Vector competence of *Glossina austeni* and *G. brevipalpis* and characterization of *Trypanosoma congolense* strains from northern KwaZulu-Natal, South Africa

By

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Declaration

I Makhosazana Yvonne Motloang, hereby declear that this thesis, submitted for the degree of Doctor of Philosophy (PhD) at the University of Pretoria, is my own work and has previously not been submitted by me for a degree at this or any other institution.

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List of Abbreviations

µg/ml:	Microgram per millilitres
µl:	Microlitre
µM:	Micromolar
AAT:	Animal African trypanosomosis
ARC:	Agricultural Research Council
BHC:	Benzene Hexachloride
Bmrng:	Boomerang
Bp:	Base pairs
DDT:	Dichloro-Diphenyl-Trichloroethane
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic acid
DNTP:	Deoxyribonucleotide triphosphate
Dpi:	Days post infection
EDTA:	Ethylenediaminetetraacetic acid
FAO:	Food and Agriculture Organization
HAT:	Human African trypanosomosis
HCl:	Hydrochloric acid
HCT:	Haematocrit centrifuge technique
HVS:	High virulent strain
I.p.:	Intraperitoneally
I.v:	Intravenously
IAEA:	International Atomic Energy Agency
ID:	Identity
KCl:	Potassium chloride
KZN:	KwaZulu-Natal
LVS:	Low virulent strain
Mg:	Milligram
$MgCl_2$:	Magnesium chloride
Ml:	Millilitre
Mm:	Millimetre
MM:	Millimolar

MST:	Median survival time
MVS:	Moderately virulent strain
MVU:	Mvuthsini
N:	Sample size
NDM:	Ndumo
NHL:	Nhlanzana
OCI:	Ocilwane
OVI:	Onderstepoort Veterinary Institute
PCR:	Polymerase chain reactions
PCV:	Packed cell volume
PP:	Patent period
PPP:	Pre-patent period
PSG:	Phosphate buffered Saline
RBC:	Red blood cell
RNA:	Ribonucleic acid
SAVP:	South African Vaccine Production
SIT:	Sterile insect technique
Taq:	*Thermobius aquaticus*
TCKZN:	*Trypanosoma congolense* KwaZulu-Natal

Dedication

This work is dedicated to my late grandmother, Mpeo Ellen Motloang; my late mother, Madipuo Adelaide Motloang and my late sister, Diatile Penelope Motloang, may their souls rest in God's undying peace; the father of my children, Mr. Martin Lesenyego Freddy Mokoena; my two beautiful angels, Lehlohonolo and Bonolo Motloang and my younger brother, Tshediso Motloang, thank you for all your support.

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To my supervisor, Prof. Abdalla Latif, I extend my heartfelt thankfulness for all the advice, guidance and support during this endevour. I also wish to say thank you to my late co-supervisor, Prof. Peter Van Den Bossche, for all the guidance and practical assistance he rendered, may his soul rest in God's peace. Thank you to my co-supervisor Dr. Ben Mans for his expertise, guidance and unlimited support. Thank you to Dr. Phelix Majiwa for the constructive inputs to this study. I am obliged to thank my friend and colleague, Dr. Justin Masumu from whom I learnt a lot about trypanosomes. To the staff of the Parasites Vectors and Vector-borne Diseases Program (Parasitology, Entomology and Khuleni Research Station in KwaZulu-Natal), thank you for every bit of assistance you rendered, being technical or moral, nothing went unnoticed. I'm grateful to Ms Chantel De Beer, PVVD Programme, for providing colony tsetse flies and assisted in drawing the maps. Dr. Lundi Ntantiso, Ms. Nomsa Letsoalo, Mr Danie De Klerk (Danie always had an answer to the impossible situation), Mr Jerome Ntshangase, Mr. Petros Gazu and Mr. Bongani Dlamini: Assistance with field work in KZN; Mr Chris Van Vuuren and Mr. Christo Troskie supplied and managed the general welfare of cattle and goats used in this study; Mr. Jonas Komape, Mr Sidwell Mafa, Mr. Isaac Kaale, Mr David Letsoalo and Mr William Maloleka took turns in assisting with handling and caring for the experimental animals. Dr. Gert Venter's constructive criticism, imputs and proof-reading of my publications are highly appreciated. Prof Tony Musoke and my fellow DST students were my family away from home; I love you guys, always.

"And we know that in all things, God works for the good of those who loves Him, who have been called according to His purpose": Romans 8:28

Summary

Tsetse-transmitted trypanosomosis (Nagana) has been the cause of cattle losses in the past and still presents a major problem to livestock owners in northern KwaZulu-Natal (KZN), South Africa. Although information on the distribution and abundance of the tsetse flies *Glossina brevipalpis* and *G. austeni* in northern KZN exists, data on related vector competence and virulence of *Trypanosoma congolense* parasites, the main cause of outbreaks, are lacking.

The aim of the first part in this study was to determine the rate of natural *T. congolense* infections in wild and colony-reared tsetse flies of the two species and their infectivity in susceptible cattle. Flies were collected from cattle dip tanks and game parks using H-traps. A total of 637 *G. brevipalpis* and 40 *G. austeni* were dissected immediately after collection to determine their infection rates. A total of 699 *G. brevipalpis* and 49 *G. austeni* flies collected at different periods from the same locations were used in xenodiagnosis experiments. Teneral colony flies were fed on infected animals under controlled conditions and dissected 21 days post infection to assess their infection rates. Of the field collected flies *G. austeni* harboured 7.5% parasites in the midgut (immature infections) and 7.5% in the proboscis (mature infections). In *G. brevipalpis*, the infection with the immature stages was lower (0.7%) and no mature infections were observed. All four batches of *G. austeni* collected from the field transmitted *T. congolense* to 3 susceptible bovines and one goat but the 10 batches of *G. brevipalpis* did not transmit any trypanosome parasites to any of the 10 susceptible bovines. Both *G. austeni* and *G. brevipalpis* colony flies acquired trypanosome infection from four infected bovines, with immature infections range of 19.6% - 33.3% (n = 534) and 0.4% - 4.2% (n = 882), respectively. However, mature infections were only found in *G. austeni* (average 3.9%).

The second part of the study aimed at characterization of *T. congolense* stocks circulating in cattle and buffalo in northern KZN, by molecular tools and using the mouse model. A total of 30 isolates were successfully recovered from the mice inoculated with parasitologically positive field blood samples,

and confirmed to belong to the genus *Trypanosoma* by PCR of the 18S ribosomal RNA gene. The identity of 21 samples was confirmed to belong to *T. congolense* Savannah by TCN-1 and TCN-2 primer sets specific for the amplification of the Savannah-type *T. congolense* and 9 isolates amplified with the *T. congolense* Kilifi-specific primers. Virulence testing in mice (6 mice per group) of 21 cattle-derived and 5 buffalo-derived *T. congolense* isolates had revealed three categories: high, moderate and low profiles based on the pre-patent period (PPP), the patent period (PP) and the median survival time (MST). In addition, the progression of parasitaemia and the development of anaemia as judged by the packed cell volume (PCV) percentage were recorded. The number of days to the percentage drop in the PCV values, PP and the MST between mice injected with the HVS, MVS and LVS was statistically highly significant ($P < 0.0001$). Overall, 46.2, 30.8 and 23.0% of the 26 strains were characterized as of high, moderate and low virulence. All of the LVS except one (6/7; 85.7%) originated from locations which are not close to game parks.

The third part of the study aimed at testing the susceptibility/resistance of Nguni cattle to virulent *T. congolense* strains selected from the “mouse model” study. The high and the low virulent strains were inoculated into 30 Nguni and 6 Friesians used as susceptible controls. A rapid development of parasitaemia was observed with both LVS and the HVS a week post infection with subsequent drop in PCV values in both cattle breeds. None of the Nguni cattle infected with the LVS required treatment while all of the Friesians were treated (PCV ≤ 19% for three consecutive days). By day 40 - 54 post-infections with HVS, 100% of Friesian cattle and only 41.7% of Nguni had received treatment. The study showed that Nguni cattle had a considerable degree of innate resistance to infection with a high virulent strain and 58.3% were able to control the progression of severe anaemia and parasitaemia similar to the well characterized trypanotolerant cattle in other parts of Africa. Eradication of trypanosomosis from affected African countries is an unrealistic goal. Efforts made in the past to control the disease through the use of trypanocidal drugs and the management of the vector have been impended

by decreasing efficacy of available drugs and the difficulties of sustaining tsetse control. Therefore, new opportunities for improved productivity are necessary and can be attempted through selection for trypanotolerance (D'leteren *et al*., 1998).

CHAPTER 1

1.1 General Introduction

Trypanosomosis is a disease that affects humans and their domestic animals in tropical and subtropical countries of the world. Trypanosomosis of economic importance has been reported in African, Asian, Central and South American continents; however, the most important forms of trypanosomosis occur in 37 sub-Saharan African countries. The disease is caused by haemoprotozoan parasites of the genus *Trypanosoma*. Trypanosomes parasitize all classes of vertebrates. In Africa, important pathogenic trypanosomes infecting livestock and humans are cyclically transmitted from one host to another by tsetse flies of the genus *Glossina* while mechanical transmission of some trypanosomes still occurs (Connor and Van den Bossche, 2004).

The history of trypanosomosis dates back to the 19th century in 1880 when Evans in India associated trypanosomes with Surra in camels and horses (Evans, 1880). Around that time, Bruce working in South Africa reported the association of the disease locally named as “Nagana” with trypanosomes (Bruce, 1895; Connor and Van den Bossche, 2004). Between 1840 and 1872, the disease was already known as “tsetse fly disease” to local natives (Connor and Van den Bossche, 2004; Emslie, 2005). In 1895, Bruce identified *Trypanosoma brucei* as the causative agent of Nagana (Steverding, 2008) and by 1909 he established that the disease was transmitted by tsetse flies. Subsequent to these findings, it was then established that *Trypanosoma* and *Glossina* species constitute a major role in the initiation of African trypanosomosis (Connor and Van den Bossche, 2004).

In Africa, the form of trypanosomosis affecting humans is termed human African trypanosomosis (HAT) also known as African sleeping sickness while that affecting domestic animals is African animal trypanosomosis (AAT) or Nagana. Both HAT and AAT have profound impacts on the health of humans

and livestock, agricultural development and nutritional resources in sub-Saharan Africa. It is estimated that over 1.3 to 5 billion US$ is lost annually due to trypanosomosis (McDermott and Coleman, 2001). In Africa, HAT is endemic to the western, central and eastern sub-Saharan countries. The acute form of HAT occurs in East Africa and is caused by *T. brucei rhodesiense*. The chronic form occurring in West and central Africa is caused by *T. b. gambiense* (Welburn *et al*., 2004). To date, no incidences of sleeping sickness have been reported in South Africa (Kappmeier *et al*., 1998), however, livestock trypanosomosis continues to deplete agricultural development and livestock production in the northern KwaZulu-Natal (KZN) Province of South Africa.

In South Africa, the tsetse (Diptera; Glossinidae) zone encompasses an area of 18 000 km^2, representing the southernmost distributional limit of this genus in Africa (Esterhuizen *et al*., 2005). This distribution continues up to the Matutuine District of Maputo Province in Mozambique. The KZN tsetse-infested area is mainly utilised for communal farming and is inhabited by *ca* 426 000 humans, 130 000 small ruminants and 360 000 cattle (Esterhuizen *et al*., 2005, 2006). Tsetse presence as vectors of trypanosomes causes a considerable loss in livestock production and agricultural development in this area (Van den Boscche *et al*., 2006).

Historically, four *Glossina* species were found in South Africa and these were *Glossina morsitans morsitans*, *G. pallidipes*, *G. austeni* and *G. brevipalpis* (Kappmeier *et al*., 1998). The former three species belong to the *morsitans* group while the latter belongs to the *fusca* group. According to Fuller (1923), knowledge of the existence of tsetse flies in South Africa begins with the year 1836. During that time, *G. m. morsitans* distribution was restricted to the northerly parts of South Africa, the northern Transvaal, while *G. pallidipes*, was the predominant species in northern KZN followed to a much lesser extent by *G. austeni* and *G. brevipalpis* (Fuller, 1923). Based on *G. pallidipes* predominance in KZN, *G. austeni* and *G. brevipalpis* were not considered as important. In essence, *G. m. morsitans* in the north-eastern Transvaal and

G. pallidipes in the northern KZN were considered the primary vectors of trypanosomes in the respective areas. *Glossina m. morsitans* was eradicated with the occurrence of rinderpest between 1896 - 1897 which cleared almost all cattle and wild Bovidae in the Transvaal, but *G. pallidipes* continued to thrive in its isolated belt as the only species capable of causing extensive epizootic in KZN (du Toit, 1954). After the end of the Second World War, *G. pallidipes* was eliminated by aerial spraying of its breeding grounds using dichlorodiphenyltrichloroethane (DDT) and benzene hexachloride (BHC) between 1945 and 1952 (Kappmeier *et al*., 1998).

Despite the eradication of *G. m. morsitans* and *G. pallidipes*, clinical cases of trypanosomosis were still diagnosed in cattle, horses and dogs between 1955 and 1989 (Bagnall, 1993). In 1990, a serious outbreak of trypanosomosis was reported in cattle at communal dip tanks in the vicinity of the Hluhluwe-Umfolozi Game Park. The infection was attributed to *T. congolense* and to a lesser extent, *T. vivax*. The treatment of cattle with trypanocidal drugs combined with tsetse control efforts using deltamethrin `pour-on' insecticide on livestock and odour-baited insecticide impregnated targets were successful in controlling the outbreak (Bagnall, 1993), however, the strategy was considered unsustainable because prevention of new infections from being introduced was not guaranteed (Kappmeier *et al*., 1998). These outbreaks changed the previous perception that *G. austeni* and *G. brevipalpis* were of minor importance in trypanosome transmission and reaffirmed that they are responsible for the cyclical transmissions in this area (Kappmeier-Green *et al*., 2007).

Among others, Mvutshini dip tank is situated close to Hluhluwe-Umfolozi Game Park. A recent study conducted at this dip tank revealed 61% of trypanosome infection prevalence in cattle suspected to be sick by owners when examined by the buffy coat technique (Van den Bossche *et al*., 2006). Because *G. brevipalpis* was caught in high densities, it was considered to be responsible for transmitting trypanosomes in cattle around the Hluhluwe-Umfolozi Game Park, thus control strategies were aimed against this species

(Kappmeier *et al*., 1998). Current surveys conducted in northern KZN indicate that the prevalence and the impact of animal trypanosomosis still pose a threat to farming communities (Van den Bossche *et al*., 2006). The disease appears to be more prevalent with severe infections in areas around the game parks where wild animals are present as natural host of trypanosomes (Van den Bossche and Vale, 2000).

All Nagana outbreaks that occurred in northern KZN had high economic impact in terms of livestock losses, production losses, cost of fly control and cost of treatment. Between 1942 and 1946, 60 000 cattle mortalities were attributed to animal trypanosomosis in northern KZN (du Toit, 1954). In response to that, a massive game eradication campaign together with the deployment of 26 000 Harris tsetse fly traps in Hluhluwe-Umfolozi Game Park reduced the disease incidence (Bagnall, 1994). However, this was short-lived following game re-proclamation which not only increased the number of wild animals but also the densities of tsetse flies. Subsequent to the 1990 outbreak, cattle dipping costs alone were estimated to be US$ 200 000 per annum while the treatment cost amounted to US$ 65 000 (Bagnall, 1994). The cost generated between 1990 and 1992 due to dipping and chemotherapeutic/ prophylactic treatment amounted to US$ 465 000 in farming areas near the game parks (Bagnall, 1994; Brown, 2008).

Although it is now known that the remaining fly species (*G. brevipalpis* and *G. austeni*) are responsible for the disease occurring in northern KZN, information on their ability to acquire and transmit trypanosomes is not available. Knowledge of this information will help identify the relationship between tsetse and trypanosomes circulating in northern KZN. From current information, *T. congolense* seemed to play a major role in the epidemiology of animal trypanosomosis in northern KZN (Van den Bossche *et al*., 2006). There is evidence that infection with *T. congolense* near the game park expresses severe clinical disease outcome compared to infections observed in areas further from the game parks. It is possible that these variations in the disease patterns are associated with the virulence profiles of the

trypanosomes affecting livestock in those various areas. Improving this knowledge on the involvement of trypanosomes on the epidemiology of livestock in northern KZN is crucial for the refinement of disease control strategies in the areas. Based on these gaps, the present study was designed to address the vectorial competence of *G. austeni* and *G. brevipalpis* and their role in the epidemiology of animal trypanosomosis and to determine the virulence of *T. congolense* strains infecting cattle in northern KZN.

CHAPTER 2
LITERATURE REVIEW

2.1. Introduction

African trypanosomosis is a debilitating disease that affects humans and livestock in the sub-Saharan African continent. The disease is mainly transmitted by tsetse flies of the genus *Glossina*. Association of tsetse flies with the transmission of African trypanosomes dates back to the discovery of the cause of Nagana by Bruce in the 19th century (Bruce, 1985). Over the years, numerous attempts have been made to understand interactions between tsetse flies and the trypanosomes they transmit (Aksoy *et al*., 2003). In the epidemiology of African trypanosomosis, the importance of understanding these interactions could help develop control methods that will interrupt their transmission cycles.

The epidemiological situation of the disease in northern KZN is influenced by the presence of protected areas such as nature reserves, game farms and parks. Wild animals in these parks provide unlimited source of food for tsetse flies. Availability of suitable habitat with abundant shady areas for resting and breeding and apt environmental conditions encourages the distribution of both tsetse species (*G. austeni* and *G. brevipalpis*). Additionally, movement of livestock to the proximity of wildlife in search of pastures contributes to disease burden in northern KZN (Van den Bossche, 2001).

2.2 The vector: *Glossina* species

2.2.1 Classification and morphology

Tsetse flies fall under the phylum Arthropoda, order Diptera, family Glossinidae with one genus, *Glossina* and three sub-genera. Each sub-genus forms a group that represents different tsetse species (Roditi and Lehane, 2008). The *fusca*, the *palpalis* and the *morsitans* groups represent the forest, riverine and savannah species, respectively (Roditi and Lehane, 2008). Although over 30 different tsetse species of *Glossina* have been identified to date, only 8 to 10 of them are considered to be of economic importance

(Vreysen, 2006). Figure 2.1 shows the classification of tsetse flies from class to species.

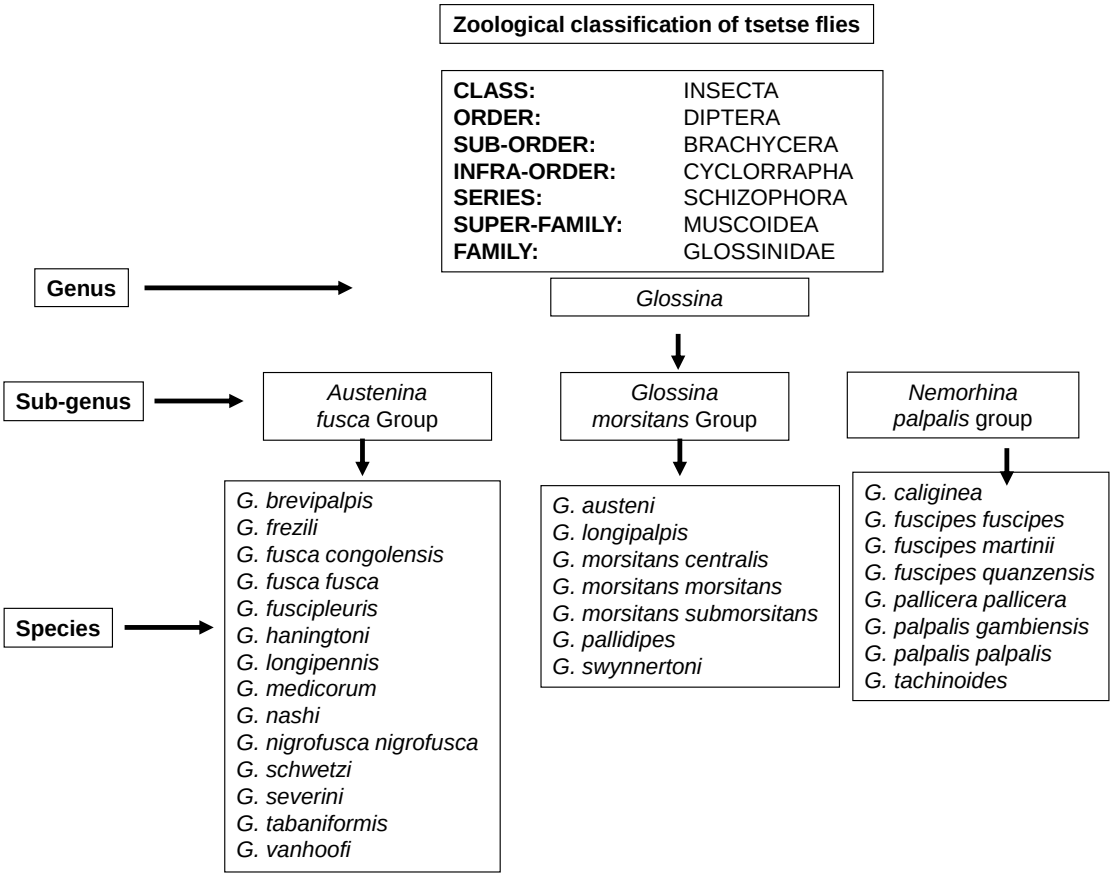


Figure 2.1: Classification of tsetse flies (Diptera: Glossinidae), (Adapted from Ford and Katondo, 1977).

