southern Rift Valley? *Infection, Genetics* and *Evolution* 69, 38–47. https://doi.org/10.1016/j.meegid.2019.01.010

Rotureau, B., Van Den Abbeele, J., 2013. Through the Dark Continent: African trypanosome development in the tsetse fly. *Frontiers in Cellular and Infection Microbiology* 3, 53. https://doi.org/10.3389/fcimb.2013.00053

Service, M., 2012. Medical Entomology for Students, 5th ed. Cambridge University Press, New York.

Shaw, A.P.M., Cecchi, G., Wint, G.R.W., Mattioli, R.C., Robinson, T.P., 2014. Mapping the economic benefits to livestock keepers from intervening against bovine trypanosomosis in Eastern Africa. *Preventive Veterinary Medicine* 14.

Shereni, W., Anderson, N.E., Nyakupinda, L., Cecchi, G., 2016. Spatial distribution and trypanosome infection of tsetse flies in the sleeping sickness focus of Zimbabwe in Hurungwe District. *Parasites and Vectors* 9, 605. https://doi.org/10.1186/s13071-016-1879-5

Simarro, P.P., Cecchi, G., Paone, M., Franco, J.R., Diarra, A., Ruiz, J.A., Fèvre, E.M., Courtin, F., Mattioli, R.C., Jannin, J.G., 2010. The Atlas of human African trypanosomiasis: a contribution to global mapping of neglected tropical diseases. *International Journal* of *Health Geographics* 9, 57. https://doi.org/10.1186/1476-072X-9-57

Simwango, M., Ngonyoka, A., Nnko, H.J., Salekwa, L.P., Ole-Neselle, M., Kimera, S.I., Gwakisa, P.S., 2017. Molecular prevalence of trypanosome infections in cattle and tsetse flies in the Maasai Steppe, northern Tanzania. *Parasites and Vectors* 10, 507. https://doi.org/10.1186/s13071-017-2411-2

Snyder, A.K., Rio, R.V.M., 2013. Interwoven biology of the tsetse holobiont. *Journal of Bacteriology* 195, 4322–4330. https://doi.org/10.1128/JB.00487-13

Solano, P., Ravel, S., de Meeûs, T., 2010. How can tsetse population genetics contribute to African trypanosomiasis control? *Trends in Parasitology* 26, 255–263. https://doi.org/10.1016/j.pt.2010.02.006

Squarre, D., Hayashida, K., Gaithuma, A., Chambaro, H., Kawai, N., Moonga, L., Namangala, B., Sugimoto, C., Yamagishi, J., 2020. Diversity of trypanosomes in wildlife of the Kafue ecosystem, Zambia. *International Journal of Parasitology: Parasites and Wildlife* 12, 34–41. https://doi.org/10.1016/j.ijppaw.2020.04.005

Squarre, D., Kabongo, I., Munyeme, M., Mumba, C., Mwasinga, W., Hachaambwa, L., Sugimoto, C., Namangala, B., 2016. Human African Trypanosomiasis in the Kafue National Park, Zambia. *PLoS Neglected Tropical Diseases* 10, e0004567. https://doi.org/10.1371/journal.pntd.0004567

Steverding, D., 2008. The history of African trypanosomiasis. *Parasites and Vectors* 1, 3. https://doi.org/10.1186/1756-3305-1-3

Swallow, B.S., 2000. Impacts of Trypanosomiasis on African Agriculture. ILRI/FAO, PAAT Technical and Scientific Series 2. Rome.

Thapa, B., Child, B., Parent, G., Mupeta, P., 2011. Tourism Demand Assessment - Kafue National Park, Zambia (Reserach).

Torr, S.J., Maudlin, I., Vale, G.A., 2007. Less is more: restricted application of insecticide to cattle to improve the cost and efficacy of tsetse control. *Medical and Veterinary Entomology* 21, 53–64. https://doi.org/10.1111/j.1365-2915.2006.00657.x

Tram, U., Ferree, P.M., Sullivan, W., 2003. Identification of *Wolbachia*--host interacting factors through cytological analysis. *Microbes and Infection* 5, 999–1011. https://doi.org/10.1016/s1286-4579 (03)00192-8

Uilenberg, G., 1998. A field guide for the diagnosis, treatment and prevention of African animal trypanosomosis.

