

**Trypanosome infection rates in Tsetse (*Glossina fuscipes fuscipes*)
in communal grazing lands in Mid-Northern Uganda**

By

Rogers Ruyu AZABO

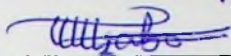
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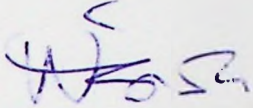
DECLARATION

I Rogers Ruyu Azabo, hereby declare that the work described in this dissertation is original and it has not been presented to any other University as partial or full requirement for the award of an academic qualification.

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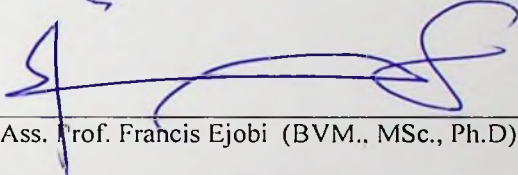
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DEDICATION

This body of work is dedicated to my Late father Lukano, mother Khadija, dear wife Immaculate, daughters Lucky and Wendy and my son David.

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TABLE OF CONTENTS

DECLARATION	i
DEDICATION	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS.....	iv
LIST OF TABLES	vii
LIST OF FIGURES.....	vii
LIST OF ACRONYMS.....	viii
SUMMARY	ix
CHAPTER 1.....	1
1.0 INTRODUCTION.....	1
1.1 STATEMENT OF THE PROBLEM	3
1.2 GENERAL OBJECTIVE.....	4
1.3 SPECIFIC OBJECTIVE	4
1.4 JUSTIFICATION.....	4
1.5 CONCEPTUAL FRAMEWORK.....	5
CHAPTER 2.....	6
2.0 LITERATURE REVIEW.....	6
2.1 Trypanosomosis	6
2.1.2 Host	11
2.1.3 LIFE CYCLE- in the mammalian hosts	11
2.1.4 LIFE CYCLE- in the invertebrate host	12
2.2 TSETSE FLIES	14
2.2.1 Host preferences	16

2.2.2 Blood meal identification methods.....	16
2.2.2.1 Serological techniques.....	16
2.2.2.2 DNA based techniques	17
CHAPTER 3.....	18
3.0 MATERIALS AND METHODS	18
3.1 STUDY AREA.....	18
3.2 TSETSE SAMPLING	18
3.2.1 DETERMINATION OF TSETSE APPARENT DENSITY	19
3.2.2 Infection rate determination	20
3.2.3 TSETSE FEEDING PREFERENCE HOSTS DETERMINATION	21
3.2.4 ANALYSIS OF TSETSE BLOOD MEALS.....	21
3.2.4.1: DNA extraction	21
3.2.4.2 DNA amplification by Polymerase Chain Reaction.....	22
3.2.4.3 Agarose gel electrophoresis.....	23
3.2.4.4 Gel extraction and purification.....	23
3.2.4.5 Sequencing	24
3.3 Data Analysis	24
CHAPTER 4.....	25
4.1: APPARENT TSETSE DENSITY	25
4.2 TRYPANOSOME INFECTION RATES IN TSETSE.....	26
4.3. IDENTIFICATION OF TSETSE BLOOD DONOR.....	27
4.3.1 DNA amplification.....	29
4.3.2 Sequencing	30
CHAPTER 5.....	31
DISCUSSIONS	31

5.1 APPARENT TSETSE DENSITIES	31
5.2 TRYPANOSOME INFECTION RATES	32
5.3 IDENTIFICATION OF TSETSE BLOOD SOURCE	33
5.4 CONCLUSIONS	33
5.5 RECOMMENDATIONS	34
REFERENCES	35

LIST OF TABLES

Table 1: Classification of the pathogenic African trypanosomes.....	14
Table 2: Number of flies caught in different seasons in the study areas and their FTD's.....	25
Table 3: Infection rates in male and female <i>G. f. fuscipes</i> in the communal grazing lands in the three districts	27
Table 4: Source of blood meal of <i>G. f. fuscipes</i> caught in each of the communal grazing lands in Kole, Apac and Lira districts.....	28

LIST OF FIGURES

Figure 1: The conceptual frame work highlighting the statement of the problem	5
Figure 2: Diagram showing the fundamental features of a trypanosome.....	8
Figure 3: Important livestock pathogenic trypanosomes.....	10
Figure 4: Dorsal view of tsetse fly	15
Figure 5: Map of Uganda showing the study sites	19
Figure 6: Seasonal variation of <i>Glossina fuscipes fuscipes</i> apparent density in communal grazing lands of Kole, Apac and Lira districts	25
Figure 7: 1 % agarose gel electrophoresis for Cyt b amplification	29
Figure 8: 1 % agarose gel electrophoresis for Cyt b amplification	29
Figure 9: 1 % agarose gel electrophoresis for Gel purified Cyt b PCR products.....	30

LIST OF ACRONYMS

AAT	African Animal Trypanosomosis
AD	Apparent Density
CFT	Complement fixation test
DALY	Disability Adjusted Life Years
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agricultural Organisation
FTD	Flies/trap/day
HAT	Human African Trypanosomosis
HCl	Hydrogen Chloride
HIT	Heamagglutination inhibition test
ICIPE	International Centre for Insect Physiology and Ecology
IR	Infection Rates
NaCl	Sodium chloride
NaLIRRI	National Livestock Resources Research Institute
NCBI	National Centre for Biotechnology Information
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
RFLP	Restriction Fragment Length Polymorphism
ROFURUM	Regional Universities Forum for Capacity Building in Agriculture
S/C	Sub-county
WHO	World Health Organisation

SUMMARY

Apparent density, Infection rates and host preferences are among the factors which are important in understanding the epidemiology of vector borne diseases and their control. A study was undertaken in the communal grazing lands of three districts in Mid-Northern Uganda to investigate the status of tsetse fly population. Tsetse apparent densities varied between 0.2-2.1 flies/trap/day across the three study areas and *Glossina fuscipes fuscipes* was the only species encountered. Tsetse flies were trapped, dissected and their organs (proboscis, salivary glands and mid-guts) were microscopically examined for trypanosome infections giving an overall infection rate of 5.6%. Out of the 376 non-teneral flies dissected and examined microscopically for infections, trypanosomes of various species were observed in 21 flies. Of the infected flies, 17 (81%) were females while 4 (19%) were males, which constituted a statistically significant difference (χ^2 , 8.048, d.f.= 1, $P= 0.002$). The infection rates were high during the wet season 3.7% than the dry season 1.9%. The infecting rate of trypanosome species were classified as 3.2% for *T.vivax*, 1.3% for *T.congolense*, 1.1% for immature (gut only) and 0% for *T.brucei*. Although the proportion of *T.vivax* (3.2%) was higher than that of *T.congolense* (1.3%), there was no significant difference between the two (χ^2 , 2.882, d.f.= 1, $P= 0.04$). Bloodmeal analysis implicated two(2) vertebrate hosts, cattle and human beings. This finding is of epidemiological importance, in relation to the cycle of transmission of human infective trypanosomes between domestic animals and man an implication that control efforts of trypanosomosis should prioritise on these two hosts.