Tsetse flies have one pair of membranous, mesothorasic, functional wings with their metathoracic modified to form halters. Their characteristic feature is the manner in which their wings overlap one another over the back of the abdomen during rest as illustrated in Figure 2.2 (Phelps and Lovemore, 2004). Another feature in the tsetse fly wings is that the rear edge lack veins as the front edge hence the wings can become increasingly damaged with age as it frays. In addition, some of these wing veins form a cell known as the hatchet cell (Figure 2.3), an exclusive characteristic feature of tsetse flies (Marquardt, 1996).

The functional mouth parts of the tsetse fly are called the haustellum. This is made up of the labium, the hypopharynx and the labrum (Figure 2.4). The haustellum is attached to the head by the thecal bulb that contains muscles to manipulate the mouth parts. When the fly is resting, the mouth parts are protected by the pair of maxillary palps. During feeding, the mouth parts are lowered from the palps to point downwards to the skin. The labium is equipped with labellar teeth situated anteriorly. The labium and the labrum form a blood-sucking tube that contains the hypopharynx (Leak *et al*., 2008).

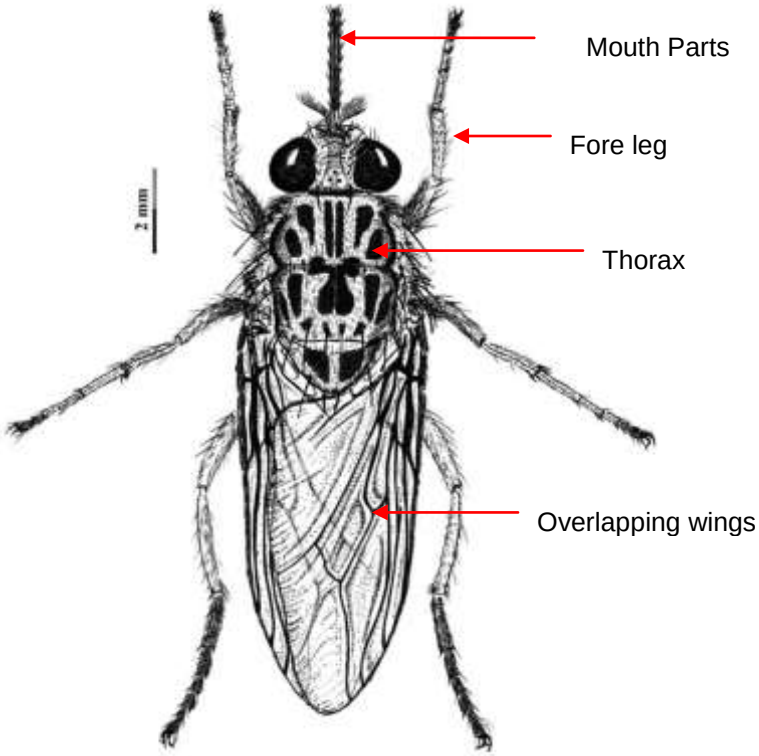


Figure 2.2: A tsetse fly showing the position of its wings during rest, (Adapted from Leak *et al*., 2008).

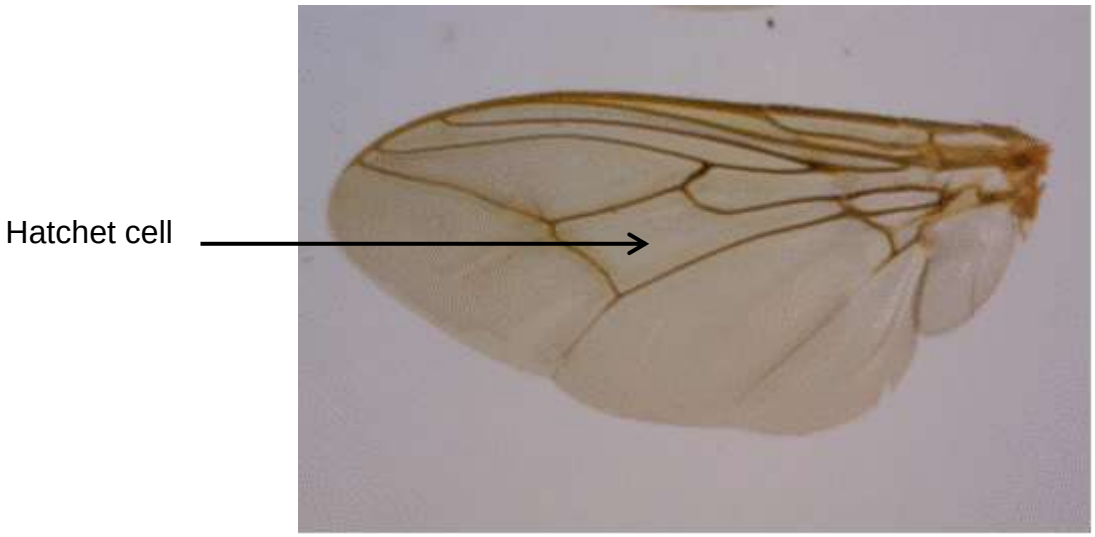


Figure 2.3: A tsetse fly wing showing a hatchet cell, (photograph courtesy of Chantel De Beer, OVI).

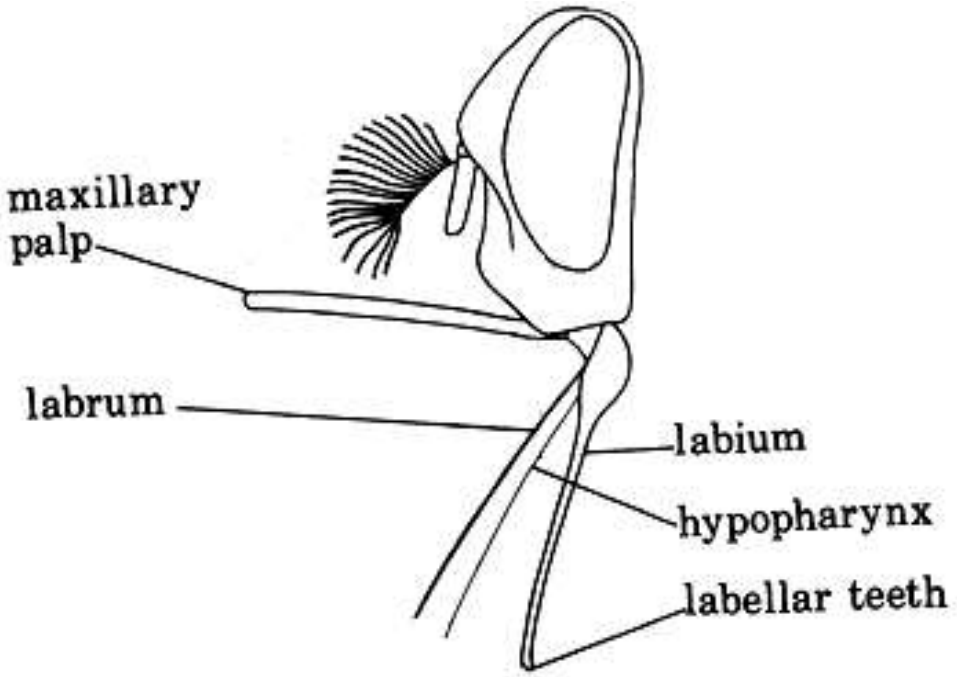


Figure 2.4: Schematic representation of the tsetse fly's mouth parts. The haustellum consists of the labium on the ventral part, the hypopharynx situated centrally and the labrum on the dorsal part of the haustellum, (Adapted from Leak *et al*., 2008).

2.2.2 Tsetse nutrition and development

Tsetse flies have distinct characteristic features that separate them from other blood-sucking biting flies. Both female and the male are obligatory blood feeders (Krinsky, 2002). They require blood for their nutritional and water needs (Hargrove, 2004). When the fly feeds, the haustellum is lowered from the palps and labellar teeth at the tip of the labium pierce the host skin and cut small blood capillaries. As the blood forms a small pool under the skin, it is sucked up through the central lumen of the haustellum while saliva containing anticoagulants is released into the wound (Phelps and Lovemore, 2004).The blood is then passed down the oesophagus into the muscular proventriculus. Blood passed from the proventriculus is stored temporarily in the crop before it is passed back into the proventriculus again (Leak *et al*., 2008). From the proventiculus, the blood proceeds into the midgut for digestion (Figure 2.5).

Unfed female tsetse flies become sexually receptive about a day after emergence while males require several meals before they are fully fertile. Female tsetse flies mate once in their lifetime. Once it becomes inseminated, it stays fertile for life and rarely mates more than once in nature although a male can mate more than once (Leak *et al*., 2008). Female tsetse flies average only about nine offspring in their lifetime (Marquardt, 1996). Unlike most biting flies which lay their eggs in a moist environment in which their larvae feed, female tsetse flies retain their eggs in the uterus until they develop into third instar larvae. During gestation, the larvae are fed from milk glands situated in the haemocoel and empty into the uterus. At least three blood meals are required for the maturation of the larva which is fully grown in 8 - 25 days. A fully developed larva is usually deposited 9 days after ovulation. Shortly thereafter, the female ovulates again within as little as 1hr after larviposition (Krinsky, 2002; Marquardt, 1996).

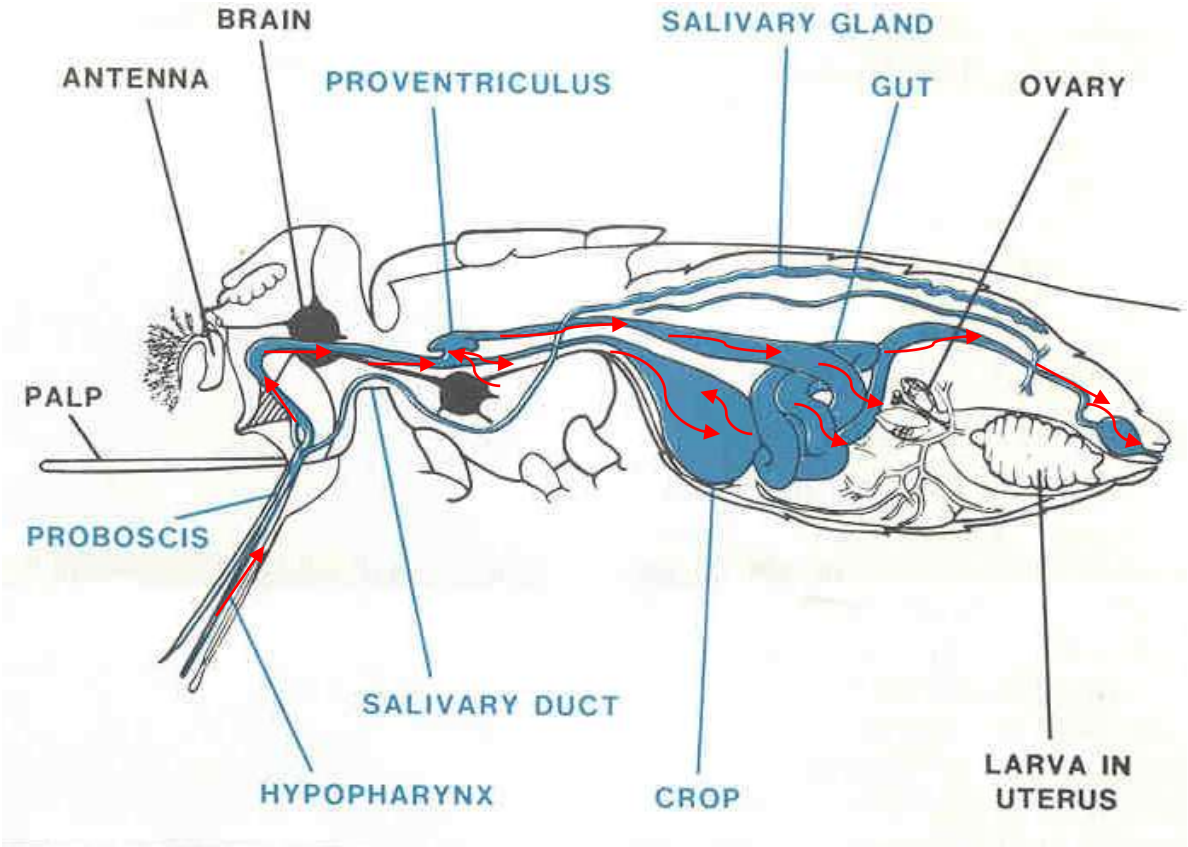


Figure 2.5: Route of blood in tsetse represented by the red arrows, (Adaprted from Leak *et al*., 2008).

2.2.3 Tsetse distribution in Africa

Tsetse fly distribution is restricted almost entirely to Africa over a land area estimated to be 10 million km^2 of the sub-tropical and tropical regions (Swallow, 2000). However, some species of tsetse, *G. fuscipes fuscipes* and *G. morsitans submorsitans,* are reported to have been caught in Saudi Arabia (Elsen *et al*., 1990). In Africa, 37 affected countries in the sub-Saharan region are rated the least developed and considered among the poorest in the world (Vreysen, 2006). These areas constitute suitable environmental conditions for tsetse survival and reproduction (Krinsky, 2002). Tsetse fly presence has a negative influence in food production, natural resource utilization and the pattern of human settlement (Nonga and Kambarage, 2009).

Tsetse flies are grouped into several tsetse belts that in some cases continue from one country to the next, across national boundaries (Leak *et al*., 2008). These belts occur between 14°N and 29°S. Each group of flies survives in a specific location with preferred habitat. Tsetse flies belonging to the *fusca* group mostly prefer the forest or dense riparian forest. The *morsitans* group is restricted mainly to savannah woodlands and those belonging to the *palpalis* group also inhabit the rain forest, but are found mostly in the riverine vegetation (Leak, 1999). The distribution of the *morsitans* and the *fusca* groups extends to the southernmost parts of the sub-Saharan countries. The southernmost distribution of the tsetse fly belt extends from Mozambique to KZN Province of South Africa.

2.2.4 Tsetse distribution in South Africa

A major determinant of the distribution of tsetse flies is the availability of suitable habitat (Van den Bossche, 2001). In KZN, the distribution of tsetse flies covers an estimated area of approximately 18 000 km^2 (Esterhuizen *et al*., 2005) in the north-eastern corner of the Province. Their distribution continues up to the Matutuine District of Maputo Province in Mozambique. The KZN tsetse-infested area is mainly utilised for communal farming and is inhabited by *ca* 426 000 humans, 130 000 small ruminants and 360 000 cattle (Esterhuizen *et al*., 2005, 2006). Their presence causes a considerable stress

to farmers in terms of livestock production and agricultural development in this area (Van den Boscche *et al*., 2006).

The distribution of *G. brevipalpis* and *G. austeni* in KZN is confined mainly to shaded areas along rivers, forests and thickets (Kappmeier-Green *et al*., 2007). Furthermore, *G. brevipalpis* is mostly found in high indigenous forests and open grassland and to a lesser extend in exotic plantations. The flies are also found in Hluhluwe-Umfolozi Game Park as well as the southern part of Great St Lucia Wetlands Park at both west and eastern lakeshore towards the south (Esterhuizen *et al*., 2005) (Figure 2.6). Towards the North, *G. brevipalpis* is more abundant in Ndumo Game Reserve and fairly limited towards the East in Tembe Game Reserve (Hendrickx *et al*., 2003).

The distribution patterns of *G. austeni* on the other hand are continuous throughout the central part of Zululand with detectable differences between the northern and southern distribution. In the northern part, *G. austeni* mainly occurs in communal areas whereas the southern distribution is represented along the lakeshores (Hendrickx *et al*., 2003). The fly populations were recorded in or adjacent to indigenous forests as opposed to humid and sub-humid coastal zones (Kappmeier, 1997).

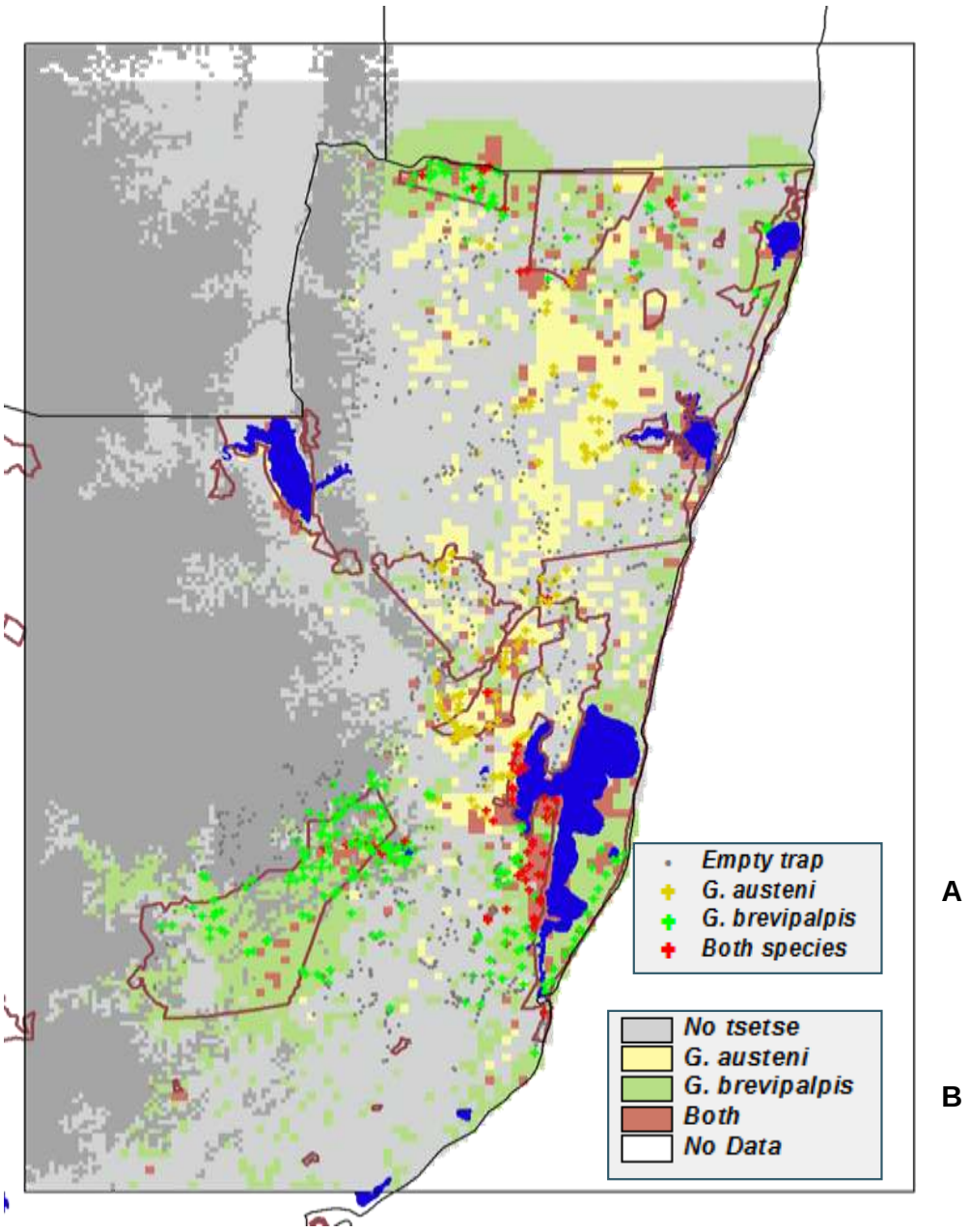


Figure 2.6: Map of the distribution of *G. brevipalpis* and *G. austeni* (Diptera: Glossinidae) in northern KwaZulu-Natal, South Africa. Legend **A** represents the actual presence of both fly species whereas legend **B** represents the prediction of the presence of the flies.