Urquhart, G.M., Armour, J., Duncan, J.L., Dunn, A.M., Junnings, F.W., 1996. Veterinary Parasitology, 2nd ed. Blackwell Publishing.

Van Den Abbeele, J., Bourtzis, K., Weiss, B., Cordón-Rosales, C., Miller, W., Abd-Alla, A.M.M., Parker, A., 2013. Enhancing tsetse fly refractoriness to trypanosome infection--a new IAEA coordinated research project. *Journal of Invertebrate Pathology* 112 Suppl, S142-147. https://doi.org/10.1016/j.jip.2012.07.020

Van den Bossche, P., de La Rocque, S., Hendrickx, G., Bouyer, J., 2010. A changing environment and the epidemiology of tsetse-transmitted livestock trypanosomiasis. *Trends in Parasitology* 26, 236–243. https://doi.org/10.1016/j.pt.2010.02.010

Vreysen, M.J., 2001. Principles of area-wide integrated tsetse fly control using the sterile insect technique. *Medecine Tropicale* (Mars) 61, 397–411.

Vreysen, M.J., Saleh, K.M., Ali, M.Y., Abdulla, A.M., Zhu, Z.R., Juma, K.G., Dyck, V.A., Msangi, A.R., Mkonyi, P.A., Feldmann, H.U., 2000. *Glossina austeni* (Diptera: *Glossinidae*) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. *Journal* of *Economic Entomology* 93, 123–135. https://doi.org/10.1603/0022-0493-93.1.123

Vreysen, M.J.B., Saleh, K.M., Lancelot, R., Bouyer, J., 2011. Factory tsetse flies must behave like wild flies: a prerequisite for the sterile insect technique. *PLoS Neglected Tropical Diseases* 5, e907. https://doi.org/10.1371/journal.pntd.0000907

Vreysen, M.J.B., Seck, M.T., Sall, B., Bouyer, J., 2013. Tsetse flies: Their biology and control using area-wide integrated pest management approaches. *Journal of Invertebrate Pathology* 112, S15–S25. http://dx.doi.org/10.1016/j.jip.2012.07.026

Wamwiri, F.N., Alam, U., Thande, P.C., Aksoy, E., Ngure, R.M., Aksoy, S., Ouma, J.O., Murilla, G.A., 2013. *Wolbachia*, *Sodalis* and trypanosome co-infections in natural populations of *Glossina austeni* and *Glossina pallidipes*. *Parasites and Vectors* 6, 232. https://doi.org/10.1186/1756-3305-6-232

Wamwiri, F.N., Changasi, R.E., 2016. Tsetse Flies (*Glossina*) as Vectors of Human African Trypanosomiasis: A Review. *Biomed Research International* 2016. https://doi.org/10.1155/2016/6201350

Wang, J., Weiss, B.L., Aksoy, S., 2013. Tsetse fly microbiota: form and function. *Frontiers in Cellular and Infection Microbiology* 3. https://doi.org/10.3389/fcimb.2013.00069

Weiss, B.L., Mouchotte, R., Rio, R.V.M., Wu, Y.-N., Wu, Z., Heddi, A., Aksoy, S., 2006. Interspecific transfer of bacterial endosymbionts between tsetse fly species: infection establishment and effect on host fitness. *Applied and Environmental Microbiology* 72, 7013–7021. https://doi.org/10.1128/AEM.01507-06

Welburn, S.C., Arnold, K., Maudlin, I., Gooday, G.W., 1993. Rickettsia-like organisms and chitinase production in relation to transmission of trypanosomes by tsetse flies. *Parasitology* 107, 141–145. https://doi.org/10.1017/s003118200006724x

Welburn, S.C., Maudlin, I., 1999. Tsetse-trypanosome interactions: rites of passage. *Parasitology Today* 15, 399–403. https://doi.org/10.1016/s0169-4758(99)01512-4