CHAPTER 1

1.0 INTRODUCTION

Tsetse flies (genus *Glossina*) are the primary vectors of trypanosomosis and the only vector capable of transmitting trypanosomes cyclically (Newstead *et al.*, 1924) . They are distributed in Africa, with the northern limit being Senegal river at 15° N to the river Nile at about 12°N and the southern limit being Coastal Angola at about 29°S (Horsfall, 1962). Their distribution is determined largely by vegetation type which in turn is influenced by temperature and humidity, with all forms of woodland, from savannah to rain forest and including agricultural plantations and modified vegetation types providing suitable habitat for one or more tsetse species. However, no vegetation type is suitable for all tsetse species (Phelps & Lovemore 1994).The abundance and distribution of tsetse flies is also closely related with the availability of hosts, with each tsetse species having its own preferred hosts. *Glossina morsitans* and *G.pallidipes* are usually associated with wild animals where Warthog, bushpig, certain Bovidae, elephant, rhinoceros and African buffalo constitute suitable hosts, while *G. palpalis* feeds extensively on reptiles. In the absence of preferred hosts tsetse flies can survive on the blood of other host species (Phelps & Lovemore, 1994).

Tsetse flies are important vectors of African trypanosomes, the causative agents of human African trypanosomiasis (HAT, sleeping sickness) as well as trypanosomosis in livestock, Nagana.The disease has for long enjoyed the dubious accolade of most important livestock disease in sub-Saharan Africa. About 45 to 60 million cattle are at risk from trypanosomosis (Chadenga, 1994; Gilbert *et al.*, 2001). Current losses due to trypanosomosis in cattle are estimated at 4.5 billion dollars per year, with about 3 million cattle deaths (Budd, 1999) and

the productivity of the survivors in terms of draft power, milk production, growth and birth rate is lowered by 10–40% (Swallow, 2000).

African animal trypanosomosis (AAT) is mainly caused by *Trypanosoma* (T). *congolense*, *T. vivax* and *T. brucei*. The disease is considered to be most important in cattle but can cause serious losses in pigs, camels, goats, and sheep. Trypanosomosis is a zoonosis; with over 60 million people living in some 250 foci at risk of contracting the disease (WHO, 1998); the estimated number of actual cases is currently at about 30,000 (WHO, 2009), most of which go untreated, while an estimated 1.5 million Disability Adjusted Life Years (DALY) are lost annually due to sleeping sickness (WHO, 2004).

Infection rates in tsetse flies are determined by the parasite, the host, the vector, and the environment. They are generally low but vary greatly according to the species of the trypanosome, with *T. vivax* ranking the highest (5%) and *T. brucei spp.* the lowest with less than 1%. (Leak, 1999).

During our routine tsetse surveys in Mid-Northern Uganda, we interacted with the natives of the area and eventually we picked interest in a scenario where livestock were kept in communal grazing lands on one side of the village and the cultivation of crop was done on the other side of the village. This presented an epidemiological situation/ circumstance in which the tsetse fly interacted with the host which was mainly livestock; large game were absent. At the same time Trypanosomosis was a problem in the area. The prevailing conditions in these areas (tsetse flies, forests, streams and swamps) provided conditions in which the susceptible hosts and infectious vector can come together long enough for transmission to occur. The pathogens, vectors and hosts occupy ecological niches determined

largely but not exclusively by the environment. Knowledge on infection in the vector is important since its level has direct influence on the epidemiology of the disease and in the planning of effective control strategies.

1.1 STATEMENT OF THE PROBLEM

Despite the above described circumstances that would favour disease transmission, there is no information about tsetse infection rates in the Mid-North region.. Tsetse flies derive their importance solely from their ability to transmit trypanosomosis to man and livestock. This is related to the risk of contracting trypanosomosis; as such there are a number of circumstances under which the degree of trypanosome risk can occur. This includes the tsetse fly species present and their density, and trypanosome infection rates in tsetse flies (MacLenna, 1980). During the developmental cycle of the trypanosome, the vector itself becomes infected and remains infective for the rest of its life. This maintains the transmission cycle and is important in the epidemiology of trypanosomosis. The present study therefore seeks to determine infection rate among tsetse flies and asses the role this plays in the epidemiology of trypanosomosis in these areas. This information will be useful while planning for control programmes.

1.2 GENERAL OBJECTIVE

To determine trypanosome infection rates in tsetse flies (*Glossina fuscipes fuscipes*) infesting the communal grazing areas within Mid-Northern zone in Uganda and delineate foci of active trypanosomosis transmission.

1.3 SPECIFIC OBJECTIVE

- i) To determine the apparent density (A.D) and infection rates in *Glossina fuscipes fuscipes* in communal grazing land.
- ii) To identify hosts for *Glossina fuscipes fuscipes* through blood meal identification in order to gain insights to the epidemiology of trypanosomosis in the area.

1.4 JUSTIFICATION

Tsetse-transmitted human/ livestock trypanosomosis is one of the major constraints to rural development in Sub-Saharan Africa. The epidemiology of the disease is largely determined by tsetse fly density and other factors like infection rates and hosts preferences (MacLenna, 1980, Katunguka, 1996, Ducheyne, 2009). Over a decade now, there has been little published information regarding apparent density and infection rates in tsetse flies in the area of study and yet people are resettling in these localities and as such the level of risk is not known to allow for proper intervention measures to ensure safety of animals and people. To restore such areas to their full potential, there is need to carryout studies on such factors which are essential to the understanding of the epidemiology of the disease in this area. This will enable informed decisions on appropriate disease control strategies in the study area. This can be by control of the disease which is aimed at either the parasite in the mammalian host (domestic livestock) by treatment with trypanocidal drugs, in order to reduce the size of the animal reservoir or removal of the vector from the environment by application of insecticides for the

purpose of reducing tsetse population and thus reducing transmission of the parasites (Waiswa, et al., 2006).