2.2.5 Vectorial competence of tsetse flies

Tsetse flies are the only known cyclic vectors of pathogenic salivarian trypanosomes in Africa (Mattioli, 1997). Known factors discussed by several authors influence the infection prevalence and contribute to the fly's susceptibility or refractoriness (Jordan, 1976; Welburn and Maudlin 1999; Roditi and Lehane, 2008). Among these factors, climate plays an important role where the light intensity determines the environmental temperature which influences the prevalence of *T. vivax* in *G. palpalis palpalis* or *G. fuscipes quanzensis* (Mattioli, 1997). In addition, the age factor may influence the readiness with which flies can be infected. Tsetse female flies generally live for about 20 - 40 days, but may have a maximum life span of 3 - 4 months. On the other hand, males survive in the wild for 2 - 3 weeks (Krinsky, 2003). Reports have indicated that older flies are more likely to be infected than younger flies. According to these reports, the longer the females of some fly species such as *G. brevipalpis* live, the more likely they will be infected with *T. congolense*. On the other hand, other species such as *G. austeni* can be readily infected while a day old and less successfully when a few days old (Jordan, 1976; Woolhouse *et al*., 1994).

Another influential factor could be the infecting parasite itself based on the complexity of the developmental cycle in the tsetse vector. In some field investigations, high prevalence of *T. vivax* infections have been reported in different tsetse populations (Mattioli, 1997). Furthermore, it has been shown that within the same trypanosome species, the capacity of infection depends on the *Glossina* species. According to Moloo and Kutuza (1988), *T. congolense* isolates from different countries became readily established in one fly species and poorly in the other.

Midgut lectins in tsetse are also reported to play a central role in determining susceptibility to trypanosomes. As such, for trypanosomes to survive and become established in the midgut, it is important that the activities of the lectins are inhibited. However, for established trypanosomes, prolonged

inhibition may prove fatal as these trypanosomes will require the activity of the same lectin for maturation to infective forms (Welburn and Maudlin, 1999).

2.3 The causative agents: Trypanosomes

2.3.1 Classification of trypanosomes

Trypanosomes are flagellated protists in the order Kinetoplastida with a relatively simple structure (Marquardt, 1996). They have a worldwide distribution and are known to parasitize all classes of vertebrates including fish, amphibians, reptiles, birds and mammals. The most important forms occur in Africa (Connor and Van den Bossche, 2004).

Trypanosome species infecting mammals fall into two groups divided into two sections. The first section, the Stercoraria, has three sub-genera, *Schizotrypanum*, *Megatrypanum* and *Herpetosoma* that represent trypanosomes produced in the hindgut and passed on by the contaminative transmission from the posteria. The section Salivaria (sub-genera *Dutonella, Nannomonas* and *Trypanozoon*) has its transmission facilitated by anterior station and inoculation (Stevens and Brisse, 2004). The salivarian trypanosome species are the only trypanosomes with the variant surface glycoproteins (VSG) therefore able to exhibit antigenic variation. The most pathogenic trypanosomes belong to the salivarian section (Table 2.1). Of the pathogenic salivarian trypanosomes infecting livestock, *T. congolense*, *T. vivax* and, to a lesser extent, *T. brucei* are regarded as major pathogens of cattle (Aksoy *et al*., 2003). Not all animal species are infected by a given trypanosome species. Certain trypanosomes infect certain animal species with various disease effects (Table 2.1).

2.3.2 The cyclical development of the salivarian trypanosomes

Trypanosomes are extracellular parasites in the blood and tissue fluid of their mammalian hosts, and require a vector to complete their life cycle. This applies for trypanosomes that are transmitted cyclically i.e. *T. congolense*, *T. simiae* and *T. brucei*. Some trypanosomes species such as *T. vivax* or *T. evansi* can be transmitted mechanically and thus do not require a vector for

the completion of their life cycle. *Trypanosoma equiperdum* also does not require a vector since it is transmitted sexually.

Table 2.1: Susceptibility of livestock species to pathogenic trypanosome species (Adapted from Soltys, 1963).

Livestock Species	Trypanosome species						
	T. congolense	***T. simiae***	***T. vivax***	***T. brucei***	***T. evansi***	***T. equiperdum***	***T. suis***
Cattle	+++	+	++	+	+	-	-
Sheep	++	+	++	+++	++	-	-
Goat	++	+	++	+++	++	-	-
Pig	+	-	-	+	-	-	+++
Horse	++	-	++	+++	++	++	-
Camel	++	-	-	++	++	-	-

+++: very susceptible, ++: susceptible, +: less susceptible, -: insusceptible

In the case of cyclically transmitted trypanosome species, the life cycle begins when the tsetse fly ingests blood stream forms (trypomastigotes) while feeding on an infected host. Subsequent to ingestion, the majority of these blood stream forms do not survive the harsh internal conditions presented by tsetse fly because of interactions with specific lectins in the insect's digestive tract (Uilenberg, 1998). Surviving trypomastigotes develop into long slender forms called epimastigotes which undergo further multiplication before they develop into infective metatrypanosomes (Vickerman *et al*., 1988). Within the fly, trypanosomes undergo cycles of development and replication in the alimentary tract and salivary gland depending on the trypanosome species involved (Table 2.2) (Aksoy, 2003). *Trypanosoma vivax* develops exclusively in the mouth parts while *T. brucei* and *T. congolense* infections are first established in the fly midgut. Subsequent maturations for *T. brucei* occur only in the salivary glands while those of *T. congolense* occur in the mouth parts (Roditi and Lehane, 2008).

Upon the bite by an infected tsetse fly, infective metatrypanosomes are injected into the mammalian host during blood meal intake. The parasites develop and multiply locally in the skin at the site of infection and may result in a skin reaction called the chancre (Dwinger *et al*., 1992; Tabel *et al*., 2000). It takes on average 5.5 days after inoculation for the chancre to show (Dwinger *et al.*, 1992). Eventually the parasite enters the blood circulation via lymph vessels and remains confined to the blood circulation throughout the infection of the host (Tabel *et al*., 2000).

Table 2.2. Classification of salivarian trypanosomes and their developmental sites in tsetse vectors (Adapted from Stevens and Brisse, 2004).

Sub-genus	Species/ Group	Development in tsetse
Nannomonas	*Congolense* group:	Midgut and proboscis
	T. congolense	
	T. simiae	
	T. godfreyi	
Duttonella	*Vivax group:*	Proboscis only
	T. vivax	
	T. uniforme	
Trypanozoon	*Brucei group:*	Midgut and salivary glands
	T. brucei	
	T. evansi	
	T. equiperdum	

2.3.3 Virulence of trypanosomes

Virulence is a variable characteristic of disease-producing micro-organisms (Joshua, 1990) influenced by a variety of factors that determines the severity of the disease. These include both the infecting parasite and the host. According to Taylor and Authié (2004), intra-species variation, especially in stock isolated from distinct geographic regions influences the pathogenicity of the trypanosome. For example, the differences expressed by *T. vivax* infections between East and West Africa, with the West African strain regarded as more pathogenic to cattle (Van den Bossche *et al*., 2011). Studies on *T. congolense* reveal that this is a complex species capable of expressing distinct virulence profiles both in mice and cattle (Bengaly *et al*., 2002a, b; Masumu *et al*., 2006). Variation in the manifestation of the disease due to infection with different *T. congolense* savannah foci reveals three categories of high, moderate and low virulence (Bengaly *et al*., 2002a; Masumu *et al*., 2006). In this regard, the severity of the disease is measured by the percentage of the decline in packed cell volume (PCV), the patent

period (PP) and the median survival time (MST) in infected mice and the survival time replaced by time to treatment in cattle (Bengaly *et al*., 2002a, b; Masumu *et al*., 2006).

A number of experiments conducted in mice to understand host-parasite interactions indicated that some strains of mice such as the Balb/c are more susceptible to infection with *T. congolense* than C57BL/6 strains (Noyes *et al*., 2009; Duleu *et al*., 2004). Considerable information on the expression of the disease caused by *T. congolense* infections on such mice now exists (Taylor and Authié, 2004). In higher vertebrates in Africa, it is well known that wildlife,,e.g. African buffalo, are generally more resistant compared to domestic ruminants and often serve as reservoirs for African trypanosomes. Moreover, different cattle breeds as well as individuals within the breed demonstrate different susceptibilities based on their origin, physical status, nutrition and environment. Generally, exotic imported breeds into tsetse-endemic areas are more severely affected than local genotypes which exhibit a range of individual susceptibility (Taylor and Authié, 2004).

2.4 Trypanosomosis (Nagana)

2.4.1 Disease characteristics

In livestock, trypanosomosis is characterized by the fluctuating presence of parasites in the blood, pyrexia, anaemia, loss of body condition, enlarged lymph nodes and lethargy in affected animals. Reduced productivity is often encountered and high mortalities are likely if animals are not treated in time (Paling *et al*., 1991; Connor and Van den Bossche, 2004). Clinical presentations range from chronic to acute symptoms; however, the range and the pathological effects are determined by the host and the parasite strain (Connor and Van den Bossche, 2004; Steverding, 2008).

Anaemia is a well-established infection-associated immunopathological feature of trypanosomosis where the degree of anaemia becomes a reliable indicator of the severity of infection (Stijlemans *et al*., 2008). In cattle,

anaemia is the key feature of the disease and persists after the parasite numbers have declined to low or undetectable levels i.e first wave of parasitaemia. Anaemia rather than parasitaemia is used as the primary indicator of when to treat the infection whereby clearing the parasites usually resolves the condition (Trial *et al*., 1992).

2.4.2 Disease diagnosis

Several techniques used to diagnose trypanosomosis include the traditional method of identifying trypanosomes in the blood using microscopy (Murray *et al*., 1977) (Figure 2.7). Microscopy, used to examine wet blood films and Giemsa-stained thin and thick blood films has its own challenges. This technique has been shown to have low sensitivity when detecting low parasite levels, which is the characteristic of the disease in large animals (Eisler *et al*., 2004).

Concentration of trypanosomes in the buffy coat using centrifugation of capillary tubes containing blood was a method developed by Woo (1970). The buffy coat/plasma interface mounted onto a slide and observed under the microscope using dark ground/phase contrast illumination has increased detection sensitivity. Murray *et al*. (1977) improved the technique by cutting the capillary tube at the buffy coat/plasma interface. This method also enables the investigator to determine the PCV and anaemia. The PCV usually gives an indication of the disease status in affected animals. In the absence of other anaemia-causing factors, low PCV level is an inevitable consequence of an infection with pathogenic trypanosomes in cattle (Van den Bossche and Rowlands, 2001).

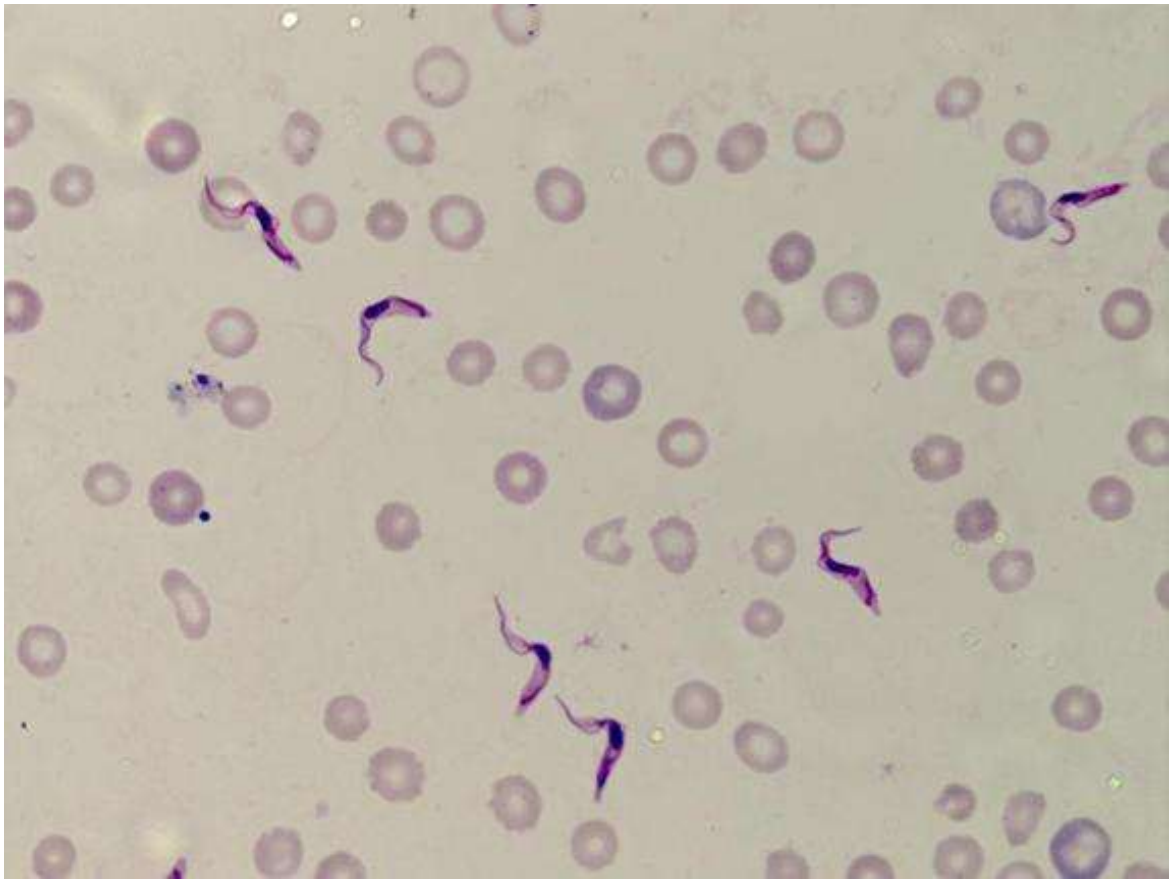


Figure 2.7: Giemsa-stained blood smear showing *T. congolense*, (Photo taken during the work on infected mice in this study).

Because previous methods of diagnosis have their limitations in terms of identifying or demonstrating parasites, more developments were made. Immunoassay methods have wide usage for the diagnosis of trypanosomosis. One of these was the development of the enzyme-linked immunosorbent assay (ELISA) (Nantulya, 1990; Luckins, 1992; Eisler *et al*., 2004). The Ab detection was based on the use of crude antigens (Ag) derived from parasites grown in mice or in tissue culture medium (Luckins, 1997) but was later criticised as being difficult to standardize Ag production and, as such, results varied between batches. Other techniques of Ag detection system were improved by Rebeski *et al*. (1999) for use under field conditions. Generally, these methods also present limitations as the demonstration of the parasites is more important to confirm active or current infections rather than humoral antibodies.

Accurate and definitive identification of trypanosomes requires the most sensitive technique. Molecular methods based on the detection and amplification of nucleic acids such as polymerase chain reaction (PCR) has proved to be the most sensitive for trypanosome detection. Molecular tools often provide another dimension to the diagnosis of the disease that would otherwise be impossible using conventional diagnostic tools (Masake *et al.*, 2002; Traub *et al*., 2005). The revolution of molecular techniques has significantly improved the sensitivity and accuracy of trypanosome diagnosis compared to the traditional parasitological methods. These tools facilitated accurate identification of different trypanosome species, subspecies and strains (Masiga *et al*., 1992). Currently, a single PCR based on internal transcribed spacer 1 of rDNA can now be used to characterise all important bovine trypanosome species (Desquesness *et al*., 2001). Furthermore, species-specific PCR primers help discriminate between species and subspecies (Geysen *et al*., 2003).

FTA card matrices preserve the parasite's DNA in the blood sample by lysing the cells and fixing DNA *in situ* to the filter paper matrix. For large molecular epidemiological surveys, the FTA cards are becoming popular for the ease

and rapid collection and archiving large number of samples. However, due to low level of parasitaemia in livestock in endemic areas the methodology should be used with care. Ahmed *et al*. (2011) found higher sensitivity if the blood was transferred onto FTA cards followed by elution in Chelex®100. A single punch from an FTA card is not sufficient to confirm the infectivity of an animal because the parasites or their DNA is not distributed evenly across the blood spot on the card. Cox *et al*. (2010) reported a higher prevalence of the parasites from repeated sampling from a single spot (86%) as compared to a single punch (9.7%).

A novel technique called loop-mediated isothermal amplification (LAMP) of DNA has been developed (Notomi *et al*., 2000). Several scientists have found this to be a useful tool to detect African trypanosomes with high sensitivity, specificity and rapidity under isothermal conditions with just a simple incubator (Kuboki *et al*., 2003; Thekisoe *et al*., 2007). Based on the high specificity, sensitivity and rapidity in which results can be obtained, this method can be very useful in areas where the application of PCR is not commonly used (Kuboki *et al*., 2003; Njiru *et al*., 2008).

2.4.3 The role of wild animals as reservoir of trypanosomes

The large populations of wild animals, particularly the wild Bovidae, play an important role in the epidemiology of trypanosomosis in Africa. Wild animals carry natural infections of *T. congolense*, *T. vivax* and *T. brucei* parasites. Through natural selection over millions of years these animals survived and managed to co-exist with trypanosomes. They have evolved in the environment of tsetse flies and their associated trypanosomes and are therefore generally more resistant to trypanosomosis (Moloo *et al*., 1999). Trypanosomes infect a wide range of wild animal hosts that serve as reservoirs of pathogenic trypanosomes and are a significant source of blood meals for many species of tsetse (Moloo *et al*., 1999). Within the game park, transmission is maintained between the wild host and the vector thus facilitating a sylvatic transmission (Van den Bossche, 2001). In this situation, trypanosomes of the same sub-species circulate in a restricted area and are

often found to have different virulence and transmissibility (Masumu *et al*., 2006). These authors concluded that such differences are thought to influence the outcome of the disease in different areas where the acute form is mostly observed in livestock exposed to the game parks. Constant encroachment of people and their domestic livestock into tsetse-infested wilderness results in the decrease in the densities of wild animal hosts (Van den Bossche, 2001). As more people increase their settlement, the number of wild animals is reduced and livestock become important diet for tsetse flies. Trypanosomes become domesticated and a domestic cycle is developed between the vector and the livestock (Van den Bossche, 2001; Masumu *et al*., 2009).

2.4.4 Disease control

Effective control of trypanosomosis can be achieved by controlling tsetse flies and the parasite (Aksoy, 2003; Jamal *et al.*, 2005). Of a few examples of successful tsetse eradication is the application of the sterile insect technique (SIT) against *G. austeni* in Zanzibar from 1994 - 1997 through the integration of various control tactics (Vreysen, 2006). Aerial spraying of chemical insecticides DDT and BHC eliminated *G*. *pallidipes* in South Africa (du Toit, 1954). Although DDT was successful in killing pest insects, the chemical was indiscriminate as other non-target organisms were at risk of being contaminated with harmful doses. For example, in a study conducted in Zimbabwe, it has been revealed that potentially harmful total DDT residues were observed in certain animal tissues and eggs of some predatory birds (Matthiessen, 1985). According to Wells and Leonard (2006), adverse health effects of DDT include reproductive and developmental failure, possible immune system effects and the widespread deaths of insects, fish and animals as a result of exposure to the chemical. Where barriers were created, long border parameters with neighbouring countries demanded regular maintenance to control re-infestation. Re-enforcement of game preservation laws resulted in spread of tsetse from one locality to another outside game reserves (Fuller, 1923).

The involvement of the central government in the control of tsetse flies and trypanosomosis has become more and more reduced. Ownership of livestock has been seen as a commercial enterprise thus directing the cost of controlling a disease such as animal trypanosomosis to be borne by the stockowner (Hargrove *et al*., 2000). While methods such as aerial/ ground spraying and sterile insect technique (SIT) may be appropriate to large areas, these methods are inappropriate to local farmers due to a possible re-invasion such that disease levels in cattle remain the same (Hargrove *et al*., 2000). Additionally, the on-going drug resistance problem on existing drugs on the market is not helping either. Based on these facts, control of tsetse and the disease mainly relies on the bait system where flies are attracted to and treated at point source. These systems consist of traps and targets and insecticide treated livestock. So far, these systems have been shown to have the advantage of causing little direct damage to the environment and of being very effective if applied properly in appropriate circumstances (Vale, 1993).