Welburn, S.C., Maudlin, I., Ellis, D.S., 1987. In vitro cultivation of rickettsia-like-organisms from *Glossina* species. *Annals of Tropical Medicine and Parasitology* 81, 331–335. https://doi.org/10.1080/00034983.1987.11812127

Wernegreen, J.J., 2012. Endosymbiosis. *Current Biology* 22, R555-561. https://doi.org/10.1016/j.cub.2012.06.010

Werren, J.H., Baldo, L., Clark, M.E., 2008. *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology* 6, 741–751. https://doi.org/10.1038/nrmicro1969

WHO, 2020a. Trypanosomiasis, human African (sleeping sickness). URL https://www.who.int/news-room/fact-sheets/detail/trypanosomiasis-human-african-(sleeping-sickness) (accessed 2.8.21).

WHO, 2020b. Report of the third WHO stakeholders meeting on rhodesiense human African trypanosomiasis. Geneva, Switzerland.

WHO, 2018. Report of the third WHO stakeholders meeting on gambiense human African trypanosomiasis elimination. Geneva, Switzerland.

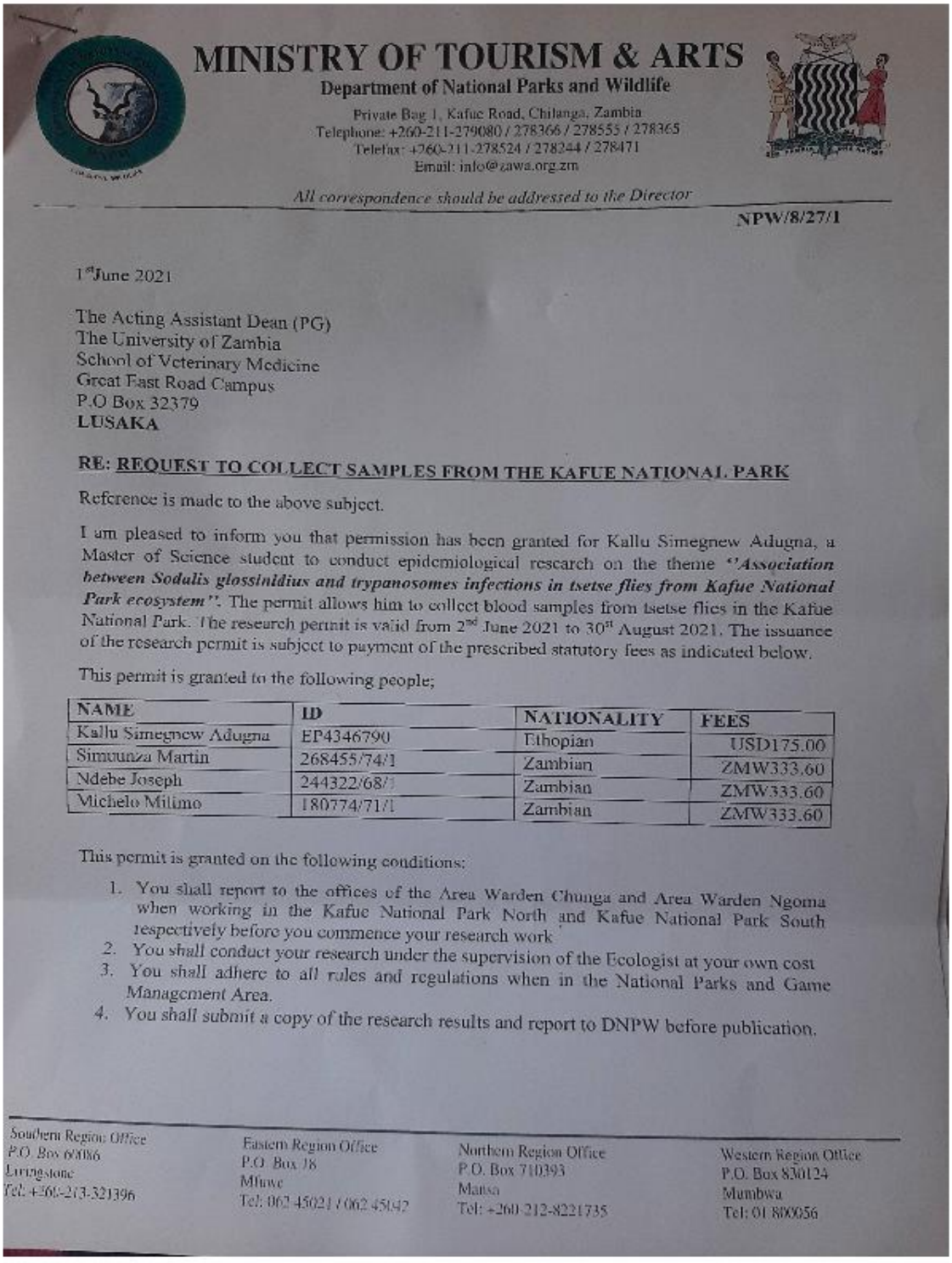
WHO, 2013. Control and surveillance of human African trypanosomiasis: report of a WHO expert committee (WHO Technical report series, no. 984). World Health Organization, Geneva, Switzerland.