1.5 CONCEPTUAL FRAMEWORK

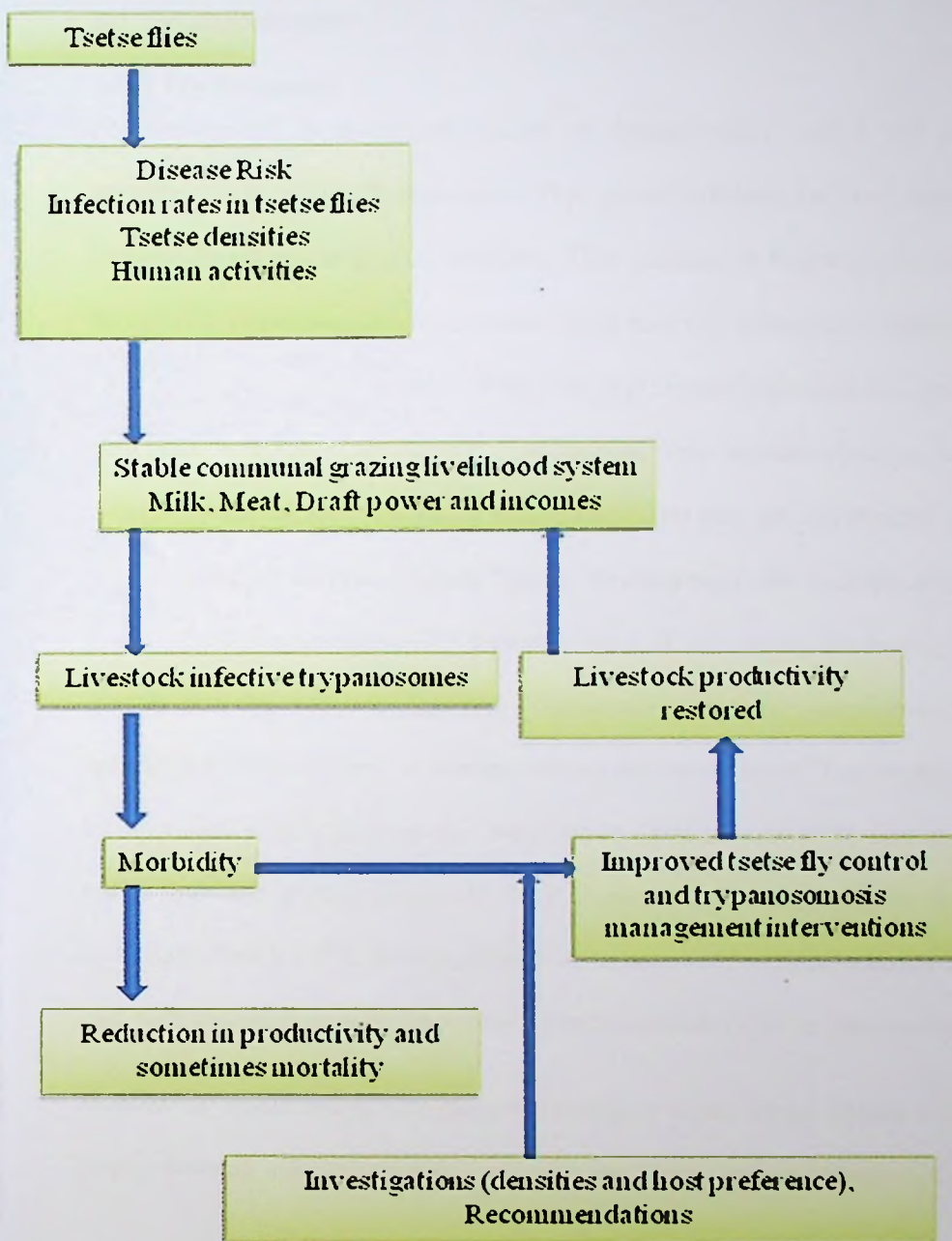


Figure 1: The conceptual frame work highlighting the statement of the problem

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Trypanosomosis

2.1.1 Trypanosomes

Trypanosomosis is a disease caused by trypanosomes, which are unicellular protozoan parasites of the genus *Trypanosoma*. This genus is divided into two major sections or groups namely the *stercoraria* and *salivaria*. This division is based on the site of production of metacyclic trypanosomes in the insect hosts and the subsequent method of infection of the mammalian hosts (Vickerman, 1976). These protozoan parasites can parasitize all classes of vertebrate with the exception of *Trypanosoma equiperdum* which is the causative agent of dourine in horses and venereally transmitted. The rest are transmitted from host to host by haematophagous insects. Usually cyclic development and multiplication of these parasites occur in different regions of digestive tract of the vector depending on the trypanosome species until the infective metacyclic trypanosomes (metatrypanosomes) are produced which are transmitted to another vertebrate host as the vector feeds. Transmission takes place either by inoculation of trypanosomes with saliva (section *salivaria*) and the incubation period varies from one species to another for *T. congolense* it is from 4 to 24 days; *T. vivax*, from 4 to 40 days; and for *T. b. brucei*, from 5 to 10 days. or by contamination of mucosa or broken skin with trypanosomes in the vector's faecal material (section *Stercoraria*); (Connor, 1994,).

Mechanical transmission also occurs whereby a biting insect passes the metacyclics (blood forms) from an infected animal to a health one in the course of interrupted feeding. The time

interval between feeds is of paramount importance for effective transmission since the trypanosomes die when the blood sample dries. This mode of transmission varies from place to place, depending on the numbers of hosts and biting insects present, and also on the species of trypanosome. For instance, large biting flies of the genus *Tabanus* and *Haematopota*, *Liperosia*, *Stomoxys*, and *Chrysops* have been implicated (Leak, 1998) even tsetse flies themselves are likely to act as mechanical vectors since they carry substantially large blood quantities (Leak, 1999). Mechanical transmission has proved to be effective in maintaining *Trypanosoma vivax* and *Trypanosoma evansi* in South and Central America, and the latter species in North Africa and Asia as well in the absence of tsetse flies. Mechanical transmission can also occur iatrogenically where poor needle hygiene is practiced (Forbes, 2004; Tudor et al., 2001). According to Jordan (1986) and Leak (1999) who extensively reviewed the subject, there is little evidence that mechanical transmission is of importance in Africa under natural conditions. Reports of field occurrence of mechanical transmission may be due to less sensitive sampling techniques that do not detect low populations of tsetse flies, besides experimental studies that support this method of transmission do not replicate natural conditions. Congenital transmission of trypanosomosis can take place in perinatal calf (Melendez et al., 1982).

The pathogenic trypanosome consists of a single cell varying in size from 8 to over 50µm. There are differences in appearance, shape and size between the various species of trypanosomes, allowing specific identification (Uilenberg, 1998). The *salivaria* group of trypanosomes may or may not have a free flagellum, the kinetoplast is terminal or sub-terminal, and the posterior end of the body is usually blunt. They develop as trypomastigotes within the mammalian host and are usually pathogenic (Mulligan, 1970).

In Africa, this group of pathogenic trypanosomes cause sleeping sickness in humans and nagana in domestic animals and undergo cyclical development which requires an invertebrate host. They comprise an outer envelope or membrane filled with cytoplasm in which various structures, including a nucleus, basal body, kinetoplast and volutin granules are suspended. The flagellum arises from the basal body in the posterior end of each trypanosome and runs the length of the organism in a fold of cell membrane called the undulating membrane. The flagellum may continue past the anterior end of the trypanosome as a whip-like free flagellum. The size, shape, extent of development of the undulating membrane and presence or absence of a flagellum vary between trypanosome species as well as between different stages in cyclical development of trypanosomes and are important characteristics in the identification of trypanosomes during microscopy (Connor, 1994). By virtue of a flagellum and undulating membrane, trypanosomes are motile. The free flagellum, when present act as a propeller by which the organism is pulled forward through blood plasma or tissue fluids. Each species has its own locomotory characteristics especially *T. vivax* which moves rapidly forward between blood cells which can be useful in the identification of trypanosomes in wet preparations during microscopy (Murray 1977; Paris et al., 1982).

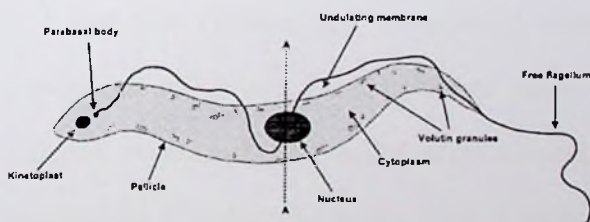


Figure 2: Diagram showing the fundamental features of a trypanosome (Leak, 1999).

Trypanosomes are classified into three subgenera on basis of their morphology and behaviour. The subgenus Duttonella is a large trypanosome varying in length from 18 to 26µm, which has an inconspicuous undulating membrane, a free flagellum in the anterior and rounded posterior end. *T.vivax* is the most common pathogenic trypanosomes in this subgenus. In wet preparations the monomorphic (all parasites similar in appearance) trypanosomes can be seen making rapid progressive movement across the field, between the blood cells. *T.vivax* infections account for the majority of trypanosomosis cases in cattle which are in close proximity to areas of natural vegetation containing wild animals at the game-cattle interface (Connor, 1994).

In cattle herds kept further away from wild game hosts, where modification of natural vegetation has occurred, the bulk of trypanosomosis cases result from infections with *T.congolense*, subgenus Nannomonas (Van den Bossche 2001), which is the smallest of the pathogenic trypanosomes, varying in length from 9 to 22µm. In wet preparations are usually monomorphic with parasites that have no free flagellum and poorly developed undulating membrane; making slow non-progressive movements between blood cells. This trypanosome arguably has the greatest impact on cattle production.