2.4.5 Trypanotolerance

Previous reports have shown that trypanotolerant animals are able to survive and remain productive under active trypanosome transmission in endemic areas (Murray *et al*., 1982). The use of trypanotolerant cattle breeds such as the N'Dama and shorthorn Baoule in an integrated control programme is recommended; however, the degree of tolerance in cattle is not comparable to that of wild Bovidae (Murray and Trail, 1984). In cases where trypanosomosis challenge is very high and is concurrent with nutritional stress, trypanotolerant cattle may develop anaemia and other clinical symptoms which may lead to death in extreme cases (Murray and Trail, 1984). The manifestations of trypanotolerance include the development of less severe anaemia and the intensity, prevalence and duration of the accompanying parasitaemia remain less compared to that observed in susceptible cattle (Murray *et al*., 1990). Therefore, based on this information, it was concluded that the ability to resist anaemia and to control parasitaemia were key indicators of the trypanotolerance trait. The mechanism of trypanotolerence (Murray *et al*., 1982; Akol *et al*., 1986; Pinder *et al*., 1988; Naessens *et al*., 2002) was related

to trypanotolerant N'Dama and Baoule developing smaller and less severe skin reaction (chancres) compared with larger and more severe reactions in the susceptible Zebu cattle at the sites of fly feeding. Trypanotolerant cattle exhibit earlier and superior immune responses and resistant animals appear to exhibit markedly less immuno-suppression of certain T-cell responses than susceptible ones and to respond slightly more efficiently to protective trypanosome antigens. Innate resistance in trypanotolerant breeds comprises at least two mechanisms, an initial stage which limits parasite growth and another involving the haemopoietic system which controls development of anaemia.

2.4.6 Nguni cattle breed

The understanding of natural/innate resistance to trypanosomosis is crucial as such breeds of cattle can be used to improve productivity in tsetse-infested areas. Trypanotolerant cattle constitute an important component in an integrated pest management system (IPM) where eradication of the tsetse flies is not considered as an option. The West African taurine breeds of cattle, namely the N'Dama and Baoule, have been characterized as trypanotolerant (Section 2.4.5). Trypanotolerance is likely a result of long survival in tsetse-infested areas. The West African N'Dama and the Nguni cattle share the same history of their origin. The migration of taurine cattle through the Nile delta to West and southern Africa dates back to 2500 - 6000 B.C. (Epstein and Mason, 1983). Cattle were found in the Luangwa Valley in Zambia by 300 B.C. and by the year 300 A.D. cattle were found in southern Africa with settled African communities including the coastal region of Natal (Bester *et al*., 2003). During the course of southward migration, cattle were exposed to harsher conditions and tropical diseases. Through the process of natural selection, the survivors were genetically adapted to their harsh environment (Bester *et al*., 2003). It is likely that the Nguni cattle possess similar traits of trypanotolerence as the West African taurine.

The Nguni is indigenous to South Africa and is known for its high fertility, short calving intervals and long reproductive lifespan. It is tolerant to tropical

diseases as well as internal and external parasites (Spickett *et al*., 1989) and is also highly adaptable to poor quality grazing and conditions of excessive heat and humidity (Bester *et al*., 2003). This adaptability provides Nguni with the unique potential to produce high quality meat and hides under ecologically controlled freeranging conditions without the use of chemicals (http://www.idc.co.za). Currently there is no available information on the level of tolerance to trypanosome infection in South African breeds of cattle. The fact that there could be some level of resistance in Nguni cattle to tropical diseases should not be overlooked.

2.5 Justification of the study

Glossina austeni and *G. brevipalpis* co-exist in many parts of northern KZN and are regarded as the main transmitters of trypanosomes. Their apparent densities and distribution patterns are reported by Esterhuizen *et al*. (2005) and Hendrickx *et al*. (2003). In South Africa, a survey conducted in the northern part of Hluhluwe-Umfolozi Game Park during 1991 - 1992 assumed that *G. brevipalpis* could be the source of infection for cattle in the farming areas surrounding the reserve (Kappmeier *et al*., 1998). However, the assumption that *G. brevipalpis* was the main vector of trypanosomosis was based on vector abundance and not on data obtained on its vectorial competence. The 1994 trypanosomosis outbreak in Ubombo district changed the perception that *G. brevipalpis* is the most important vector as it was not collected in that area (Kappmeier, 1997). These findings suggested that both tsetse species are implicated in the transmission and therefore, there was a need for a closer look at their capacity to transmit trypanosomes.

Two species of pathogenic trypanosome infecting cattle, i.e. *T. congolense* and *T. vivax*, occur in KZN with the former species being most prevalent (Van den Bossche *et al*., 2006; Mamabolo *et al*., 2009; Gillingwater *et al*., 2010). Genetic variation between *T. congolense* sub-species and the fact that isolates belonging to the same sub-species express different virulence has been shown by Masumu *et al*. (2006). Furthermore, different disease expressions have been associated with the sylvatic and domestic cycles of

transmissions where a more acute form of trypanosomosis resulting in high disease prevalence and cattle mortalities occurs in livestock kept near the game parks (Van den Bossche, 2001). On the other hand, the form of the disease where livestock serves as reservoir, i.e domestic cycle, presents a milder course of infection (Masumu *et al*., 2009) due to domestication of trypanosomes (Van den Bossche, 2001). The research conducted to update the current trypanosomosis situation in KZN lacks information on the level of virulence expressed by trypanosomes circulating in the study area. In South Africa, *T*. *congolense* has been revealed to have two of the four previously reported genotypic types, *T. congolense* Savannah-type and *T. congolense* Kilify-type (Mamabolo *et al*., 2009). Of the two types, *T. congolense* Savannah have been found to be the most predominant and associated with the occasional outbreaks of the disease in endemic areas (Van den Bossche *et al*., 2006). Because *T. congolense* populations circulating in livestock are composed of various genotypes (Masake *et al*., 1988; Kihurani *et al*., 2000; Masumu *et al*., 2009) it is important to determine the number of strains circulating in affected areas. Identification of the primary vector and understanding the virulence profiles of trypanosome strains circulating in KZN will provide a basis for prioritizing control strategies aimed both at the vector and the parasite.

2.6 Objectives of the study

2.6.1 General objectives

The main objectives of this study were to investigate the role of some important factors in the epidemiology of trypanosomosis in cattle in KZN. These included: firstly, the assessment of the vectorial competence of *G. austeni* and *G. brevipalpis* caught in the wild and of tsetse colonies kept and maintained at Onderstepoort Veterinary Institute (OVI) insectaria, and secondly, to characterize by molecular tools and by *in vivo* models *T. congolense* strains isolated from buffalo inside Hluhluwe-Umfolozi Game Park, from cattle kept at the edge of the game park and cattle kept away from the game park.

2.6.2 Specific objectives

1. To assess, under controlled conditions, the vectorial capacity and competence of wild and laboratory-reared *G. austeni* and *G. brevipalpis.*
2. To characterize by molecular tools *T. congolense* strains obtained from buffalo and cattle.
3. To assess the virulence of *T. congolense* isolates collected from buffalo and cattle in KZN using the mouse model.
4. To determine the susceptibility of Nguni and Friesian cattle for *T. congolense* strains previously characterized as of low and high virulence (from objective 3).

CHAPTER 3

Vector competence of *G. austeni* and *G. brevipalpis* for *T. congolense* in KwaZulu-Natal, South Africa

3.1 Introduction

The tsetse zone in South Africa encompasses an area of 18 000 km^2, representing the southern distributional limit of this genus in Africa (Esterhuizen *et al*., 2005). Of the four tsetse species (Diptera: Glossinidae) historically found in South Africa, only *Glossina austeni* (*morsitans* group) and *G*. *brevipalpis* (*fusca* group) still exist in the northern parts of KZN. The flies' habitat is confined to protected nature reserves, game parks, indigenous forests and river beds (Kappmeier *et al*., 1998). The other two species, namely *G*. *m. morsitans* and *G*. *pallidipes*, which were considered to be the most efficient vectors of trypanosomes in general, had been eradicated in 1897 and 1954, respectively (du Toit 1954). *Glossina austeni* and *G. brevipalpis* were not considered to be important vectors of trypanosomosis in South Africa (Fuller, 1923).

Despite the eradication of *G. m. morsitans* and *G. pallidipes*, clinical cases of trypanosomosis were still diagnosed in cattle, horses and dogs between 1955 and 1989 (Bagnall, 1993). In 1990, a serious outbreak of the disease in cattle, locally referred to as Nagana, was reported at dip tanks in the vicinity of the Hluhluwe-Umfolozi Game Park and the infection was attributed to *Trypanosoma congolense* and *T. vivax*. The treatment of cattle with trypanocidal drugs, combined with tsetse control efforts using deltamethrin `pour-on' insecticide and odour-baited insecticide-impregnated targets, was successful in controlling the outbreak (Bagnall, 1993), but the strategy was considered unsustainable because tsetse fly reinvasion could not be prevented (Kappmeier *et al*., 1998). The temporary nature of this approach was evidenced by the recurrence of trypanosomosis, which has reverted to the high levels seen in 1990, before these temporary control measures were instituted (Van den Bossche *et al*., 2006). The recurrence of outbreaks

changed the previous perception that *G. austeni* and *G. brevipalpis* were of minor importance in trypanosome transmission and reaffirmed that they are responsible for the cyclical transmission of Nagana (Kappmeier & Nevill, 1999; Kappmeier-Green *et al*. 2007). Subsequent tsetse surveys conducted from 1991 to 1999 revealed a relatively higher prevalence and abundance of *G. brevipalpis* compared to that of *G. austeni* in the affected areas (Bagnall, 1993; Kappmeier, 2000). Recently, a study conducted at the Mvutshini dip tank found trypanosome infections in 61% of suspected sick cattle using the buffy coat procedure (Van den Bossche *et al*., 2006). The survey indicated that Nagana is still prevalent in KZN and that *T. congolense* was the dominant causative organism. Of the two tsetse species, *G. brevipalpis* was found to be more abundant and therefore believed to be responsible for causing the disease in cattle around the Hluhluwe-Umfolozi Game Park. The importance of a species as a vector is not determined by only its abundance, but also by its vector competence; that is, its ability to become infected and transmit pathogens (Leak, 1999).

There are two key stages in the interaction of some trypanosomes (e.g. *T. brucei* or *T. congolense*) with tsetse flies, namely the initial establishment of infection in the midgut and the subsequent maturation of the trypanosomes to produce infective stages in the salivary glands or oesophagus (Aksoy *et al*., 2003). Trypanosomes undergo a complex life cycle between the mammalian host and the insect vector. The mechanism of refractoriness and susceptibility of tsetse flies to trypanosome infection are not fully understood (Akoda *et al*., 2008). In the epidemiology of African trypanosomosis, the proportion of infected tsetse flies in a population is of considerable importance. The current study was therefore conducted to identify the principal vector of trypanosomes in both field and controlled experimental conditions by comparing the vector competence of *G. austeni* and *G. brevipalpis* in transmitting *T. congolense* to cattle.

3.2 Materials and Methods

3.2.1 Field collection of tsetse flies

Glossina brevipalpis and *G. austeni* were collected between 2006 and 2008 from areas known to be endemic with Nagana in northern KZN (Figure 3.1). Flies were collected using the H-trap designed for collecting these two species (Kappmeier, 2000). Traps were baited with a mixture of 1-octen-3-ol and 4-methylphenol, dispensed from heat-sealed sachets. The traps were emptied daily and flies were transported in a cooler box to the Agricultural Research Council (ARC) Field Research Station at Kuleni. Collected flies were divided into two groups, of which one was immediately used to determine the infection rate and the other group was used in xenodiagnosis experiments. Flies were couriered to the ARC-Onderstepoort Veterinary Institute (OVI) laboratory, situated 600 km from the collection areas.

3.2.2 Determination of infection rate in field tsetse flies

A total of 442 *G. brevipalpis* and 40 *G. austeni* flies were dissected immediately after collection and examined for trypanosome infections. An additional 195 *G. brevipalpis* specimens were dissected after transmission experiments to confirm the results of the infectivity testing in cattle. *Glossina austeni* numbers fed on susceptible animals ranged between 6 and 20. Most of the flies died before they could be used, probably due to stress of transportation and adaption to new environment. Dissections were conducted according to the method of Lloyd and Johnson (1924) and described by Leak *et al*. (2008). Flies were dissected to expose immature infections (non-infective trypanosome forms) in the midgut and mature infections (infective trypanosome forms) in the proboscis (Jordan, 1976; Van den Bossche *et al*., 2004). The respective organs were placed onto microscope slides containing droplets of phosphate buffered saline with glucose (PSG) and covered with cover slips.

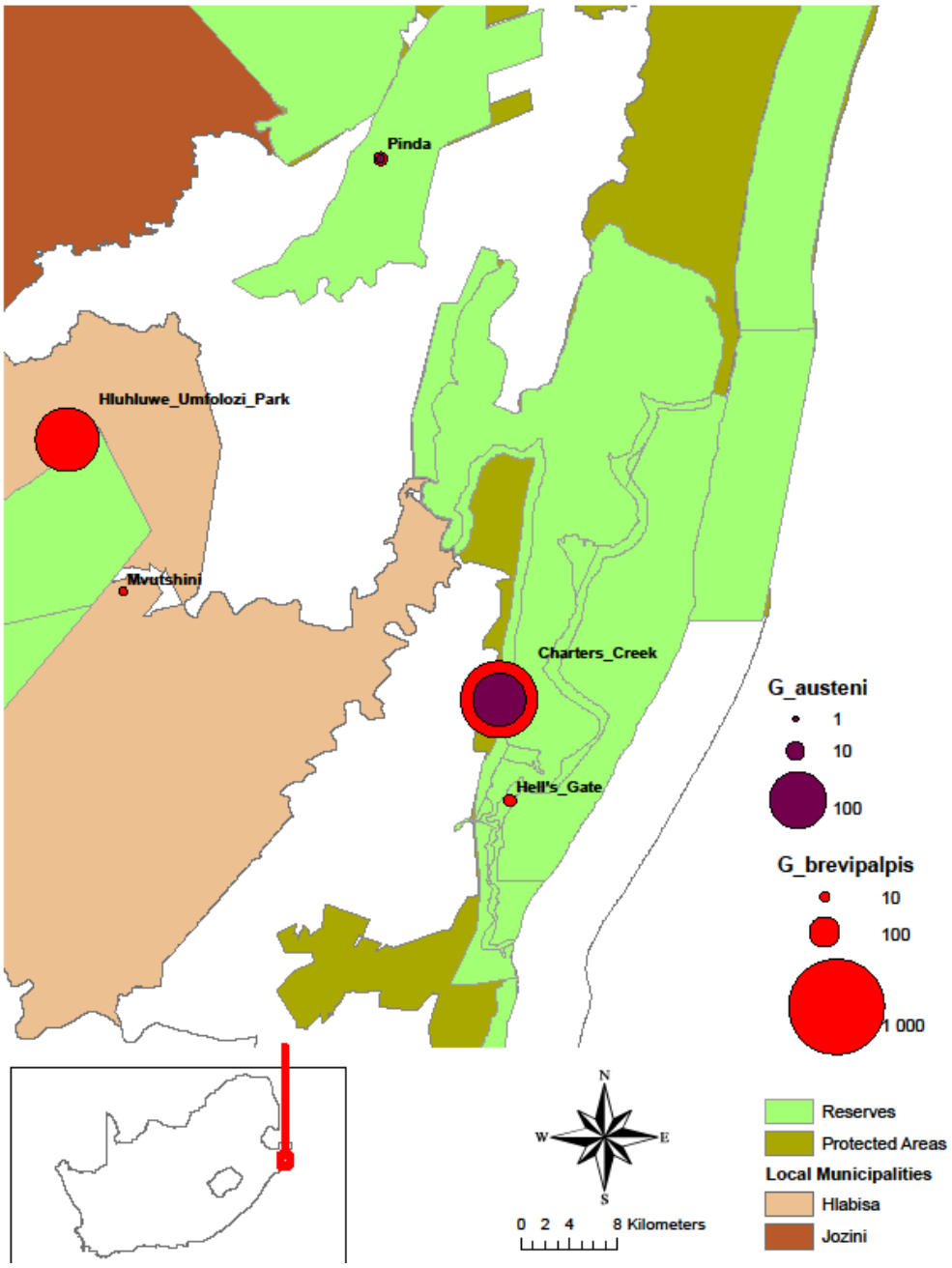


Figure 3.1: Location of sites for the collection *G. brevipalpis* and *G. austeni*. On the legend, the purple and the red circles represent the number of *G. austeni* and *G. brevipalpis* flies caught at a particular site ranging between 1 and 100 and 10 and 1000 respectively.

Infection rates were determined by direct observation of prepared slides under a compound microscope using a 10X eyepiece and a 25X objective.

3.2.3 Experimental infection and monitoring of susceptible animals using field flies

To demonstrate the infectivity of field-collected flies in cattle, 10 batches of *G. brevipalpis* (699 flies in total) and four batches of *G. austeni* (49 flies in total) were used in the transmission experiments. The batch sizes were determined by the number of flies caught in an area on a specific date. For each area, the number of collections ranged from 16 to 180 and 6 to 40 for *G. brevipalpis* and *G. austeni*, respectively. Flies were fed on animals as mentioned in Section 3.2.5. Where the number of flies per batch exceeded 20, extra cages were used. Each batch was allowed to feed on a susceptible Nguni bovine, except for one batch of *G. austeni*, which was fed on a goat. The animals were monitored daily for the development of trypanosome parasitaemia, fever and PCV over 30 days. Blood was collected from the tail vein into heparinised capillary tubes. Capillary tubes were spun in a haematocrit centrifuge machine for 5 minutes to separate the plasma, the buffy coat and the red blood cells. One of the spun haematocrit tubes was used to estimate the PCV on the haematocrit reader for each animal. After estimation of the PCV, the same tube was cut 1 mm below the buffy coat zone and extruded on to the microscope slide, covered with a cover slip and examined by dark-ground phase contrast illumination (Murray *et al*., 1977). Trypanosome scores were determined according to Paris *et al*. (1982). An animal was declared parasitologically negative when at least 50 microscopic fields were observed and no parasites were detected. Animals were considered anaemic and required treatment if PCV dropped to 18% for 3 consecutive days. Animals received treatment with 3.5 mg/kg diminazene aceturate (Berenil ^{TM}).

3.2.4 Colony tsetse flies

The ARC-OVI houses well-established colonies of *G. austeni* and *G. brevipalpis*. These colonies were established in 2002 with seed materials

of *G. austeni* obtained from the Tsetse and Trypanosomosis Research Institute in Tanga, Tanzania, and *G. brevipalpis* supplied by the Insect Pest Control Sub-Programme of the International Atomic Energy Agency (IAEA) laboratories in Seibersdorf, Austria. Colony flies were maintained at 75% relative humidity and 24 °C and fed on artificial membranes using high-quality gamma-irradiated bovine blood according to the standard operating procedure of the FAO/IAEA (2006). The field-collected flies were also maintained under the same conditions as the colony-reared flies before being fed on susceptible animals.

3.2.5 Experimental infection of colony flies using infected animals

The artificial infection of colony flies was carried out to compare the results with the infection rate in field-collected flies. Each of four susceptible cattle was infected intravenously with (1×10^6) parasites using four different *T. congolense* isolates. Three of the isolates (I, II and BmrngK2) were isolated from cattle at Boomerang Farm and the fourth (MVU10) from cattle at the Mvuthsini Community dip tank. All isolates were maintained in mice. Molecular characterisations of all four isolates were performed according to the method described by Geysen *et al*. (2003). The infected cattle were monitored daily for the development of trypanosome parasitaemia and fever, whilst PCV values were also determined. Flies were fed on cattle when parasites were detected in the buffy coat preparations. Experimental cattle were clipped on their flanks and localised areas shaved with a scalpel blade to expose the skin as shown in Figure 3.2 (Akol & Murray, 1983). Prior to feeding on infected animals, flies were kept in the tsetse colony on holding trays as shown in Figure 3.3. Flies were confined in cages in groups of not more than 20 and allowed to feed until fully engorged (Figure 3.4 and 3.5). Thereafter, engorged flies were maintained on artificial feeding membranes for 21 days before dissection (as described for the field collected flies, Section 3.2.2).

Figure 3.2: Nguni calves shaved on their back in preparation for tsetse fly feeding.

Figure 3.3: Tsetse colony of *G. austeni* and *G. bervipalpis* kept at the ARC-OVI: used for vector competence study.

Figure 3.4: Feeding of tsetse on infected cattle.

Figure 3.5: Engorged tsetse flies following feeding on cattle.

3.2.6 Statistical analyses

Variation in infection prevalence between isolates and tsetse flies was analysed using the statistical program GenStat® (Payne *et al*., 2007). Testing was done at the 5% significance level, with $p < 0.05$ used as the cut-off for statistical significance.

3.2.7 Ethical considerations

Animal ethics approval for the experiments was obtained from the Animal Ethics Committee of the OVI Ref. (07/20/C174) and Animal Use and Care Committee of the University of Pretoria, Faculty of Veterinary Science, Ref. (VO56-09).

3.3 Results

3.3.1 Infection rate in field-collected flies

A total of 637 *G. brevipalpis* and 40 *G. austeni* specimens collected from the field were dissected and their infection prevalence assessed (Table 3.1). About 7.5% of immature and mature trypanosome infections were found in the midgut and proboscis of *G. austeni*. On the other hand, only 0.7% of immature infections were found in the midgut of *G. brevipalpis* and none in the proboscis.