WHO, 1998. Control and surveillance of African trypanosomiasis : report of a WHO expert committee (WHO Technical report series, no. 881). World Health Organization, Geneva, Switzerland.

Wilke, A.B.B., Marrelli, M.T., 2015. Paratransgenesis: a promising new strategy for mosquito vector control. *Parasites and Vectors* 8, 342. https://doi.org/10.1186/s13071-015-0959-2

8.0. APPENDICES

8.1. Permission Letter from Department of National Parks and Wildlife



MINISTRY OF TOURISM & ARTS

Department of National Parks and Wildlife

Private Bag 1, Kafue Road, Chilanga, Zambia
Telephone: +260-211-279080 / 278366 / 278555 / 278365
Telefax: +260-211-278524 / 278244 / 278471
Email: info@zawa.org.zm

All correspondence should be addressed to the Director

NPW/8/27/1

1st June 2021

The Acting Assistant Dean (PG)
The University of Zambia
School of Veterinary Medicine
Great East Road Campus
P.O Box 32379
LUSAKA

RE: REQUEST TO COLLECT SAMPLES FROM THE KAFUE NATIONAL PARK

Reference is made to the above subject.

I am pleased to inform you that permission has been granted for Kallu Simegnew Adugna, a Master of Science student to conduct epidemiological research on the theme ***"Association between Sodalis glossinidius and trypanosomes infections in tsetse flies from Kafue National Park ecosystem"***. The permit allows him to collect blood samples from tsetse flies in the Kafue National Park. The research permit is valid from 2nd June 2021 to 30st August 2021. The issuance of the research permit is subject to payment of the prescribed statutory fees as indicated below.

This permit is granted to the following people;

NAME	ID	NATIONALITY	FEES
Kallu Simegnew Adugna	EP4346790	Ethopian	USD175.00
Simoonza Martin	268455/74/1	Zambian	ZMW333.60
Ndebe Joseph	244322/68/1	Zambian	ZMW333.60
Michelo Milimo	180774/71/1	Zambian	ZMW333.60

This permit is granted on the following conditions:

1. You shall report to the offices of the Area Warden Chunga and Area Warden Ngoma when working in the Kafue National Park North and Kafue National Park South respectively before you commence your research work
2. You shall conduct your research under the supervision of the Ecologist at your own cost
3. You shall adhere to all rules and regulations when in the National Parks and Game Management Area.
4. You shall submit a copy of the research results and report to DNPW before publication.

Southern Region Office
P.O. Box 60886
Livingstone
Tel: +260-213-321396

Eastern Region Office
P.O. Box 78
Mfuwe
Tel: 062 45021 / 062 45042

Northern Region Office
P.O. Box 710393
Mansa
Tel: +260-212-8221735

Western Region Office
P.O. Box 830124
Mumbwa
Tel: 01 800056

5. You shall be escorted into the park by a Wildlife Police Officer at all times and at your own cost.
6. Unmanned Aerial Vehicles shall be used in this study upon clearance from Civil Aviation Authority in Zambia
7. The researcher shall not obtain or attempt to obtain patent coverage on the samples (unmodified derivatives or progeny) without prior written consent of DNPW.
8. The researcher shall promptly notify DNPW in writing of any invention and discoveries arising out of the use of the samples. DNPW and the Researcher shall jointly decide on protection and commercialization of such inventions and discoveries and take into account DNPW contributions including its provision of the samples.
9. The samples shall not be sold, distributed or otherwise made available to any other third party or stored at any other facility for any purpose.
10. The researcher will leave duplicate samples in the custody of DNPW.
11. The permit is subject to any other written laws of Zambia

Kindly note that you may be requested to make an oral presentation to DNPW of your research findings and its conservation and management implications.