The subgenus Trypanozoon comprises 5 members: i) *T.brucei rhodesiense* and *T.brucei gambiense*, which are the aetiological agents of east and west African human sleeping sickness respectively ii) *T.equiperdum* the cause of the venerally transmitted equine disease dourine, iii) *T. evansi*, the aetiological agent resulting in trypanosomosis in several mammal species in North Africa, Asia, central and South America and iv) *T.brucei brucei* which is the third pathogenic trypanosome species diagnosed in trypanosomosis infections in cattle in sub-Saharan Africa, but which results in disease of greater severity in dogs and horses. *T.brucei*

brucei infections are usually polymorphic with long slender (23 to 30µm) forms with no free flagellum, short stumpy (17 to 22µm) forms with no free flagellum that are adapted to life in the intermediate tsetse host and intermediate parasite forms with a free flagellum. Parasite movement in wet preparations is usually slow but progressive.

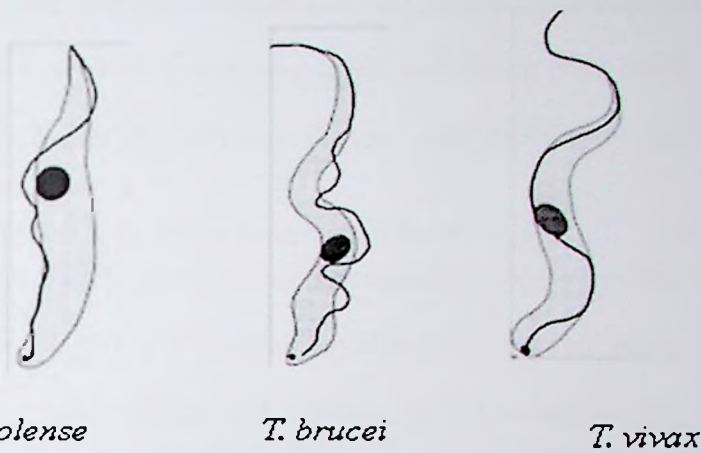


Figure 3: Important livestock pathogenic trypanosomes (Connor,1994, Leak,1998, Uilenberg, 1998)

Infection rates in tsetse are determined by the parasite, the host, the vector, and the environment. They are generally low but vary greatly according to species of trypanosome, with *T. vivax* ranking the highest (5%) and *T. brucei spp.* the lowest (1%), Leak, 1999).

2.1.2 Host

Cattle-infective trypanosomes circulate in a variety of wildlife hosts, which generally tolerate infections or have a state of pre-immunity. Small ruminants, equines, pigs, dogs and cats are also susceptible to some species of cattle-infective trypanosomes (Rodastitis et al., 2000). The existence of reservoir and alternative hosts complicates the epidemiology of African trypanosomosis, making it difficult to manage and perhaps infeasible to eliminate the disease. Susceptibility of cattle to trypanosomosis depends on their breed, age, behaviour, previous exposure and health status; of these the most important factor is the breed (Hoare, 1972).

2.1.3 LIFE CYCLE- in the mammalian hosts

When metacyclic trypanosomes are inoculated by tsetse flies into the mammalian hosts, they cause the development of a nodular swelling the 'chancre' at the site of the bite (Fairbairn and Godfrey, 1957; Willett and Gordon, 1957; Gordon and Willett, 1958; Connor, 1994; Leak 1998 and Uilenberg, 1998). The inoculated trypanosomes undergo morphological changes characterized by drastic reduction in volume and cell membrane areas of the mitochondria. The trypanosomes are transformed into long slender bloodstream forms (Harmseen, 1973; Vickerman, 1970; Brun et al.; 1984). The mitochondrial changes are reported to be the expression of changes in respiration (Langley, 1975; Baker, 1977; Ghiotto et al.; 1979 and Leak, 1998). The respiratory metabolism of the slender bloodstream forms are wholly glycolytic involving glucose 6-phosphate oxidase enzymes but the metacyclic and stumpy bloodstream forms show evidence of mitochondrial oxidative decarboxylation (Bowman et al., 1972; Harmsen, 1973 and Leak 1998).

2.1.4 LIFE CYCLE- in the invertebrate host

Although only a few tsetse species are implicated as vectors of trypanosomes in Africa, there is no evidence that any species is incapable of cyclical transmission of trypanosomes. Several of the factors that are known to affect fly infection would be expected to affect all fly species equally, however, Maudlin, (1982) reported that fly infection by *T.congolense* has some genetic controlling aspects.

The general course of development of the salivarian trypanosomes in their vector, *Glossina* spp, has been known for many years. The *Nannomonas* subgenera develop in the midgut and the proboscis of the tsetse flies while the *Duttonella* subgenus completes its development in the proboscis only. The *Trypanozoon* subgenus undergoes the most complicated route of development passing through the midgut, salivary glands and the proboscis (Hoare, 1972). Under natural conditions, *T.congolense* has a very low (0.6-10%) infection rate in the tsetse flies but in experimental situations, the infection rate can be as high as 35% (Hoare,1972). Factors that determine the trypanosome infection rates in the tsetse flies have been reported by many workers amongst which the age and species of the fly, nature of the first bloodmeal and temperature are important (Harley,1971, Baker,1974 and Leak 1998). If tsetse flies ingest trypanosomes in the blood while feeding, the trypanosomes must adapt themselves to life inside the tsetse flies. According to Leak (1998) the process of adaptation in *T.brucei* is effected by an enzyme transformation (Glucose-6-phosphate oxidase) while the parasites are still in the crops of the flies. He believed that the age of the flies at the time of infection was important and in young flies, the crops would still be small thus retaining blood longer allowing time for the enzyme transformation to act on the trypanosomes. Similar

observations stressing the importance of age and low temperature as major factors on *T.brucei* infection rate in tsetse flies had been reported by Otieno et al., (1983).

In *T.congolense* and *T.vivax* little is known about the bloodstream forms pre-adapting to life in the vector (Baker, 1977). During the developmental cycles of trypanosomes especially in the Trypanozoon subgenus there is an alternative proliferation and regression of the mitochondrial structure. This complex process physiological alterations undertaken by trypanosomes during their developmental cycles is believed to be controlled by kinetoplast DNA (Vickerman and Preston, 1976).

The bloodstream stumpy forms of *T.brucei* spp. which are reported to be infective to the tsetse flies show evidence of mitochondrial oxidation decarboxylation and they have greatly increased mitochondrial volume (Brun et al., 1984). After transformation there is an initial multiplication of procyclic trypomastigotes in the midgut. The promastigotes later invade the ectoperitrophic space and reach the proventriculus where they continue to multiply. The proventricular forms finally migrate to the proboscis where they transform into the epimastigotes and multiply further to give rise to the infective metacyclic trypanosomes (Hoare, 1972 and Baker, 1974). During the long process of development the trypanosomes are reported to cross three barriers before reaching the metacyclic stage: in the midgut, penetration of the peritrophic membrane and migration to the proboscis or salivary glands (Leak, 1998).

The reports on the duration from the time the fly ingests to the time the fly becomes infective have been conflicting. The prepatent period of *T.congolense* in the fly is reported to 19-53 days (Buxton, 1955 and Hoare, 1972). It is important to note that Jenni (1977) obtained mature infection of *T.brucei* in *G.morsitans* in 9 days. This is in sharp contrast to the popular

belief that *T.congolense* takes a shorter time to develop in the fly than the brucei group (Buxton,1955 and Hoare,1972). This point stresses the importance of temperature, age and species of the tsetse flies and the trypanosome species in determining the duration and the rate of infection in tsetse flies.