3.3.2 Infectivity of field flies in susceptible animals

Assessment of vector competence was conducted to confirm the results of the infection rates with trypanosomes in *G. brevipalpis* and *G. austeni* collected from game parks and communal dip tanks in KZN (Table 3.2). The feeding of flies in the 10 batches (total 699) of *G. brevipalpis* (as many as 180 per animal) did not produce any infections in susceptible bovines. On the other hand, transmission with *G. austeni* was achieved with all the 4 fly batches in 3 bovines and a goat. Trypanosomes were detected in buffy coat samples within 14, 17 and 22 days for cattle and 15 days for the goat. All animals received treatment subsequent to clinical signs of anaemia.

Table 3.1: Prevalence of trypanosome parasites in midgut and proboscis of field-collected *G. austeni* and *G. brevipalpis.* N (sample size). *P*-value < 0.05 were used as the cut-off for statistical significance.

	G. austeni	***G. brevipalpis***	***P*-value**
	40	637	
Number (%) of flies with immature infection (midgut)	3 (7.5%)	5 (0.7%)	0.009
Number (%) of flies with mature infection (proboscis)	3 (7.5%)	0 (0.0%)	<0.001

Table 3.2: Infectivity of *G. austeni* and *G. brevipalpis* collected from different field sites and fed on susceptible hosts under controlled conditions. PPP (pre-patent period), (* Treatment day with Berenil™ after PCV dropped to 18% for 3 days).

Cattle ID	Infection date	Fly species	Collection site	Number Fed	PPP (days to treatment)*
8306	26-Nov-06	*G. austeni*	C. Creek	9	22 (64) *
8461	28-Jan-07	*G. austeni*	C. Creek	14	17 (46) *
755	19-Nov-08	*G. austeni*	C. Creek	6	14 (58) *
8024 (goat)	28-Oct-06	*G. austeni*	C. Creek	20	15 (35) *
8383	15-Jul-06	*G. brevipalpis*	C. Creek	40	no infection
8379	15-Jul-06	*G. brevipalpis*	Hluhluwe	40	no infection
8269	26-Nov-06	*G. brevipalpis*	C. Creek	100	no infection
8340	26-Nov-06	*G. brevipalpis*	Hluhluwe	120	no infection
8462	28-Jan-07	*G. brevipalpis*	C. Creek	54	no infection
8465	28-Jan-07	*G. brevipalpis*	Hluhluwe	50	no infection
8467	28-Jan-07	*G. brevipalpis*	C. Creek	64	no infection
751	01-Aug-08	*G. brevipalpis*	Hluhluwe	35	no infection
755	01-Aug-08	*G. brevipalpis*	C. Creek	180	no infection
756	01-Aug-08	*G. brevipalpis*	Hell's Gate	16	no infection

3.3.3 Xenodiagnoses using colony flies

To confirm the infection rate results obtained from the wild tsetse flies, laboratory-controlled fly infectivity experiments were conducted. A total of 534 colony-reared *G. austeni* and 882 *G. brevipalpis*, fed on experimentally infected parasitaemic cattle, were dissected to reveal their infection status. Results showed that *T. congolense* parasites from all four isolates (BoomerangI, BoomerangII, BmrngK2 and MVU10) became established in the midgut of both *G. austeni* (19.6% - 33.3%) and *G. brevipalpis* (0.4% - 4.2%) (Table 3.3). The overall average of infected flies, 22.6% (*n* = 121) immature infections and 3.9% (*n* = 21) mature infections were found in *G. austeni* and only 1.9% (*n* = 17) immature infections, were seen in *G. brevipalpis* and no parasites were detected in the proboscis.

When immature infections in the midgut of *G. austeni* were considered, there was no significant difference (*P* = 0.1582) between the four *T. congolense* isolates. However, there was a significant difference (*P* = 0.0254) in the immature infection prevalence of these isolates in *G. brevipalpis*. Higher values of midgut infections were observed from infections with BoomerangI and BoomerangII (4.3% and 2.6%, respectively) than from MVU10 and BmrngK2; 0.4% and 0.5%, respectively, for *G. brevipalpis* (Table 3.4). In contrast, the number of *G. austeni* with mature infections of the four isolates differed significantly (*P* = 0.007). *Glossina austeni* infected with BoomerangI and BoomerangII isolates had a higher infection prevalence (9.5% and 8.2%, respectively) compared to infections with isolates MVU10 and BmrngK2 (2.6% and 1.6%, respectively) (Table 3.4). Mature infections were not detected in the proboscis of any *G. brevipalpis*.

Table 3.3: *Trypanosoma congolense* infection rate in midgut and proboscis of *G. austeni* and *G. brevipalpis* colony flies fed on infected cattle (calculated *P*-values).

		G. austeni	*G. brevipalpis*	
***T. congolense* Isolate**	**Experimental tsetse groups**	***N* (%)**	***N* (%)**	***P*-value**
BoomerangI	Number dissected	63	163	–
	Flies with infection in midgut	21 (33.3)	7 (4.2)	< 0.001
	Flies with infection in proboscis	6 (9.5)	0 (0.0)	< 0.001
BoomerangII	Number dissected	85	274	–
	Flies with infection in midgut	20 (23.5)	7 (2.5)	< 0.001
	Flies with infection in proboscis	7 (8.2)	0 (0.0)	< 0.001
MVU10 (Mvutshini)	Number dissected	193	247	–
	Flies with infection in midgut	42 (21.7)	1 (0.4)	< 0.001
	Flies with infection in proboscis	5 (2.5)	0 (00.0)	0.016
BmrngK2 (Boomerang)	Number dissected	193	198	–
	Flies with infection in midgut	38 (19.6)	2 (1.0)	< 0.001
	Flies with infection in proboscis	3 (1.5)	0 (0.0)	0.119
	Total midgut infections	121 (22.6)	17 (1.9)	–
	Total proboscis infections	21 (3.9)	0 (0.0)	–

Table 3.4: *Trypanosoma congolense* infection prevalence in the midgut and proboscis of infected colony *G. austeni* and *G. brevipalpis* flies. MVU (Mvutshini) and Bmrng (Boomerang) isolates.

		Isolates				
Species	**Stage**	**BoomerangI**	**BoomerangII**	**MVU10**	**BmrngK2**	***P*-value**
G. austeni	immature	33.3%	23.5%	21.8%	19.7%	0.1582
	mature	9.5%[a]	8.2%[a]	2.6%[b]	1.6%[b]	0.0072
G. brevipalpis	immature	4.3%[a]	2.6%[a]	0.4%[b]	0.5%[b]	0.0254
	mature	0.0%	0.0%	0.0%	0.0%	-

[a], higher infections; [b], lower infections

3.4 Discussion

In the present study, dissections of wild flies indicated that only 0.7% of *T. congolense* infections were found in the midgut of *G. brevipalpis* which did not develop to the mature infective stage. In contrast, the infection rate in the midgut and the proboscis of *G. austeni* was significantly higher, with 7.5% detected in both organs. Similarly, results of the dissections of colony tsetse flies showed higher infection prevalence in *G. austeni*, 22.6 and 3.9% compared to 1.9 and 0.0% infections in *G. brevipalpis* in the midgut and proboscis respectively. These results are in accordance with those reported by Gillingwater *et al*. (2010) who found high infection prevalence in the midgut compared to very low infections in the proboscis. According to Gillingwater *et al*. (2010), the predominant tsetse species they collected was *G. brevipalpis*. These authors observed an average tsetse midgut infection rate of 20%.It is nonetheless not clear to which tsetse species the 20% infection prevalence observed in the midgut belonged. However, the 1.6% mature infection in the proboscis could have most probably been observed in *G. austeni*. The percentage of mature infections found in wild tsetse flies is usually extremely low (< 1%) which is in accordance with the study by Gillingwater *et al*. (2010) and the present study. Mamabolo *et al*. (2010) detected trypanosome DNA in 89% of the flies examined but the results did not specify from which species. These findings by Mamabolo *et al*. (2010) are not consistent with our findings and those of Gillingwater *et al*. (2010). A plausible explanation for the presence of trypanosomes in the *G. brevipalpis* could be attributed to contamination during dissections. This was evident when the injected suspension of macerated fly organs did not produce any viable infections in mice but tested positive by PCR (Mamabolo *et al*., 2010). This part of their study will be in accordance with our findings showing that *G. brevipalpis* is a poor vector.

Factors contributing to the observed low infection rate in *G. brevipalpis* from the field are not fully understood. However, previous reports indicate that factors such as age may influence the readiness with which flies can be infected. According to Harley (1966), the longer the female of some fly

species such as *G. brevipalpis* lives, the more likely they are to be infected with *T. congolense* as older flies are more likely to be infected than younger flies (Harley 1966; Jordan 1976; Woolhouse *et al*., 1994). In the present study, the age of the two *Glossina* species collected in the field was not determined. However, it was anticipated that due to the higher population densities of *G. brevipalpis* in areas close to the Hluhluwe-Umfolozi Game Park, where high infection prevalence in cattle persists (Gillingwater *et al*., 2010; Van den Bossche *et al*., 2006), the latter species would be highly infected which was not the case. The H-trap used to capture both tsetse fly species in South Africa seems to work well for *G. brevipalpis* (Kappmeier *et al*., 1999), however, the possibility that the very same trap may be attracting younger uninfected *G. brevipalpis* flies than older infected flies has not been investigated. On the other hand, for transmissibility studies with colony-reared flies, both *G. austeni* and *G. brevipalpis* were fed a day after emergence and both were able to establish infections in the midgut, however, trypanosomes could not develop to maturity in *G. brevipalpis*. It is not known whether the difference in the proportions of infected *G. austeni* and *G. brevipalpis* in this study is due to genetic factors, however, there is evidence that this is possible. Mattioli (1997) sited several authors who investigated and reported large variation in the proportion of trypanosome infected flies in different species of *Glossina*. According to these authors, sensitivity to trypanosome infections in tsetse is genetically modulated amongst *Glossina* species and also amongst individuals of the same species.

Notwithstanding the extremely low densities of *G. austeni* caught in this study, higher infection prevalence was obtained from both the field and colony flies. Furthermore, the feeding of less than ten flies produced severe infections in cattle and a goat and animals had to be treated. Colony-reared teneral *G. austeni* were able to acquire and transmit *T. congolense* which is in agreement with previous work that *G. austeni* can be readily infected when they are one day old and less successfully later (Jordan 1976; Ward 1968). Whether the relatively low numbers of *G. austeni* collected in this study was associated to the low natural population density or the inefficiency of the H-

trap to catch this species still remains to be investigated. However, it is clear from the findings of this study that *G. austeni* is the major vector of trypanosomes in northen KZN. Similar observations were reported by Gaturaga *et al*., (1989) when they collected only 33 flies over a period of one year, despite a high trypanosome infection rate (22%) amongst cattle. They attributed these low catches of *G. austeni*, assumed to be the major vector, to the inefficiency of the biconical traps used.

Interestingly, flies infected with isolates BoomerangI and II exhibited more midgut infections in *G. brevipalpis* and more mature infections in *G. austeni* than those infected with isolates MVU10 and BmrngK2. It seems that both fly species are more susceptible to infection with BoomerangI and II isolates; however, *G. brevipalpis* is refractory to subsequent parasite maturation occurring in the proboscis. Goossens *et al*. (2006) found a very low prevalence of *T. congolense* in cattle (0.8%) on Mafia Island, Tanzania, where *G. brevipalpis* is the only tsetse fly species encountered and widely distributed. They attributed the low prevalence to a combination of factors, such as frequent use of prophylactic treatment of cattle with trypanocidal drugs, a low feeding frequency of *G. brevipalpis* on cattle and the low vectorial capacity of the fly. In contrast, Wilson *et al*. (1972) found the infection rate with *T. congolense* in field-collected *G. brevipalpis* to be about 2% in their study in Uganda. The injection of the infected proboscis collected from these flies produced patent infection in mice and thus demonstrated the ability of *T. congolense* to mature in the proboscis.

3.5 Conclusion

A wealth of entomological data has been collected over the years in KZN, which were used by Hendrickx *et al*. (2003) to produce distribution and prediction maps. However, parallel data on the epidemiology of the disease have not been generated to support the intention of the veterinary authorities to control or eradicate tsetse flies from South Africa. The results from the current study support the findings of Goossens *et al*. (2006) that *G. brevipalpis*, is not the main vector of *T. congolense* in KZN, despite its

higher abundance, whereas *G. austeni* has been shown to have a higher vector competence. Focus should, therefore, be directed towards the control of *G. austeni* in the province whilst more research is still needed to develop more efficient traps to monitor the population dynamics of this species before, during and after control operations.

CHAPTER 4

Molecular characterization and virulence of *Trypanosoma congolense* isolates in mice circulating in cattle and African buffaloes in KZN, South Africa

4.1 Introduction

Virulence can be defined as the ability of a microorganism to produce disease and cause damage to its host. It is measured by the severity of the disease it causes or death. African trypanosomes exhibit a wide range of virulence in the vertebrate host (Joshua, 1990), while the severity of pathological effects are influenced by variables that involve both the host and the infecting trypanosome (Murray and Morrison, 1978; Taylor and Authie, 2004). Some host species, breeds or individuals within the breed exhibit innate resistance to infections with certain species or strains of a trypanosome. Trypanosome infections can become established in a host but with considerable variation in susceptibility (Murray and Morrison, 1978). In mice, inbred strains have been shown to have a range of susceptibility to infection with African trypanosomes (Morrison *et al*., 1978) and Balb/c mice strains are highly susceptible compared to the other mice strains (Duleu *et al*., 2004).

Trypanosoma congolense was associated with recent outbreaks in cattle and found to be more prevalent than the other pathogenic trypanosomes in northern KZN (Van De Bossche *et al*., 2006; Mamabolo *et al*., 2009; Gillingwater *et al*., 2010). Although extensive research is being conducted to understand the ecology of flies in South Africa, information on the different genotypes of *T. congolense* circulating in northern KZN was obtained from only one study (Mamabolo *et al*., 2009). One of the two objectives of this chapter was to characterize different trypanosome isolates into species and of *T. congolense* to genotypic level by molecular tools. Isolates obtained were related to the livestock-wildlife interface in which case samples were collected from cattle at different communal dip tanks neighbouring game reserves, dip

tanks and other locations situated farther away from game reserves and from buffaloes inside the game parks.

The DNA sequence analysis has revealed that *T. congolense* comprises several genotypic groups which were previously designated as strains (Majiwa *et al*., 1985; Majiwa, 1992). The genetic component of *T. congolense* differs among clones and stocks of the species and four *T. congolense* genotypes exist, i.e. the Savannah, Riverine-forest, Kilifi and Tsavo- types (Majiwa, 1992; Majiwa *et al*., 1993; Reifenberg *et al*., 1997). A fifth “new type” found in a relic forest in the Gambia can be added (McNamara *et al*., 1991). These genotypes have also been shown to have different virulence profiles. Bengaly *et al*. (2002b) compared the pathogenecity of three genotypes in cattle. The results of their study indicated that the Savannah-type was the most virulent, the Riverine-forest-type was of low pathogenecity while Kilifi-type was non-pathogenic. The Kilifi-type was also shown in earlier studies to be of low pathogenecity in goats (Paling *et al*., 1987). Currently, the only taxonomic groups identified in both vectors and mammalian hosts in South Africa belong to the savannah-type and the Kilifi-type of *T. congolense* (Mamabolo *et al*., 2009). The second objective of the study was to assess the pathogenecity of different *T. congolense* isolates collected from livestock-wildlife interface as well as from cattle kept farther away from game parks.

4.2 Materials and methods

4.2.1 Collection of blood samples from cattle

Blood was collected from cattle at 3 communal dip tanks neighbouring game reserves (Mvutshini, Ocilwane, Ndumo), one dip tank situated farther away (Nhlanzana), one commercial farm (Boomerang). Cattle were bled from tail veins and blood collected into 10 ml tubes containing EDTA as anticoagulant as indicated in Figure 4.1. To keep the blood as fresh as possible, each labelled EDTA tube containing a blood sample was placed in a cooler box containing crushed fine ice. All samples were transferred to the laboratory within 2 hours of collection for further processing.

Additionally, trypanosomes were isolated from infected blood collected from buffaloes from Hluhluwe-UMfolozi Game Park in KZN. Blood stabilates were obtained from the Faculty of Veterinary Sciences, Department of Veterinary Tropical Diseases, University of Pretoria.

4.2.2 Inoculation of mice with blood collected from the field

Balb/c female mice aged between 6 - 8 weeks were obtained from the South African Vaccine Production (SAVP). They were kept under standard animal housing facilities in an airconditioned room (22 - 23 °C), with a relative humidity of 60 - 70%. Mice were fed mouse pellets and supplied with clean water *ad libitum*. Only blood from cattle in the field which was found infected with trypanosomes by the buffy coat examination was used. Mice were inoculated intra-peritoneally (i.p.) with 0.5 ml of infected blood (Paris *et al*., 1982). Mice were then monitored daily for the development of parasitaemia using wet blood smears. Smears were prepared by snipping 1 mm of the tail tip, allowing a drop of blood to touch the microscope slide. The blood droplet was immediately covered with a cover slip to allow blood to spread evenly underneath and was observed under the light microscope at 40X magnification. The parasitaemia scores were estimated using the formula developed by Herbert and Lumsden, (1976). A slide was considered negative when 50 microscopic fields were examined and no trypanosomes were detected. Mice were declared uninfected when parasites could not be detected for 30 successive days. A drop of blood from each infected mouse with an estimated parasitaemia of 10^7 - 10^8 trypanosomes per microliter was preserved on FTA® Elute micro card for DNA extraction (Section 4.2.3). Furthermore, highly parasitaemic mice (10^7 - 10^8 trypanosomes/ml) were euthanized by i.p. injection of pentobarbitone sodium 200 mg/ml and blood was collected to prepare stabilates for future use.

Figure 4.1: Sampling: Blood collected from the tail vein of cattle into EDTA tubes while cattle were restrained in crush pens.

4.2.3 Preparation of FTA® cards using blood from infected mice

Buffy coats were prepared (Section 4.2.3) from positive mice and applied to labelled FTA® Elute micro cards (Whatman, Schleicher & Schuell, Germany) containing four printed circles that allowed preservation of four samples. However, to avoid contamination each sample was assigned a separate card. The filter papers were then air-dried and stored in sealed plastic bags at room temperature for future use.

4.2.4 Preparation of blood stabilates from infected mice

Infected mice with high parasitaemia were euthanized and, blood was collected from the heart using a 2 - 5 ml syringe and a 21 gauge needle flushed with heparin. On average, 8-12 week old Balb/c mouse would yield 1-2 ml of blood. One part of 20% Dimethyl Sulfoxide (DMSO) was mixed with three parts of the collected infected blood. This mix was then dispensed into labelled cryogenic vials (Greiner Bio-One, Germany) in aliquots of 500 µl. Trypanosomes in the cryogenic vials were frozen overnight at -80 °C and later transferred to -180 °C liquid nitrogen until use.

4.2.5 DNA extraction from FTA® cards

DNA was extracted from FTA cards containing the preserved buffy coat spots from parasitologically positive mice. Extraction was performed according to the manufacturer's instructions. Briefly, a 3 mm sample disk was removed from the centre of the circle containing the buffy coat spot with the aid of 3 mm Harris Uni-Core device (Whatman®, Germany). The buffy coat spot was transferred into a 1.5 µl microfuge tube on 500 µl of sterile water. The contents of the tubes were pulse-vortexed three times for five seconds and the disk transferred to a 0.5 ml microfuge tube containing 30 µl of sterile water. The tubes were briefly centrifuged and incubated for 30 minutes at 95 °C on a heating block. After incubation, samples were centrifuged for 30 seconds and the disk was removed from the tube and discarded. The resultant DNA was stored at -20°C.

4.2.6 DNA amplification using the polymerase chain reaction (PCR)

DNA was amplified using a single primer pair that targets the 18S ribosomal sub-unit of all trypanosomes according to Geysen *et al*. (2003). For this amplification, the reaction was carried out in a final volume of 25 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.8 µM of each of the primers and 0.5 U *ExTaq* polymerase (Takara Bio Inc, Japan) and 2.5 µl of eluted DNA. The tubes were placed in the thermocycler and incubated for 4 minutes at 94 °C in the initial denaturation step. This was followed by 40 cycles of 1 minute at 94 °C, 1.5 minutes at 58 °C and 2 minutes at 72 °C.