Yours sincerely,

Chuma Simukonda DSc
DIRECTOR – NATIONAL PARKS AND WILDLIFE

Cc: Acting Assistant Director-Research and Veterinary Services
Cc: Senior Warden – Kafue Region

8.2. Ethical Clearance from UNZABREC

UNIVERSITY OF ZAMBIA
BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: +260 977925304
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
Federal Assurance No. FWA00000338

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia
E-mail: unzarec@unza.zm
IRB00001131 of IORG0000774

20th October 2021

Your REF. No. 1865-2021

Dr. Simegnew Adugna Kallu,
University of Zambia,
Department of Veterinary Medicine,
PO Box 50110,
Lusaka.

Dear Dr. Kallu,

RE: ASSOCIATION BETWEEN SODALIS GLOSSINIDIUS AND TRYPANOSOMES INFECTIONS IN TSETSE FLIES FROM KAFUE NATIONAL PARK ECOSYSTEM, ZAMBIA (REF. NO. 1865-2021)

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 20th August, 2021. The proposal is **approved**. The approval is based on the following documents that were submitted for review:

a) **Study proposal**
b) **Questionnaires**
c) **Participant Consent Form**

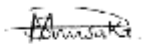
APPROVAL NUMBER : REF. 1865-2021

This number should be used on all correspondence, consent forms and documents as appropriate.

- **APPROVAL DATE : 19th October 2021**
- **TYPE OF APPROVAL : Standard**
- **EXPIRATION DATE OF APPROVAL : 18th October 2022**
  After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the UNZABREC Offices should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING**: All SAEs and any other serious challenges/problems having to do with participant welfare, participant safety and study integrity must be reported to UNZABREC within 3 working days using standard forms obtainable from UNZABREC.
- **MODIFICATIONS**: Prior UNZABREC approval using standard forms obtainable from the UNZABREC Offices is required before implementing any changes in the Protocol (including changes in the consent documents).

- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the UNZABREC using standard forms obtainable from the UNZABREC Offices.
- **NHRA:** You are advised to obtain final study clearance and approval to conduct research in Zambia from the National Health Research Authority (NHRA) before commencing the research project.
- **QUESTIONS**: Please contact the UNZABREC on Telephone No. +260977925304 or by e-mail on unzarec@unza.zm.
- **OTHER**: Please be reminded to send in copies of your research findings/results for our records. You are also required to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study. Use the online portal: unza.rhinno.net for further submissions.

Yours sincerely,



Sody Mweetwa Munsaka, BSc., MSc., PhD
CHAIRPERSON
Tel: +260977925304
E-mail: s.munsaka@unza.zm

8.3. Approval Letter from NHRA

NATIONAL HEALTH RESEARCH AUTHORITY
Paediatric Centre of Excellence, University Teaching Hospital, P.O. Box 30075, LUSAKA
Chalala Office Lot No. 18961/M, Off Kasama Road, P.O. Box 30075, LUSAKA
Tell: +260211 250309 | Email: znhrasec@nhra.org.zm | www.nhra.org.zm

Ref No: NHRA000001/16/11/2021 **Date: 16th November, 2021**

The Principal Investigator,
Simegnew Adugna Kallu,
University of Zambia
Lusaka, Zambia.

Dear Simegnew Adugna Kallu,

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Association Between Sodalis Glossinidius and Trypanosomes Infections in Tsetse Flies from Kafue National Park Ecosystem, Zambia".**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, University leadership, and all key respondents.

Yours sincerely,

Prof. Godfrey Biemba
Director/CEO
National Health Research Authority