Table 1: Classification of the pathogenic African trypanosomes

Subgenus	Species/group	Development/transmission
<i>Duttonella</i>	<i>Vivax</i> group:	In tsetse: proboscis only
	<i>T. vivax</i>	Can also persist by mechanical transmission
	<i>T. uniforme</i>	
<i>Nannomonas</i>	<i>Congolense</i> group:	
	<i>T. congolense</i>	In tsetse: midgut and proboscis
	<i>T. simiae</i>	Not known to maintain itself by mechanical transmission
	<i>T. godfreyi</i>	
<i>Trypanozoon</i>	<i>Brucei</i> group:	In tsetse: midgut and salivary glands
	<i>T. brucei brucei</i>	
	<i>T. evansi</i>	
	<i>T. equiperdum</i>	(Venereal transmission)

2.2 Tsetse flies

Tsetse flies are haematophagous dipterids belonging to the family *Glossinidae* and genus *Glossina*. They have one pair of functional membranous wings with characteristic 'hatchet'-shaped discal cell, and are folded one on top of the other at rest in contrast to other biting flies

uch as the *Tabanus* and the *Stomoxys* species. The proboscis is a distinct, thin and long structure attached to the bottom of the head and pointing forward at rest while the antennae have bristles on the aristae. The above features are useful in the identification of the fly Fig 4.



Figure 4: Dorsal view of tsetse fly

They seek their host through olfactory (volatile substances in urine and breath) and optical (movement and colour stimuli) (Phelps & Lovemore 1994; Kappmeier et al. 1999a, 1999b and 1999c). Blood is the source of hydration and energy, and does not allow sufficient energy for long sustained flight, which influences the behaviour patterns of the fly. After emerging from their puparia the unfed (teneral) tsetse flies immediately seek a blood meal. If this happens to be from an infected animal with trypanosome parasitaemia, then flies may become

infected after which they act as biological vectors transmitting the infection to animals or humans they subsequently feed on at 3-5 days intervals. The trypanosomes become established in the mouth parts, midgut, salivary glands or a combination of these depending on the species of trypanosome involved.

Their longevity, mobility, and frequent feeding make tsetse flies highly efficient vectors, but the low rate of population growth means even small increases in mortality rate can result in population decline and even extinction (Hargrove, 2005).

2.2.1 Host preferences

Different tsetse fly species have different host preferences. Therefore, tsetse host preference in an area is an important parameter in the epidemiology of trypanosomosis as it determines, whether an individual tsetse fly species is an efficient vector of human or animal trypanosomosis (Staak, et al., 1986; Makumi, et al., 1996; Clausen, et al., 1998).

2.2.2 Blood meal identification methods

Blood meal identification of a vector is important in identifying its natural sources of food (Boakye, et al., 1999. Clausen, et al., 1998) as this improves our knowledge in understanding the transmission cycle of trypanosomosis in the study area and will also help in the planning and monitoring the success of tsetse and trypanosomosis control programmes (Staak, et al., 1986; Clausen, et al., 1998).

Analysis of bloodmeal samples can be serological or by DNA based techniques.

2.2.2.1 Serological techniques

The serological identification of tsetse bloodmeals depends on the ability of the antisera to recognize only the unique proteins of the host blood (Staak et al., 1986; Clausen et al., 1998; Gomes et al., 2001).

The serological tests are Precipitation test (Weitz, 1960; 1963), Heamagglutination inhibition test, HIT(Weitz, 1960; 1963), Complement fixation test, CFT (Staak et al., 1986) and the Enzyme-linked immunosorbent assay, ELISA (Edrissian and Hafizi, 1982; Beier et al., 1988; Clausen et al., 1998; Gomes et al., 2001). However, serological tests are limited by the specificity of the antisera and by cross reactions that may occur with antiserum of poor quality, particularly as many hosts of tsetse are closely related species (Emsile and steinberg, 1973; Clausen et al., 1998).

2.2.2.2 DNA based techniques

Recent developments in molecular biology have allowed a significant increase in the accuracy and reliability of bloodmeal identification, and DNA-based molecular markers are now being harnessed for typing arthropod bloodmeal. These markers have been used for the analysis of vertebrate hosts DNA obtained from the gut of mosquito (Mukabana et al., 2002). DNA based techniques also have a major significance in the identification of bloodmeal. These techniques include polymerase chain reaction (PCR), restriction fragment length polymorphism analysis (RFLP), DNA probe hybridisation and nucleotide sequencing. Recent and accurate methods for arthropods bloodmeal identification include molecular techniques that detect mammalian DNA in the gut of tsetse flies (Boakye et al., 1999; Torr et al., 2001) and a modified heteroduplex PCR-based method by Njiokou et al.,(2004). The latter is a modification of Boakye et al.,(1999) method of identification of blood meals in haematophagous Diptera by cytochrome B heteroduplex analysis.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 STUDY AREA

The study was undertaken in the communal grazing lands in the districts of Apac (Balodilo in Chegere S/C), Lira (Awie Alem in Agweng S/C) and Kole (Amonya in Aboke S/C) which comprise part of mid-northern Uganda. Basing on the reports of animal trypanosomosis cases that were originating from these areas (District Veterinary Reports), the sub-counties in the districts of Apac, Lira and Kole were selected by simple random procedure Fig 5. Within each sub-county, a list of villages was generated which constituted the sampling frame from which the study villages were selected by simple random procedure. Most parts of these Communal grazing lands are covered with open savanna grasslands. The dominant tree species are *Acacia seyal*, *Acacia sieberiana*, *Acacia tortilis*, *Combretum* spp and *Butyrospermum paradoxum*, Lantana is too widely spread, it's a recognized tsetse habitat . The main activities of the people in these areas are farming and livestock keeping where grazing is done communally.

3.2 TSETSE SAMPLING

Two surveys were carried out; one in March (dry season) and another between May and July (wet season) 2011. Sixteen odour baited biconical traps (Challier and Laveissiere, 1973) were deployed every 100m apart in the watering areas, along the water courses and the grazing areas in the shade of trees (to reduce fly mortality due to heat) following a Transect. Each sampling was point georeferenced for consecutive sampling. The traps were set for 8 days in each study area and fly collections made every 24 hrs. The harvested flies were transported in

apparent density which is often expressed as the number of tsetse caught per trap per day was calculated by dividing the total number of tsetse captured ($\sum F$) by the product of the number of traps used to catch them (T) and the number of days for which traps were operational (D)

$$FTD = \sum F / T \times D.$$

a method described by Rogers (1983). These estimates of apparent density can be used for estimating disease risk.

3.2.2 INFECTION RATE DETERMINATION

Non teneral flies (these are flies which have taken a blood meal) were dissected for trypanosome infections according to the rapid technique of Penchenier & Itard (1981). The dissecting instruments were cleaned between each dissected organ and the dissected fly by rinsing with distilled Water and in normal saline to avoid carry over contamination. The proboscis was Separated first, then the salivary glands and the midgut (including the proventriculus). A cover slip was then put on each part of the slide where each of the organ from a single fly were placed and examined for trypanosomes under a microscope at 400 x magnification. Each organ was confirmed positive or negative by an experienced field technician. Parasitological interpretation relied on the localization of the trypanosomes in the fly (Lloyd and Johnson, 1924). When parasites (trypanosomes) were found only in the proboscis, it was considered to be *Duttonella*, whereas both in the proboscis and mid-gut was considered to be *Nannomonas* and Similarly in salivary gland were considered to be *Trypanozoon*. While Presence of the trypanosomes in midgut only was classified as immature.

The infection rate was calculated as the number of tsetse flies infected divided by the total number of tsetse flies dissected over a given period multiplied by 100.