Additionally, characterization of all isolates into species and sub-species was conducted using oligonucleotide primers that amplify satellite DNA monomers of each species or subgroup (Masiga *et al*., 1992; Sloof *et al*., 1983; Masake *et al*., 1997; Majiwa and Otieno, 1990). A list of primers and their expected product sizes are shown in Table 5.1. For species-specific detection of trypanosomes, the PCR reaction mix contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.4 µM of each of the primers and 0.5 U *ExTaq* polymerase (Takara Bio Inc, Japan) and 2 µl of eluted DNA. Cycling conditions were set as follows: initial denaturation step at 94 °C for 3 minutes followed by 30 cycles of 1 minute at 94 °C, 1 minute at 60 °C and 1 minute at 72 °C. DNA amplifications were carried out in an Eppendorf Mastercycler Thermal Cycler.

Amplified products were resolved by electrophoresis through a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide. Separated PCR products were observed using a gel documenting system (BIORAD, Quantity One 4.6.3) prior to photography and species identified according to their molecular sizes.

Table 4.1: Primer sequences for the amplification of all African trypanosomes (18S rRNA) and those targeting specific satellite regions and their predicted amplification product sizes.

Specificity of primers	**Forward primer**	**Reverse primer**	**Product size (bp)**	**Reference**
African trypanosomes	5'- CAA CGA TGA CAC CCA TGA ATT GGG GA- 3'**(18ST nF2)**	5'-TGC GCG ACC AAT AAT TGC AAT AC- 3' **(18ST nR3)**	750	Geysen *et al*. (2003)
African trypanosomes	5'- CAA CGA TGA CAC CCA TGA ATT GGG GA- 3'**(18ST nF2)**	5'-GTG TCT TGT TCT CAC TGA CAT TGT AGT G- 3' **(18ST nR2)**	650	Geysen *et al*. (2003)
T. brucei	5'- CGA ATG AAT ATT AAA CAA TGC GCA GT- 3'**(TBR1)**	5'- AGA ACC ATT TAT TAG CTT TGT TGC- 3'**(TBR2)**	177	Sloof *et al*. (1983)
T. congolense Kilifi	5'- GTG CCC AAA TTT GAA GTG AT- 3'**(TCK1)**	5'-ACT CAA AAT CGT GCA CCT CG-3' (**TCK2**)	294	Masiga *et al*. (1992)
T. congolense Savannah	5'- TCG AGC GAG AAC GGG CAC TTT GCG A- 3'**(TCN-1)**	5'-ATT AGG GAC AAA CAA ATC CCG CAC A- 3' **(TCN-2)**	341	Majiwa *et al*. (1993)
T. vivax	5'- TCG CTA CCA CAG TCG CAA TCG TCG TCT CAA GG- 3' **(TVMF)**	5' CAG CTC GGC GAA GGC CAC TTG GCT GGG GTG- 3' **(TVMR)**	400	Masake *et al*. (1997)

4.2.7 Assessment of virulence of *T. congolense* strains

Trypanosoma congolense stabilates were removed from liquid nitrogen and allowed to thaw at room temperature before mouse inoculation. About 250 µl of each isolate was injected i.p into each of the two mice. Infected mice were monitored for clinical symptoms and parasitaemia of trypanosomosis (Section 4.2.2). When the parasitaemia was estimated to reach 10^7 - 10^8 trypanosomes/ml in infected mice, tail-blood was extracted and diluted into PSG to achieve a concentration of 10^5 parasites in a total volume of 0.2 ml. This volume was injected i.p. in six Balb/c mice for each isolate. An additional group of six mice used as negative control was injected i.p. with 0.2 ml of PSG.

Virulence categories were determined according to the criteria adopted from Bengaly *et al*. (2002a) and Masumu *et al*. (2006). Twenty-one cattle-derived and five buffalo-derived *T. congolense* isolates were characterized for virulence in mice. Isolates were classified as strains following their different virulence profiles. Strains were divided into four categories of high, moderate, low and non-virulent profiles based on the pre-patent period (PPP), days from infection to first identification of parasites in the blood; the patent period (PP), the period during which trypanosomes can be detected in the blood of infected mice during the course of the experiment and the median survival time (MST), i.e. time from treatment at which half of the infected mice that developed parasitaemia had died. In addition, the progression of parasitaemia and the development of anaemia as judged by the PCV percentage were recorded. Parasitaemia was recorded daily for the first two weeks and thereafter every two days. Parasitaemia scores were estimated according to the protocol of Herbert and Lumsden, (1976). Death was recorded every day for all experimental and control groups. For PCV, blood was collected from all mice before infection and every second day for the first two weeks and once a week for up to 90 days post infection (p.i.). Blood was collected from the tail into heparinised haematocrit centrifuge tubes and the PCV measured using the haematocrit reader.

Different virulence profiles of each trypanosome strain were categorised based on the PPP, PP, percentage drop in PCV and MST of infected mice in each group. Strains with the survival time of less than 14 days (two weeks) were considered extremely virulent while those with a survival time of more than two weeks up to a month were regarded as moderately virulent. Those that had a survival time of more than 30 days were characterised as low virulent strains. Not all mice developed parasitaemia in some groups therefore when trypanosomes were not detected in one or two mice in a group, the MST was considered for all parasitaemic mice in that group. Similarly with the low virulent strain-treated mice, when parasites were not detected in wet blood smears for up to 30 days p.i., only the MST of the parasitaemic mice in that group were considered. In addition, the maximum average parasitaemia of each group was recorded at the end of each experiment. Furthermore, a percentage drop in PCV was calculated by subtracting the final PCV value at the end of the experiment from the initial PCV value at the onset of the experiment per group. The resultant difference was then divided by the initial PCV value and multiplied by 100 to get the percentage drop.

4.3 Results

4.3.1 Molecular identification of *Trypanosoma* isolates

A total of 30 isolates were infective to mice and successfully recovered; four of these could not survive the second passage in mice. Results of the DNA amplification using a single primer pair that targets the 18S ribosomal sub-unit gave major bands between 800 and 900bp. There was an observable size difference between the Kilifi and Savannah controls indicated in Figure 4.2 on lane 9, 10; 19, 20 and 29, 30 respectively. Based on this figure, different isolates already gave profiles that corresponded to either of the two controls. For example, isolates in lane 1 - 7 were in line with the Savannah control in lane 10 while sample in lane 16 - 18 aligned with the Kilifi control in lane 19. Products were run against a 100 bp molecular marker, O'GeneRuler from Fermentus. Known Kilifi (OVIKZNTT/7098/07) and Savannah (*T. congolense* IL1180) were used as controls.

To confirm the identity of the *Trypanosoma* DNA amplified with the 18S rRNA primer pairs, an additional amplification was conducted using primers complementary to the conserved region of trypanosomal small subunit ribosomal RNA (ssu rRNA) genes to amplify variable segments of the gene. Amplification of DNA samples using species-specific PCR primers for the Savannah-type*T. congolense* (Majiwa *et al*., 1994), gave major bands around 350 bp for 19 and five isolates from cattle and buffaloes respectively. The results of the products obtained with Savannah-specific primers are illustrated in Figure 4.3. Amplification of samples that yielded products with the Savannah-specific primers did not give any products when the Kilifi-type*T. congolense*, *T. brucei, T. equiperdum* and *T. vivax* specific primers were used. However, amplification with the Kilifi-specific primers gave products of 300 bp for samples BmrngK4, BmrngK9 and BmrngK5 and the three others (BmrngK6, 7 and 8), which were not amplified with savannah-specific primers in the previous run.

Other than the controls for each specific primer set, no products were obtained when the samples were amplified with *T. vivax*, *T brucei* and *T. equiperdum*-specific primers. However, *T. brucei* oligonucleotide primers amplified *T. equiperdum* control DNA. Both controls had a product size of about 180 bp (results not shown).

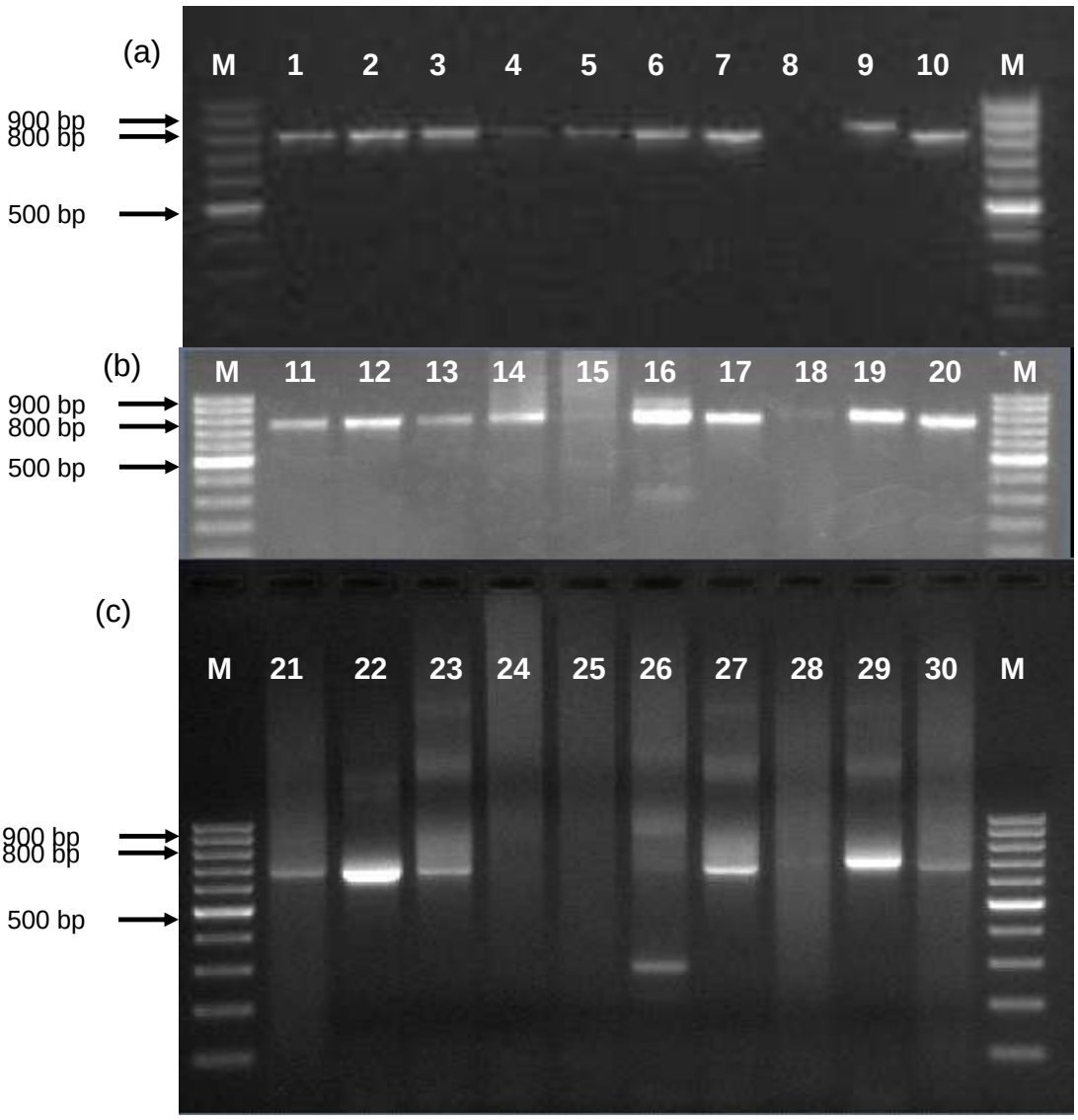


Figure 4.2: A 1.5% ethidium bromide-stained agarose gel image showing results of the PCR products following amplification of some *Trypanosoma* species DNA samples with the 18S rRNA primers (18ST nF2 and 18ST nR3). The figure is sub-divided into three panels represented by (a), (b) and (c).The DNA of different trypanosomes was loaded in each lane as follows: M, 100 bp molecular marker; 1-30: 1, BmrngK1; 2, BmrngK2; 3, BmrngK3; 4, NDMH2; 5, NDMT1; 6, NJB1; 7, OCI; 8, H_2O, 9, 7098 Kilifi; 10,IL1180 Savannah; 11, MVU1; 12,MVU2; 13, MVU3;14, MVU4; 15, NJT1; 16, NJT2; 17, BmrngK4, 18, BmrngK5, 19, 7098 Kilifi, 20, IL1180 Savannah; 21, MVU5; 22, MVU6; 23, MVU7; 24, BmrngK6; 25, BmrngK7; 26, MVU8; 27, MVU9; 28, MVU10; 29, 7098 Kilif and 30, IL1180 Savannah.

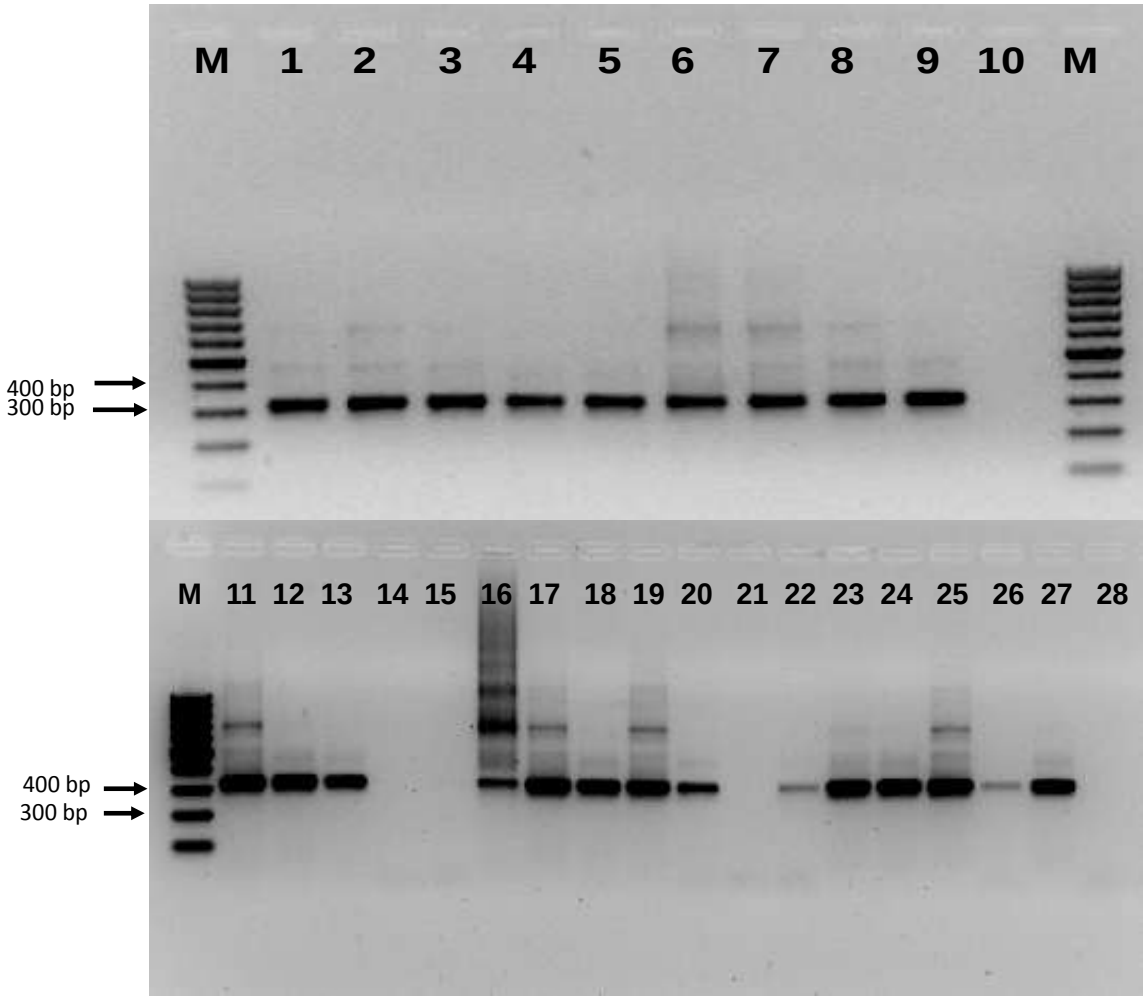


Figure 4.3: Ethidium bromide-stained gel photo following DNA amplification of some Trypanosoma species DNA with TCN-1 and TCN-2 Savannah-specific primers. Lane M, 100 bp molecular marker; 1, BmrngK1; 2, BmrngK2; 3, BmrngK3; 4, NDMH2; 5, NDMT1; 6, NJB1; 7, OCI; 8, TCIL1180(0.01ng/µl); 9, TCIL1180 (0.1 ng/µl); 10, 7098 Kilifi; M, Marker; 11, BmrngA1; 12, BmrngK2; 13, BmrngK3; 14, BmrngK4; 15, BmrngK9; 16, MVU1; 17, MVU2; 18, MVU3; 19, MVU4; 20, MVU5; 21, BmrngK5; 22, MVU6; 23, MVU7; 24, MVU8; 25, NJT1; 26, MVU9; 27, MVU10 and 28, NJT2.

4.3.2 Virulence characterization of *Trypanosoma congolense* strains in mice

The averages of PPP, maximum parasitaemia, percentage drop in PCV, PP and MST for each group are shown in Tables 4.2a, b and c, for HVS, MVS and LVS Savannah sub-type respectively and (d) for the LVS Kilifi sub-type. The overall average of the values of individual mice for each category is also shown.

Table 4.2: Mean and standard deviations of the pre-patent period (PPP), average drop in PCV values (days to drop) , patent period (PP) and median survival time (MST) and maximum parasitaemia calculated for individual groups infected with Savannah-type *T. congolense* (a) high (b) moderately and (c) low virulent strains. (*): buffalo-derived strains; (d): Kilifi-type *T. congolense*.

(a)

Strain	Ave PPP ± stdev	Max para	% drop in PCV (days)	Ave PP ± stdev	Ave MST ± stdev	Category
TCKZN1*	8.8 ± 1.0	8.3	21.3 (11)	4.7 ± 1.8	12.5 ± 2.3	HVS
TCKZN3*	9.5 ± 1.6	8.4	33.5 (12)	5.0 ± 0.9	13.0 ± 0.8	HVS
TCKZN4*	6.7 ± 0.8	8.4	25.6 (7)	4.0 ± 1.7	9.0 ± 1.0	HVS
BMRNG/A1	10.0 ± 0.0	8.2	40.1 (9)	3.0 ± 1.0	13.0 ± 1.0	HVS
MVU2	8.0 ± 0.0	8.4	25.5 (9)	5.0 ± 1.0	12.0 ± 1.0	HVS
NDMT1	5.0 ± 1.0	9.0	41.7 (7)	7.0 ± 3.0	10.0 ± 3.0	HVS
MVU5	5.0 ± 1.0	8.4	27.5 (7)	7.0 ± 2.0	11.0 ± 3.0	HVS
MVU6	6 .0 ± 2.0	5.6	19.1 (7)	2.0 ± 2.0	6.0 ± 0.0	HVS
MVU7	4.0 ± 1.0	5.6	2.2 (3)	4.0± 1.0	6.0 ± 0.0	HVS
MVU9	6.0 ± 2.0	8.4	48.8 (11)	10.0 ± 3.0	13.0 ± 2.0	HVS
MVU10	6.0 ± 2.0	8.3	55.2 (11)	8.0 ± 1.0	12.0 ± 0.0	HVS
OCI	7.8 ± 0.4	9.0	21.6 (9)	6.0 ± 3.0	12.5 ± 3.0	HVS
Overall Ave.	**6.8 ± 2.3**	**8.0 ± 1.1**	**30.2 ± 14.5 (8.6 ± 2.5)**	**5.4 ± 2.7**	**11.0 ± 2.7**	

(b)

Strain	Ave PPP ± stdev	Max para	% drop in PCV (days)	Ave PP ± stdev	Ave MST ± stdev	Category
TCKZN2*	8.5 ± 3.0	8.7	20.8 (7)	7.0 ± 5.2	14.5 ± 6.4	MVS
TCKZN5*	6.2 ± 1.3	9.0	27.3 (7)	9.3 ± 6.6	14.0 ± 6.5	MVS
MVU1	7.0 ± 0.0	9.0	38.1 (16)	17.0 ± 3.0	22.0 ± 3.0	MVS
MVU8	10.0 ± 4.0	8.0	9.6 (11)	7.0 ± 4.0	17.0 ± 2.0	MVS
BMRNG/K1	15.5 ± 4.0	7.2	9.1 (11)	5.0 ± 2.0	18.5 ± 5.4	MVS
NDMH2	5.0 ± 1.0	8.7	18.5 (14)	17.0 ± 9.0	24.0 ± 3.0	MVS
MVU3	6.0 ± 0.0	8.7	17.5 (13)	22.0 ± 7.0	28.0 ± 7.0	MVS
Overall Ave.	**7.9 ± 3.8**	**8.5 ± 0.6**	**20.1 ± 10.1 (10.7 ± 3.8)**	**11.5 ± 7.3**	**21.0 ± 6.0**	

(c)

Strain	Ave PPP ± stdev	Max para	% drop in PCV (days)	Ave PP ± stdev	Ave MST ± stdev	Category
BMRNG/K2	9.0 ± 0.0	8.7	26.9 (14)	21.0 ± 6.0	30.0 ± 6.0	LVS
NJB1	8.0 ± 1.0	7.5	27.9 (35)	38.0 ± 9.0	43.0 ± 6.0	LVS
BMRNG/K3	12.0 ± 3.0	8.9	25.2 (24)	37.0 ± 13.0	53.0 ± 12.0	LVS
NJT1	7.0 ± 0.0	8.9	25.7 (21)	31.0 ± 7.0	38.0 ± 7.0	LVS
MVU4	8.0 ± 3.0	8.3	23.2 (13)	23.3 ± 11.0	35.0 ± 9.0	LVS
Overall Ave.	**8.7 ± 2.6**	**8.5 ± 0.6**	**25.8 ± 1.8 (21.4 ± 8.9)**	**30.0 ± 11.3**	**36.0 ± 11.2**	

(d)

Strain	Ave PPP ± stdev	Max para	% drop in PCV (days)	Ave PP ± stdev	Ave MST ± stdev	Category
BMRN/K5**	12.3 ± 5.6	5.9	6.2 (69)	10.0 ± 11.0	64.0 ± 0.0	LVS
NJT2**	4.0 ± 0.0	8.3	31.4 (29)	43.0 ± 6.0	48.0 ± 6.0	LVS
Overall Ave.	**8.2 ± 5.8**	**7.1 ± 1.7**	**18.8 ± 17.9 (48.5 ± 29.0)**	**26.4 ± 18.9**	**56.0 ± 10.3**	

Overall, all of the HVS and the MVS strains and 71.4% (5/7) of the LV belonged to Savannah-type *T. congolense*. Two out of 7 (28.6%) which showed LV characteristics belonged to the Kilifi-type of *T. congolense*. The results of this study showed that 42.8% (9/21), 23.8% (5/21) and 33.3% (7/21) of the strains obtained from cattle expressed HV, MV and LV properties, respectively. Sixty (3/5) and 40.0% (2/5) of the buffalo strains expressed HV and MV, respectively. In all, 52.0% (13/25) of the strains were collected from 3 communal dip tanks neighboring game reserves (Mvushini, 40.0%; Ocilwane, 4.0% and Ndumo, 8.0%), one dip tank situated farther away (Nhlanzana, 8.0%), one commercial farm (Boomerang, 20.0%) and from buffaloes (Hluhluwe-Umfolozi Game Park, 20%). Location of the sites is shown in Figure 4.4.