That is to say Infection rate(IR)

$$\frac{\text{Number of tsetse flies infected with trypanosomes}}{\text{Total Number of tsetse flies dissected over a given period}} \times 100$$

3.2.3 TSETSE FEEDING PREFERENCE HOSTS DETERMINATION

From the trapped flies, those whose Midguts Contained sufficient blood residues, were dissected out of the abdomen using a clean scalpel and clean forceps. The guts were then squashed on whatman filter paper No.1 which was Labeled into eight sections using a pencil, air dried and then placed in airtight containers containing silica gel (Clausen et al., 1998; Boid et al., 1999). The blood specimens were sent to ICIPE Nairobi, Kenya for analysis using Polymerase chain reaction (PCR).

3.2.4 ANALYSIS OF TSETSE BLOOD MEALS

The Polymerase chain reaction (PCR) was used; primarily using DNA-based techniques and mitochondrial cytochrome b gene as the molecular marker.

3.2.4.1: DNA extraction

The protocol used for this extraction was the Ammonium Acetate Precipitation Method. This protocol was adapted from Sheffield Molecular Genetics Facility as described by Bruford and others (1998).

Briefly A small piece (1mm in diameter) of the blood meal sample was excised using sterile scalpel and put into sterile 1.5 mL eppendorf tubes then 250 μ L of Digsol Buffer (20 mM EDTA, 120 mM NaCl, 50 mM Tris HCl, 1% SDS, pH 8.0) and 10 μ L of 10 mg/mL Proteinase K was added. The mixture was vortexed for 30 sec and incubated at 37 °C overnight (12-14 hrs). After the incubation, vortexing was done for 30 sec, then 300 μ L of 4M ammonium acetate was added and the mixture vortexed again for 30 sec. This was followed with an incubation step at room temperature for 15 min while vortexing for 30 sec during this period. The mixture was then centrifuged at 15,000 rpm for 15 min and the supernatant transferred into a clean and sterile 1.5 mL eppendorf tubes where 1 mL of

absolute ethanol was added, then the tube inverted 10-20 times, gently, and centrifugation done at 15,000 rpm for 15 min. The ethanol was poured off with care taken not to lose the pellet. Immediately, 900 μ L of 70% cold ethanol (stored at -20 °C) was added and the tube inverted gently 10-20 times to rinse the DNA pellet. Centrifugation was done at 15,000 rpm for 5 min and the ethanol poured off. A second wash with cold 70% ethanol was done. Centrifugation was done at 15,000 rpm for 5 min to ensure that the DNA pellet stuck at the bottom of the tube and was not lost during the pouring off of ethanol. After the final ethanol wash, was poured of, the tubes were allowed to stand at room temperature, under a lamp for 30 min to dry the pellet. Once fully dry, the DNA pellet was resuspended by adding 50 μ L of TE buffer (10 mM Tris HCL, 0.1 mM EDTA, pH 7.4). The tubes were gently flicked to bring the pellet into solution. If the pellet was too dry, the tubes were placed in a water bath set at 37 °C for 30 min while flicking the tubes after every 10 min. The resuspended solution contained DNA which was stored at -20 °C for later use in PCR and subsequently stored at -80 °C for long term storage. All the centrifugation steps were performed in BIOFUGE fresco centrifuge (DJB Lab care Ltd, UK).

3.2.4.2 DNA amplification by Polymerase Chain Reaction

Amplifications were carried out for the mitochondrial Cytochrome b gene fragment of the isolated DNA. Typical cycle conditions were as follows: Initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 40 sec, annealing at 56°C for 45 sec, and primer elongation at 72°C for 1 min followed by a final extension at 72°C for 10 min. The PCR was carried out in a total volume of 20 μ L containing 10 pmoles of each primer, (Fw 5' CCC CTC AGA ATG ATA TTT GTC CTC A 3' and Rv 5' CCA TCC AAC ATC TCA GCA TGA TGA AA 3', respectively), 10X GenScript PCR Buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl), 1 unit *Taq* DNA polymerase (GenScript Corporation) and 1 μ L of DNA template. This gave a target region of ranging between 383 and 500 Bp. The PCR was done in a programmed thermal cycler (PTC-100 Programmable Thermal Controller (96-well), MJ Research, Gaithersburg).

3.2.4.3 Agarose gel electrophoresis

A 1% agarose gel was prepared by dissolving 1 gm of agarose powder into 100 ml of 1 X TAE buffer, the mixture stirred and boiled in a microwave, then ethidium bromide (50 mg/μl) was added and the gel allowed to cool before being poured into the casting trays in order to polymerize. The PCR product (4 μl), was mixed with the loading dye and samples loaded into the gel, alongside a molecular weight DNA marker. Electrophoresis was set at 70 volts for 1.3 hour, followed by visualization of the DNA under UV-illumination. The gel photo was analyzed and documented using the software; KODAK Gel Logic 200 Imaging System (Raytest GmbH, Straubenhardt).

3.2.4.4 Gel extraction and purification

This was prepared using the Quick Clean 5M Gel Extraction Kit II from GenScript (GenScript Corporation, Piscataway, NJ), following the manufacturer's instructions. A sharp sterile scalpel was used to excise the band of interest from the agarose gel. The gel slice was placed into a sterile 1.5 ml eppendorf tube and weighed. Three volumes of binding solution II was added into the tubes (100 mg~100 μl). This was then incubated at 55°C for 10 minutes with occasional shaking until the gel slice completely dissolved. Then one volume of isopropanol was added and mixed. The mixture was then transferred into a clean spin column and centrifuged at 6,000 rcf for 1 minute (BIOFUGE fresco centrifuge, DJB Lab care Ltd, UK). The flow-through was discarded, and then 500 μl of binding buffer II was added into the column, centrifuged at 12,000 rcf for 1 minute, flow through discarded, and then 750 μl of wash buffer was added into the column, incubated at room temperature for 5 minutes then centrifuged at 12,000 rcf for 1 minute. The flow-through was discarded and the spin column was again spun at 12,000 rcf for 1 minute to get rid of any residual wash buffer. Finally, the column was transferred to a clean 1.5 ml micro centrifuge tube and 35 μl of elution buffer was added to the center of the column membrane and incubated at room temperature for 1 minute. The DNA was then eluted by centrifugation at 12,000 rcf for 1 min. Five microlitres of the purified products was be loaded onto a 1% ethidium bromide-stained agarose gel to confirm recovery of the DNA.

3.2.4.5 Sequencing

The purified PCR products were then sent to Macrogen Inc for sequencing. The successfully sequenced samples were then cleaned and queried at the GenBank database in NCBI for identification of the tsetse blood meal donors.

3.3 Data Analysis

The data was entered into Microsoft excel and analyzed by SPSS Version 16.0 software at 95% confidence interval. The overall infection rates of trypanosomes in *G. fuscipes fuscipes* were calculated as the number of microscopically positive flies divided by the total number of dissected flies multiplied by 100. The infection rate between the male and female as well as between the different parasites were subjected to Chi-square test.

The successfully sequenced samples were then cleaned and queried at the GenBank database in NCBI for identification of the tsetse blood meal sources.

CHAPTER 4

RESULTS

4.1: APPARENT TSETSE DENSITY

A total of 707 flies were caught across the three districts and *Glossina fuscipes fuscipes* was the only tsetse species encountered during the surveys carried out in the districts of Apac, Kole and Lira . The apparent tsetse density across the three study areas during the dry and wet seasons (Table 2), generally show more flies were caught during the wet season than the dry season, Table 2.

Table 2: Number of flies caught in different seasons in the study areas and their FTD's

	Round	Kole	Apac	Lira	Total
Catches	Dry	199	27	75	301
	Wet	272	26	108	406
FTD		Kole	Apac	Lira	Total
	Dry	1.5547	0.2109	0.5859	0.7839
	Wet	2.1250	0.2031	0.8438	1.0573

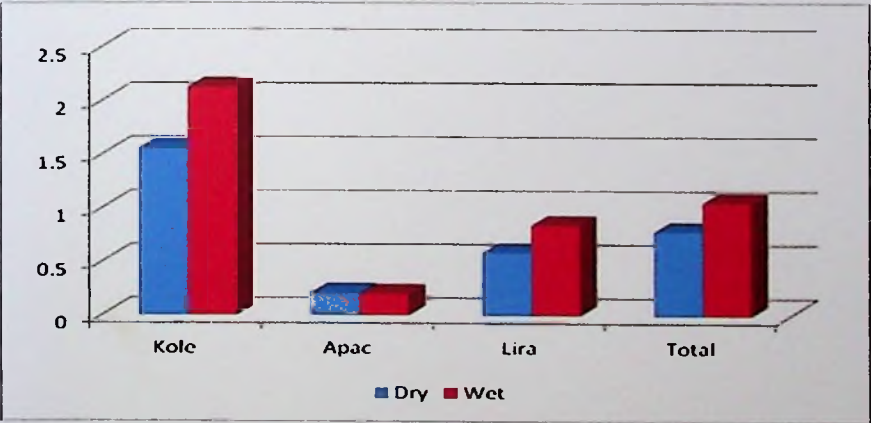


Figure 6: Seasonal variation of *Glossina fuscipes fuscipes* apparent density in communal grazing lands of Kole, Apac and Lira districts

4.2 TRYPANOSOME INFECTION RATES IN TSETSE

The trypanosome infection rates in tsetse for the three districts are shown in Table 2. Out of the 376 non-teneral flies dissected and examined microscopically for infections, trypanosomes of various species were observed in 21 flies giving an overall infection rate of 5.6%. Of the infected flies, 81% were females while 19% were males. The difference was found to be significant (χ^2 , 8.048, d.f.= 1, $P= 0.002$). The infection rates were high during the wet season 3.7% than the dry season 1.9% as shown in Table 3. By the dissection method of Lloyd and Johnson (1924), the trypanosomes encountered belonged to Duttonella and these were classified as *Trypanosoma vivax* and those in Nannomonas as *Trypanosoma congolense*, immature (gut only) and none in Trypanozoon subgenus (*T.brucei*). The infection rate of trypanosome parasites were found to be 3.2% for *T.vivax*, 1.3% for *T.congolense*, 1.1% for immature (gut only) and 0% for *T.brucei*. More flies were infected by *T.vivax* (3.2%) than *T.congolense* (1.3%), however with no statistical significance (χ^2 , 2.882, d.f.= 1, $P= 0.04$).

Table 3: Infection rates in male and female *G. f. fuscipes* in the communal grazing lands in the three districts and FTD's

District	Season	Sex	Total catch	FTD's	No. dissected	No. infected	Trypanosome species (%)			
							Tv	Tc	Tb	Immature
Kole	Dry	M	75		38	0(0)	0(0)	0(0)	0(0)	0(0)
		F	124		84	4(4.8)	3(75)	1(25)	0(0)	0(0)
		Total	199	1.55	122	4(3.3)	3(2.5)	1(0.8)	0(0)	0(0)
	Wet	M	137		58	2(3.4)	2(100)	0(0)	0(0)	0(0)
		F	135		94	8(8.5)	3(37.5)	3(37.5)	0(0)	2(25)
		Total	272	2.13	152	10(6.6)	5(50)	3(30)	0(0)	2(20)
Apac	Dry	M	9		4	0(0)	0(0)	0(0)	0(0)	0(0)
		F	18		9	0(0)	0(0)	0(0)	0(0)	0(0)
		Total	27	0.21	13	0(0)	0(0)	0(0)	0(0)	0(0)
	Wet	M	13		4	0(0)	0(0)	0(0)	0(0)	0(0)
		F	13		9	0(0)	0(0)	0(0)	0(0)	0(0)
		Total	26	0.20	13	0(0)	0(0)	0(0)	0(0)	0(0)
Lira	Dry	M	26		16	1(6.3)	0(0)	1(100)	0(0)	0(0)
		F	49		33	2(6.1)	0(0)	0(0)	0(0)	2(100)
		Total	75	0.59	49	3(6.1)	0(0)	1(33.3)	0(0)	2(66.7)
	Wet	M	41		9	1(11.1)	1(100)	0(0)	0(0)	0(0)
		F	67		18	3(16.7)	3(100)	0(0)	0(0)	0(0)
		Total	108	0.84	27	4(14.8)	4(100)	0(0)	0(0)	0(0)
Total			707	D 0.78 W 1.06	376	1(5.6)	12(57.1)	5(23.8)	0(0)	4(19.0)

T.v = *Trypanosoma vivax*, *T.c* = *Trypanosoma congolense*, *T.b* = *Trypanosoma brucei*

D= Dry, W= Wet

4.3. IDENTIFICATION OF TSETSE BLOOD SOURCE

A total of 40 bloodmeals were collected from *Glossina fuscipes fuscipes* during the study, and of these 27 (67.5%) samples were confirmed to be the host blood. Thirteen, 13(32.5%) samples which were successfully sequenced then cleaned and queried at the GenBank database in NCBI for identification of the tsetse blood meal source were invalid and did not get accession results because there was a lot of background interference. Among the hosts, the predominant tsetse blood meal source identified from these samples based on nucleotide sequences was cattle (*Bos taurus/Bos indicus*) with about 74.1% (20) of the meal. Human beings made about 25.9 % of the tsetse meal as shown in Table 4.

Table 4: Source of blood meal of *G. f. fuscipes* caught in each of the communal grazing lands in the three districts

Species identified	Study districts			Total	GenBank accession numbers(cytb)
	Kole (n = 17)	Apac (n = 3)	Lira (n = 7)		
Cattle	12	2	6	20	AJ931337.1, AY172562.1, AY682377.2 EU365345.1, EU760476.1, GU367344.1 HM596472.1, HQ025805.1, JN817307.1
Human beings	5	1	1	7	AY509658.1, JQ703928.1, JX303864.1

Twenty seven (67.5%) of the 40 bloodmeals were identified. The total number of bloodmeals analyzed per the study site is in the parenthesis. The data also contains cytb GenBank accession numbers for representative sequences given, which were used to identify the species.

Out of the seventeen bloodmeals analyzed from Kole area, twelve, 12(70.6%) were from cattle and five, 5(29.4%) from human beings. Of the three bloodmeals from Apac, two, 2 (66.7%) were from cattle and One, 1(33.3%) from human being. Hosts identified from seven (7) blood fed *G.f.fuscipes* in Lira, six, 6 had fed on cattle which accounted for 85.7% while one, 1(14.3%) had fed on human being.

4.3.1 DNA amplification

A total of 40 blood meals were worked on and they are detailed below.



Figure 7: 1 % agarose gel electrophoresis for Cyt b amplification

M: 100 Bb DNA ladder (Fermentas). **Lanes 1 – 23:** Blood meal samples collected from Lango area (Mid-Northern Uganda). **Lanes 24 and 27:** Positive control (cow). **Lanes 25 and 26:** negative control.