When comparing the results of the HVS, MVS and LVS in terms of the development of parasitaemia, there was no significant difference (P = 0.1828) in the overall mean parasitaemias. Similarly, there was no significant difference (P = 0.2143) between the three categories when the PPP was compared. Once parasitaemia was detected, it continued until all the infected parasitaemic mice were dead in the HVS and MVS categories or the experiment was terminated in the LVS of the Savannah-type *T. congolense*. On the other hand, parasitaemia in the two groups infected with the Kilifi-type *T. congolense* dropped gradually, and then the parasites disappeared and could not be detected in the buffy coat examination.

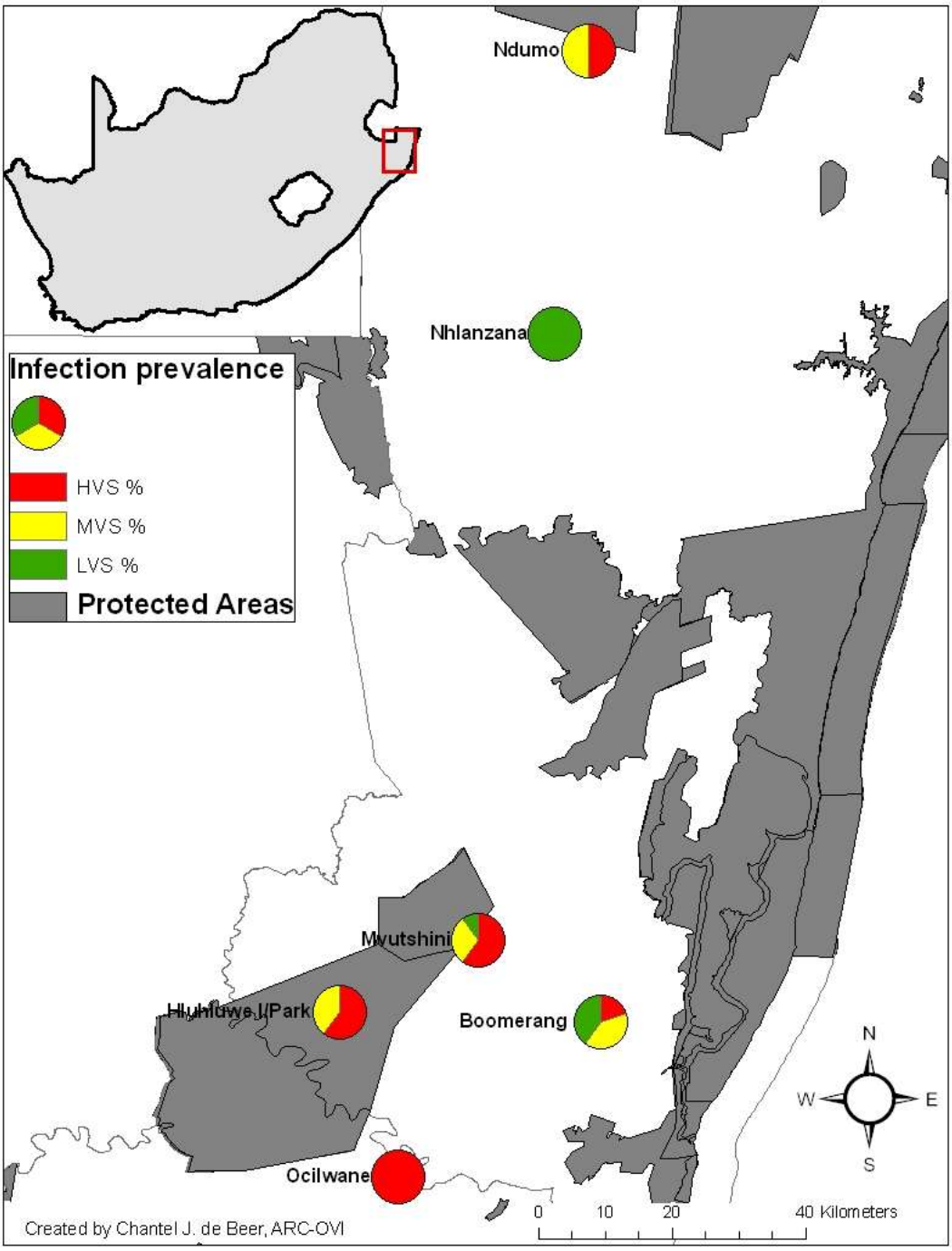
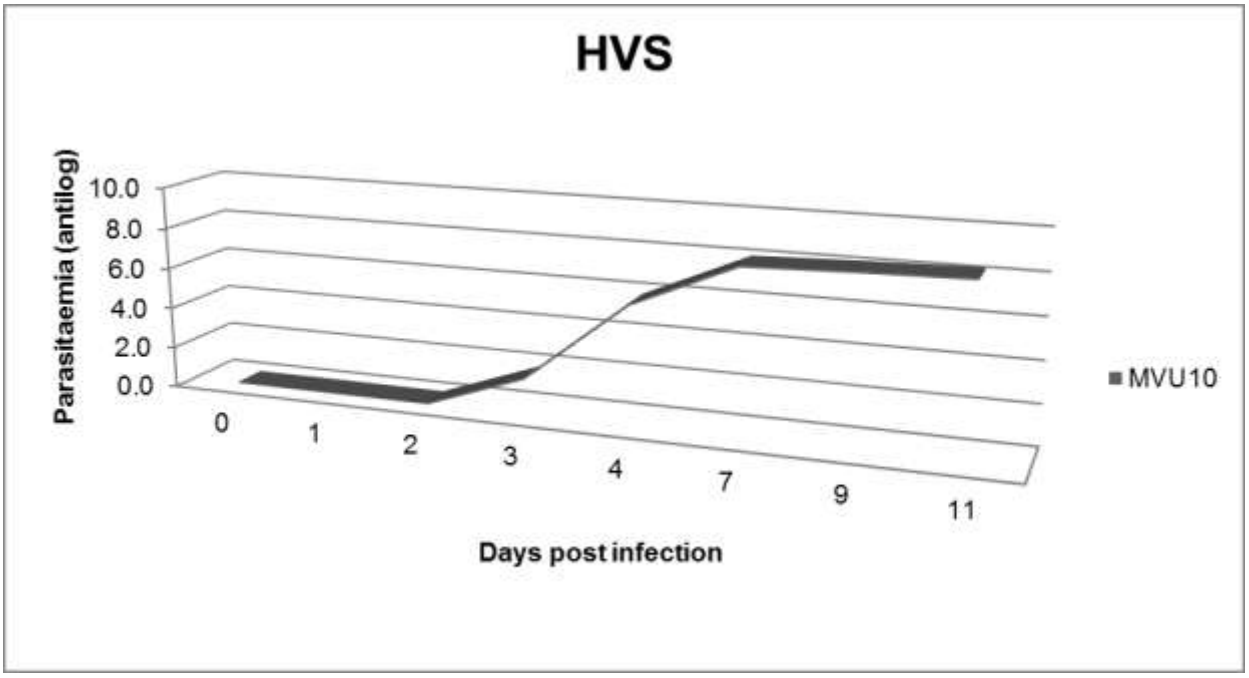


Figure 4.4: Location of the communal dip tanks, game reserves and the commercial farm in the study area and strain categories percentages represented as pie charts.

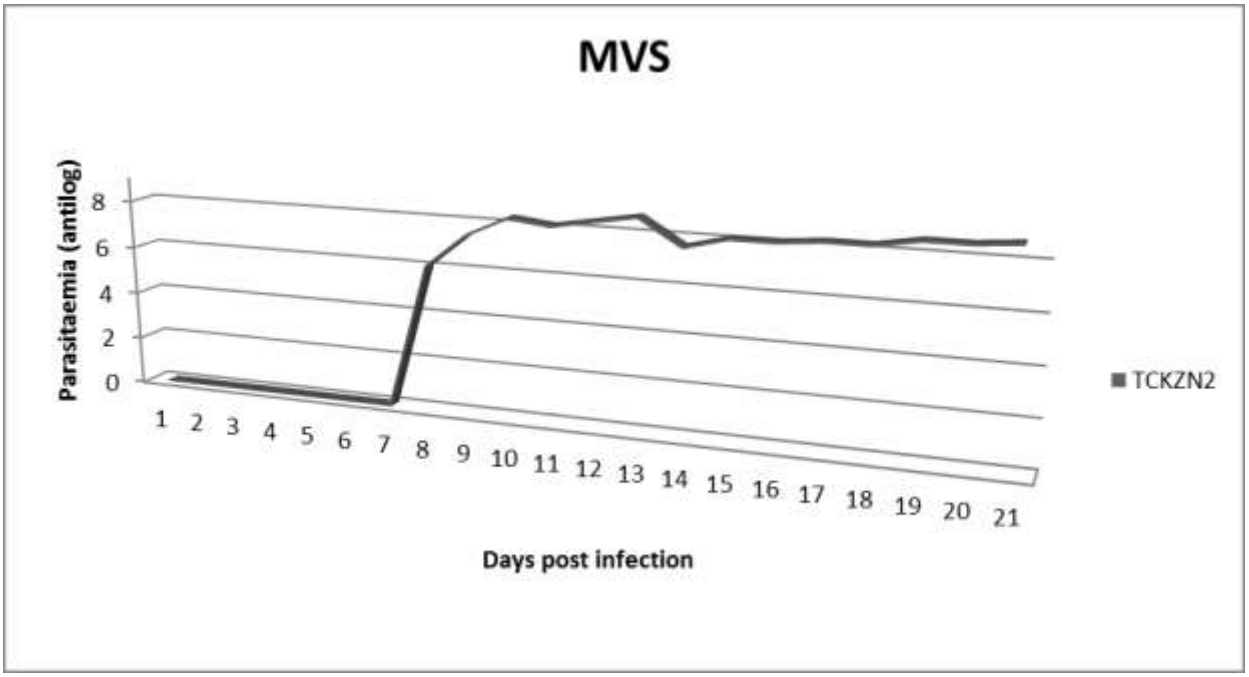
Figures 4.5 a, b, c and d show the trend in development of parasitaemia in 3 representative of the categories. All mice infected with the HVS died when the parasitaemia peak was at maximum level. For mice infected with the MVS, the parasitaemia peak stabilized at the maximum level which extended for a longer duration (up to a month) and all animals died during this phase. On the other hand, some of the mice infected with the LVS maintained high levels of parasitaemia for more than a month which remained detectable until the experiment was terminated. Kilifi-type *T. congolense* maintained low parasitaemia and self-cured for the remainder of the experiment.

The summary of overall means, standard deviations and the *p*-values of virulence parameters for the HVS, MVS and LVS are shown in Table 4.3. The overall means of the PP between the three categories was highly significant ($P < 0.0001$). Comparison of the overall PCV % drop between HVS, MVS and LVS was not statistically different ($P = 0.2114$). However, there was a high significant difference ($P < 0.0008$) in the number of days to effective anaemia in the three virulence categories. The duration to PCV percentage drop was 8.6, 10.7 and 29.0 in the HVS, MVS and LVS, respectively. The differences in the MST between the HVS (12 days), MVS (21) and LVS (44) were highly significant ($P < 0.0001$). There were no significant differences in PPP, maximum parasitaemia and percentage drop in PCV between the mice in the three virulent categories.

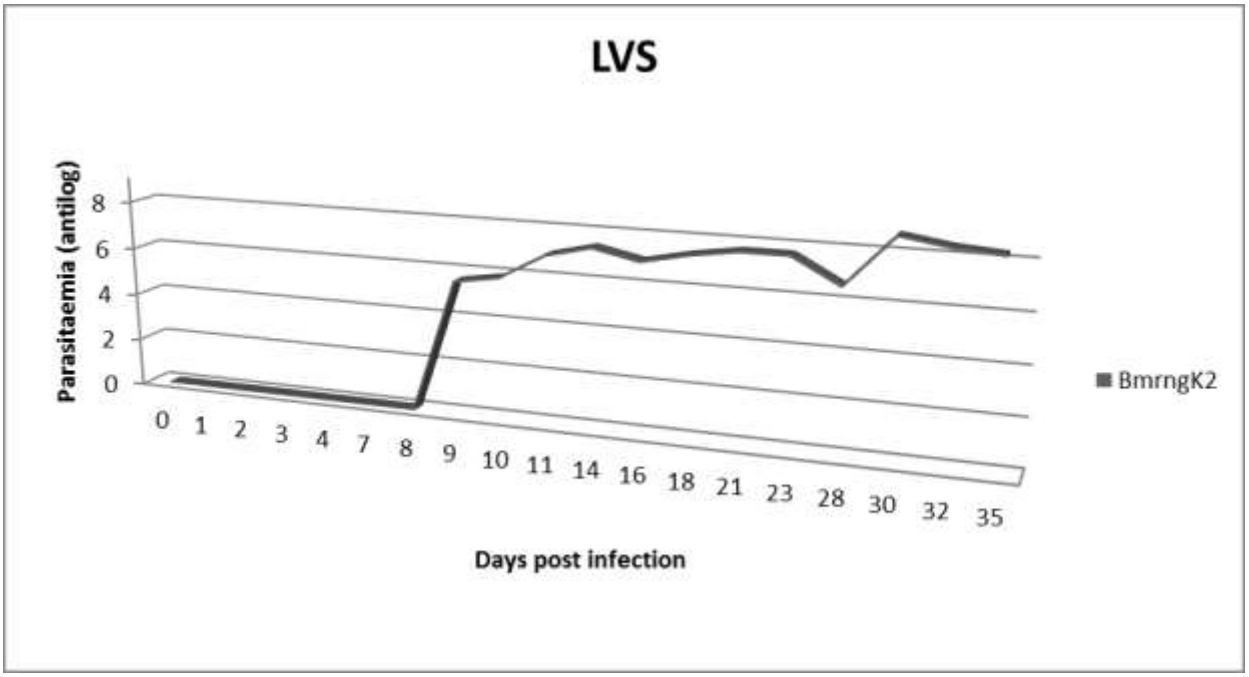
(a)



(b)



(c)



(d)

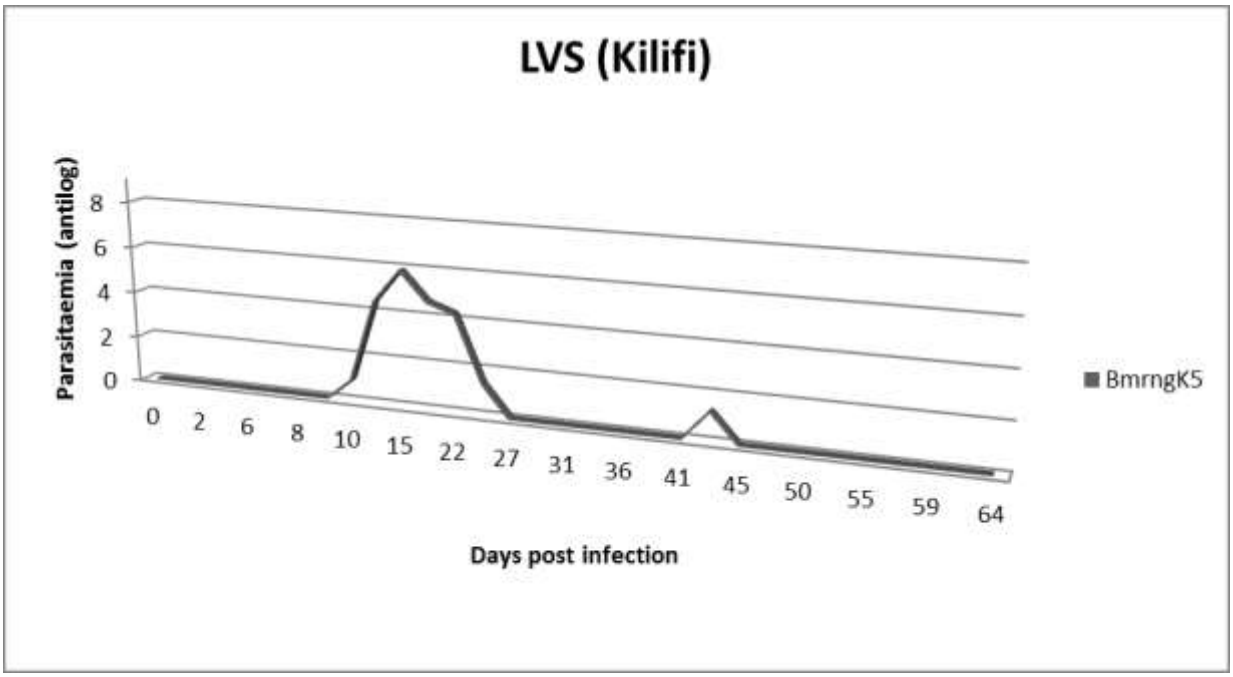


Figure 4.5: Trends in parasitaemia in the 3 *T. congolense* Savannah and the Kilifi -type.

Table 4.3: Overall average, standard deviation and *p*-values for the pre-patent period (PPP), maximum parasitaemia, percentage drop in PCV, days to drop in PCV, patent period (PP), and median survival time (MST) for HVS, MVS and LVS Savannah-type *T. congolense* and LVS for Kilifi-type *T. congolense*.