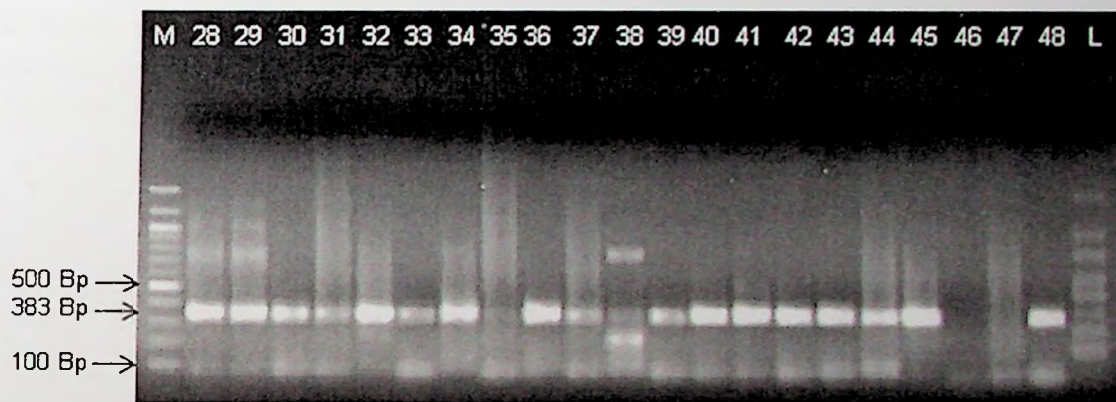


Figure 8: 1 % agarose gel electrophoresis for Cyt b amplification

M: 100 Bb DNA ladder (Fermentas). **Lanes 28 – 44:** Blood meal samples collected from Lango area (Mid-Northern Uganda). **Lanes 45 and 48:** Positive control (cow). **Lanes 46 and 47:** negative control.

Excellent amplification was obtained from most of the domestic samples. The aim of the PCR was to recover the correct gene of interest (Cyt b) and since this was achieved, the samples were gel purified and sent for sequencing to facilitate for the host identification. The gel purified results is as illustrated below.



Figure 9: 1 % agarose gel electrophoresis for Gel purified Cyt b PCR products

M: Low Molecular Weight DNA ladder (New England Biolabs). **Lanes 1– 38:** Blood meal samples collected ready for sequencing.

4.3.2 Sequencing

The successfully sequenced samples were queried from the GenBank database at NCBI and the tsetse blood meal donors identified.

CHAPTER 5

DISCUSSIONS

This study was undertaken to determine the status of the tsetse vector, *Glossina fuscipes fuscipes* population which is the main tsetse species in Mid-Northern Uganda as this knowledge helps understanding the epidemiology of trypanosomosis in these areas (Waiswa, 2006; Bitew, 2011) and delineate the active foci. The epidemiology of the disease is largely determined by tsetse fly density and other factors like infection rates and hosts preferences (Katunguka, 1996, Ducheyne, 2009). To restore such areas to their greatest potential, there is need to carryout studies on such factors which are essential to the understanding of the epidemiology of the disease. This will enable to prioritize decisions on appropriate disease control strategies in the study areas.

5.1 APPARENT TSETSE DENSITIES

The apparent densities varied between 0.2 – 2.1 flies/trap/day across the three study districts. The observed fluctuation in apparent densities (Fig.6) showed that *Glossina fuscipes fuscipes* was abundant during the wet seasons across the three districts. This is in agreement with previous works reported in Mukono(Katunguka,1996) and other parts of Africa ,Somalia,(Mohammed and Diarra,1987), Ethiopia (Msangi,1999) and Cote d'ivore, Togo, Gabon and DRC(Leak et al., 1987). The fly abundance during the wet season may be attributed to the environmental conditions(temperature and humidity) which might have reduced the fly mortality. These estimates of apparent density show that the disease risk in the study area is of a significance importance .

5.2 TRYPANOSOME INFECTION RATES

The overall trypanosome infection rate of 5.6% recorded included the immature infections that accounted for 19.05 % of the total infections. Discarding them would have influenced the overall infection rate (Van Wettere, 1975). The mature and immature infection rates observed in this study probably reflected a population of *Glossina fuscipes fuscipes* with high vectorial capacity. The infection rate recorded in the flies in this study is higher than the 0.52 % and 1.55% obtained from the previous study by (Kutuza & Okoth, 1981; Waiswa, 2006,) in *G.f.fuscipes* in Kigulu and South eastern Uganda (Kamuli, Mukono, Tororo) respectively. It is possible that the infections would have been higher, if a more sensitive method such as the dot-ELISA (Bossempem, 1996) and PCR (Morlais et al., 1998; Ouma et al., 2000) was used. A high *Duttonella*(*T.vivax*) infection was observed than *Nannomonas*(*T.congolense*) and no *Trypanozoon* (*T.brucei*) subgenus detected. This is in agreement with the previous work done by (Waiswa, 2006; Lonjino,et al., 2007; Bitew, 2011). It is known that natural infections with *T.congolense* -type of parasites are generally low, with characteristically low parasitaemia than those of *T.vivax* (Stephen,1986). It is therefore possible that such scanty parasites are rarely picked up by the flies during feeding which may account for the inability of the parasites to get established in the vector. Failure to get *T.brucei* infection in flies is not surprising because detection of natural infections is rare (Ahmed, 2000; 2006),especially by the conventional diagnostic dissection method utilized in this study (Lloyd & Johnson 1924; Penchenier & Itard 1981).

From the present study, female *Glossina fuscipes fuscipes* were more infected than males and the difference was statistically significant $P=0.02$. This was in agreement with the previous study done elsewhere in the same species in Ethiopia (Bitew, 2011). The difference in

infection could probably be due to the feeding habits of the sexes. Male flies feed every 3-4 days (Davies, 1977) while females take blood meals regularly due to their role in reproduction (Moloo, 1976) which increases their chances of feeding on an infected host and acquiring infection.

5.3 IDENTIFICATION OF TSETSE BLOOD SOURCE

Tsetse distribution and abundance is associated with the host presence and its distribution. During this study, forty (40) blood meals were collected since the stationary traps used catch only hungry flies which are seeking for the host. During the investigations, 67.5% of the collected blood was confirmed to be host blood and the predominant tsetse host across the three districts was cattle. This may be attributed to the absence of large game in these areas. The other host implicated across the study districts was human beings. This was similar to the work done in South eastern Uganda (Waiswa, 2006). In the study areas there are a number of activities which take place like charcoal making, firewood collection and also footpaths in these grazing areas through which people from other areas cross to reach their gardens or market. These activities increase the fly/man contact. Therefore, *Glossina fuscipes fuscipes* could be of epidemiological importance in case an infected animal, human or reservoir game animals are introduced in these areas accidentally in transmitting the zoonotic human trypanosomosis and there is need to use approaches like live bait technology on cattle using deltamethrin to control this species since cattle have been found to be a predominant host.

5.4 CONCLUSIONS

A better understanding of the epidemiological factors which influence trypanosomosis transmission in these communal grazing lands is necessary for the planning of an appropriate tsetse and trypanosomosis control strategies. The present results showed that;

- i) There is a high infection rates of trypanosomes in *Glossina fuscipes fuscipes* and the females are more infected than males. These infections are more in the wet season than the dry season. This implies that the flies in those areas are a potential source of trypanosomes
- ii) *T.vivax* was a more common parasite than *T.congolense* and *T.brucei* was the least.
- iii) The hosts for *Glossina fuscipes fuscipes* vary and depend on the availability; abundance and behaviour of the animal host. In addition to cattle, human beings were implicated as tsetse host and this can play an important role in the epidemiology of human trypanosomosis in case an infected animal, human being or wild game reservoir is accidentally introduced in these communal grazing areas. This could be considered in other tsetse and trypanosomosis control strategies. In other words live bait technology can be applied during intervention.

5.5 RECOMMENDATION

Further studies should be undertaken in order to give priority in the control of tsetse flies and other biting flies to alleviate the trypanosomosis problem in the study area.

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