	Overall mean ± Stdev				
Parameters	**HVS**	**MVS**	**LVS**	**LVS (Kilifi)**	***P*-value**
PPP	6.8 ± 2.3	7.9 ± 3.8	8.0 ± 4.3	8.2 ± 5.8	0.363
Max Parasitaemia	8.0 ± 1.1	8.5 ± 0.6	8.0 ± 1.2	7.1 ± 1.7	0.2747
% drop in PCV	30.2 ± 14.5	20.1 ± 10.1	23.8 ± 8.2	18.8 ± 17.9	0.3791
Days to drop in PCV	8.6 ± 2.5	10.7 ± 3.8	29.1 ± 19.2	48.5 ± 29.0	< 0.0001
PP	5.4 ± 2.7	11.5 ± 7.3	29.2 ± 12.4	26.4 ± 18.9	< 0.0001
MST	11.0 ± 2.7	21.0 ± 6.0	44.0 ± 13	56.0 ± 10.3	< 0.0001

4.4 Discussion

Pathogenic trypanosomes affecting cattle, i.e. *T. congolense* and *T. vivax* were recently identified from cattle, wild animals and tsetse flies in norther KZN (Van den Bossche *et al*., 2006; Mamabolo *et al*., 2009; Gillingwater *et al*., 2010). Of the two trypanosome species, *T. congolense* has been implicated to play a significant role in the epidemiology of cattle trypanosomosis. The sub-inoculation of mice in the current study was conducted to successfully propagate *T. congolense* isolates and possibly eliminate *T. vivax* as the wild isolates of this species has failed to develop and establish in mice (Eisler *et al*., 2004). The trypanosomes recovered from mice belonged to the *Nannomonas* subgenus which readily grows in laboratory rodents (Joshua, 1990; Morrison *et al*., 2010).

Characterization of 30 trypanosome field isolates was successful using the mouse model and confirmation of the species and types by PCR analysis of the 18S rRNA gene and species-specific PCR. The analysis confirmed the identity of 24 isolates (80.0%) as *T. congolense* Savannah and six (20.0%) as Kilifi type. These results confirm recent findings by Mamabolo *et al*. (2009) and Gillingwater *et al*. (2010) that Kilifi type occurs in northern KZN in low percentages. The last authors found that the prevalence of the Savannah and Kilifi-type *T. congolense* were 37% and 2.7%, respectively. Both studies present a certain degree of mixed infections of the Savannah and the Kilifi type. This is due to the fact that molecular characterization in both studies was conducted on field blood samples. In the present study, isolates used were characterized following inoculation in mice and species-specific PCR in order to eliminate the possibility of mixed infections in future experiments (Chapter 5).

Although the Kilifi type was reproduced in mice, this sub-species was nevertheless not as readily amplified as the Savannah type in the mouse model. From these results, it cannot be concluded that Kilifi grows poorly in mice, i.e. problem related to host preference because of the small number of

samples used. However, the low virulence characteristics of this sub-species observed in the present study is in accordance with that reported by Bengaly *et al*. (2002a) when they established that Kilifi-type *T. congolense* expresses non-virulent properties in mice. More studies still need to be conducted to understand the host-parasite relationships.

Results of the virulence of the 24 isolates suggested a range of clinical outcomes within *T. congolense* sub-genotype (Savannah) ranging from high, moderate to low virulence as observed previously (Bengaly *et al*., 2002a). In the present study, all isolates of the two sub-species multiplied in mice but their progression in the host differed. There was no remission or self-cure following the prepatent period in the Savannah strains, however, where infections were due to the Kilifi type, the course of the disease was brief and the infection cleared within weeks and remained so throughout the experimental observation. None of the mice in this latter group died suggesting that these were of extremely low or of non-virulence nature in mice.

Other studies found the PPP for the HVS to be two to four days shorter than the results obtained in this study (Bengaly *et al*., 2002a; Masumu *et al*., 2006). In the present study, there was a significant difference in the PPP between the HVS, and the LVS. No significant differences in the PPP were, however, found between the HVS and the MVS, or between the MVS and the LVS. A plausible reason is that genetic variation in trypanosomes of the same species from distinct geographic origin could influence the outcome of infection (Taylor and Authié, 2004).

In animals infected with trypanosomes, anaemia plays a key factor in determining the severity of the infection (Noyes *et al*., 2009). The change in red blood cell (RBC) count was observed in infected mice. In these animals, RBC count decreased below ~ 60% of pre-infection values, while that of the uninfected controls remained stable. In mice infected with the HVS and MVS, the RBC kept falling and the decline in PCV was an indication that death in

these groups correlated with severe anaemia. For example, in groups of mice under the category of HVS, there was an overall 30% decline in PCV values in less than two weeks compared to 22% decline in less than a month for the MVS. For mice groups in the LVS category, overall PCV decline of 24% was evident in two months (59 days post infection). This therefore confirms that the animals in the three categories behaved differently in terms of the severity of anaemia depending on the virulence of the trypanosome isolates.

Of the 26 characterized strains in mice, 26.9% (7/26) were found as LVS. All of the LVS except one (6/7; 85.7%) originated from locations which are not close to game parks and/or specifically neighbouring game parks which keep buffaloes. These include all of the samples obtained from Nhlanzana (3/3), 60.0% (3/5) from Boomerang and only one from Mvushini dip tank (1/10), which is located at the vicinity of Hluhluwe-Umfolozi Game Park. Van den Bossche *et al*. (2006) reported that 60% of cattle presented as Nagana-infected by the owners were found to harbour *T. congolense* and all were in poor body condition. The virulence profile obtained in the current study confirms the findings of this report and that high disease impact occurs near game areas (Van den Bossche, 2001; Ntantiso, 2012). The only strain obtained from Ocilwane, located at the southern part of Hluhluwe-Umfolozi Game Park, was also allocated to the HVS. Ntantiso (2012) found in his study at this dip tank that Nagana was acute in affected cattle and 100% of positive cattle were anaemic. On the other hand, he also reported that cattle at Boomerang had very high trypanosome infection rates but the level of anaemia in the cattle herd did not exceed 27% as compared to 62% anaemia in cattle at Mvutshini dip tank. The proportion of highly virulent strains remains significantly higher in the sylvatic transmission cycle (Van den Bossche *et al*., 2011). This is believed to be influenced by the evolution of trypanotolerance in wildlife which selects trypanosomes for higher parasite replication rates. This situation maximises the production of the transmission forms which increases the virulence of the strains in susceptible animals near the parks (Van den Bossche *et al*., 2011). Results of the present study indicate that high proportions of highly virulent strains were circulating in the game parks (as

shown in buffalo in Hluhluwe-Umfolozi Game Park) and in cattle herds kept near the game park like Mvutshini and Ocilwane and Ndumo dip tanks (neighbouring Ndumo Game Park) which support the above statement.

The small proportion (20.0%) of strains obtained from Boomerang commercial cattle farm expressed high virulence properties requires further explanation. This is not surprising as the farm is located farther away from Charter's Creek (8 km), supposedly the closest game reserve to the farm. It is highly possible that cattle on the farm constituted an important food source for the tsetse flies and thus acted as the main reservoirs. According to Van den Bossche *et al*. (2011), in areas where tsetse are highly dependent on cattle, pathogenic and lethal strains of trypanosomes are likely to be rare and an endemic status is created. The low pathogenicity seen circulating in cattle at Boomerang is therefore indicative of maintenance of low virulent strains. Virulent trypanosome strains necessitate constant treatment or induce higher mortalities in susceptible animals (Masumu *et al*., 2009) they often get eliminated in endemic areas. According to Van den Bossche, (2001), the endemic type of the disease occurs as a consequence of the domestication of trypanosomes in an area where large game animals are rare or absent. This endemic form is characterised by low genotype variability that gives a more homologous challenge as compared to the epidemic type of the disease encountered near the game parks where sylvatic transmission occurs.

4.5 Conclusion

The results based on characterization of the 26 *T. congolense* strains obtained in the present study, suggested the presence of HVS inside and in areas along the game reserves. Isolates from Mvutshini, Ocilwane and Ndumo dip tanks expressed severe disease symptoms as opposed to those from other dip tanks situated away from the game reserves. Although the pre-patent periods were found to be longer, which in turn contributed to longer survival periods, the observations in this study were in accordance with those of Bengaly *et al*. (2002a) and Masumu *et al*. (2006). The disease status

related to the sylvatic and domesticated cycles of transmission were also demonstrated in this study as suggested previously (Van den Bossche, 2001; Van den Bossche *et al*., 2011). The highly virulent strains killed all infected mice rapidly during the first peak of parasitaemia while mice infected with low virulent strains reacted extremely mild where some groups of mice had to be euthanized while still parasitaemic at the end of the experiment.

CHAPTER 5

Susceptibility of Nguni and Friesian cattle to *Trypanosoma congolense* strains of high and low virulence originating from cattle in KwaZulu-Natal

5.1 Introduction

The pathogenicity of a parasite is commonly determined by its infectivity and its virulence in a host animal. Infectivity is defined as the ability of a parasitic organism to multiply and be maintained in a given host, while virulence refers to the capacity of a parasite to damage and cause disease to its host (Leak, 1999). All trypanosome species infecting livestock are of great economic importance to the livestock industry. The degree of virulence of trypanosome species varies between and within the species. However, the infectivity of some trypanosomes, for example, *T. vivax*, is difficult to interpret because of their limited infectivity for laboratory animals (Joshua, 1986). Geographic distribution also influences virulence in that some strains can become more pathogenic in one area and less or non-pathogenic in the other (Stephen, 1986).

In the northern KZN, trypanosomosis constitutes one of the important constraints to livestock development. *Trypanosoma congolense* has been implicated as the species responsible for the occasional outbreaks (Van den Bossche *et al*., 2011). In the previous chapter, various strains of high, moderate and low virulence were determined using a standard protocol in mice. There was a need to compare the pathological effect induced by such strains in cattle. Currently, no information exists on the level of tolerance or susceptibility to trypanosome infections in Nguni cattle, mostly owned by the resource-poor farmers in the region and Friesians as a susceptible breed of cattle. Thus, this study was conducted to determine the susceptibility of Nguni and Friesian cattle to Savannah-type*T. congolense* strains of high and low virulence.

5.2 Materials and Methods

5.2.1 Experimental animals

A total of 36 cattle comprising 30 indigenous Nguni and six Friesian cattle (Figures 5.1, 5.2), aged between 2 - 2.5 years with no history of exposure to animal trypanosomosis were used. All experimental cattle were kept under controlled conditions for three months before the study. During this period, the PCV and the rectal temperature were recorded daily for each animal. The experimental infections of animals were done in the OVI insect-free facility. They were fed grass hay supplemented with cattle pellets (commercial) and water supplied *ad libitum*. Additionally, four inbred Balb/c mice were used in this study and cared for as mentioned (Section 4.2.2). Animal ethics approval was obtained from the OVI and University of Pretoria Animal Ethics Committee and Animal Use and Care Committee, respectively.

5.2.2 Experimental infection of cattle with high and low virulent strains

Two *T. congolense* strains previously characterized as HVS (MVU10) and LVS (BmrngK2) were propagated into two Balb/c mice each as described (Section 4.2.7). When the parasitaemia reached $10^{7.8}$ - $10^{8.1}$ trypanosomes/ml (Herbert and Lumsden, 1976), a drop from the tail blood was diluted with PSG to achieve a concentration of 10^5 trypanosomes in a total volume of 5 ml infective dosage. The 30 Nguni cattle were divided into three groups: two groups of 12 each and one control group of six. Friesians were divided into two groups of three each. One group of Nguni and one of Friesians were injected i.v. with 5 ml of HVS and cattle in the second groups received similar dose of the LVS. The Nguni control group received i.v injection of 5 ml of PSG.

Figure 5.1: Experimental Nguni cattle obtained from ARC-OVI Nguni breeding herd, Kaalplaas farm.

Figure 5.2: Experimental Friesian cattle.

5.2.3 Clinical examination of experimental cattle

Cattle were monitored daily for morning rectal temperature, PCV and parasitaemia as described (Section 3.2.5). Trypanosome scores were determined according to Paris *et al*. (1982). An animal was declared parasitologically negative when a minimum of 50 microscopic fields of a buffy coat smear were examined and no parasites were detected. Infected cattle with a PCV of 19 % or less for three consecutive days were considered as suffering from severe anaemia and required treatment (Achukwi *et al*., 1997). Cattle were treated with 3.5 mg/kg diminazene aceturate (Berenil ^{TM}) and excluded from further analysis. All remaining cattle were treated similarly at the end of the observation period, i.e. 61 days after infection.

5.3 Results

It should be noted that one animal in the Friesian LVS group had received, by mistake, a dose of HVS resulting in four animals in the HVS and two in the LVS, respectively.

5.3.1 Temperature

Temperature sheets for the 5 groups of cattle are shown in Figures 5.3 - 5.7. Generally, cattle did not show high temperature for more than a day. Seven (58.3%) of HVS infected Nuguni group had temperatures of 39.9 - 40^{o}C for one day on day 7 (3 animals), day 8 (2), days 13 and 14 (2). Three animals in the LVS infected Nguni group had high temperatures (40^{o}C each) on days 9, 11, and 15. Friesian cattle in the LVS group showed normal temperatures for all the observation period while two (2/4) of the HVS group showed high temperature for one day on day 15.

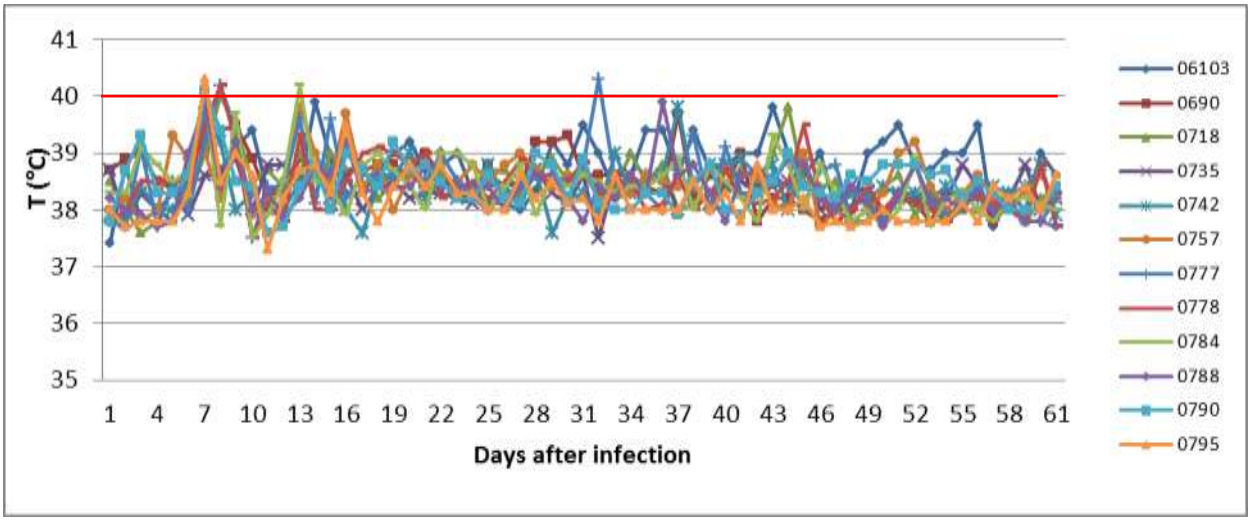


Figure 5.3: Temperature of individual Nguni infected with HVS.

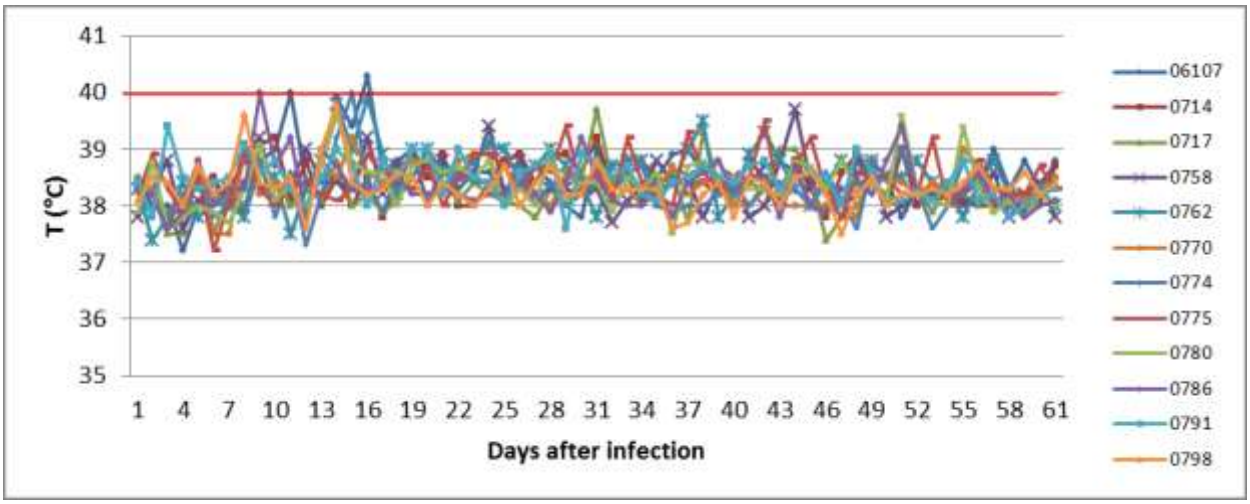


Figure 5.4: Temperature of individual Nguni infected with LVS.

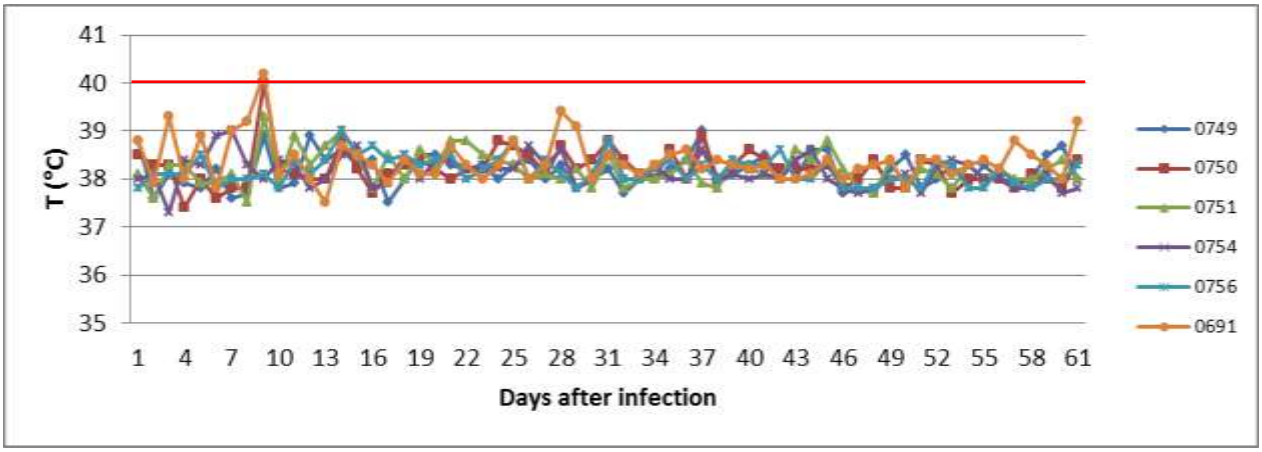


Figure 5.5: Temperature of individual Nguni controls.

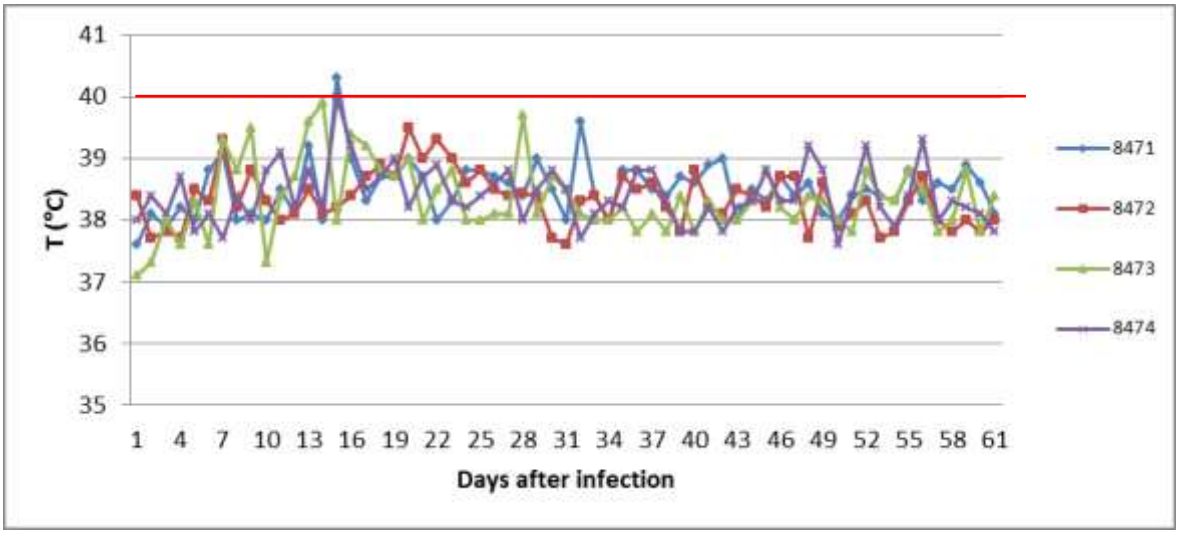


Figure 5.6: Temperature of individual Friesians infected with HVS.

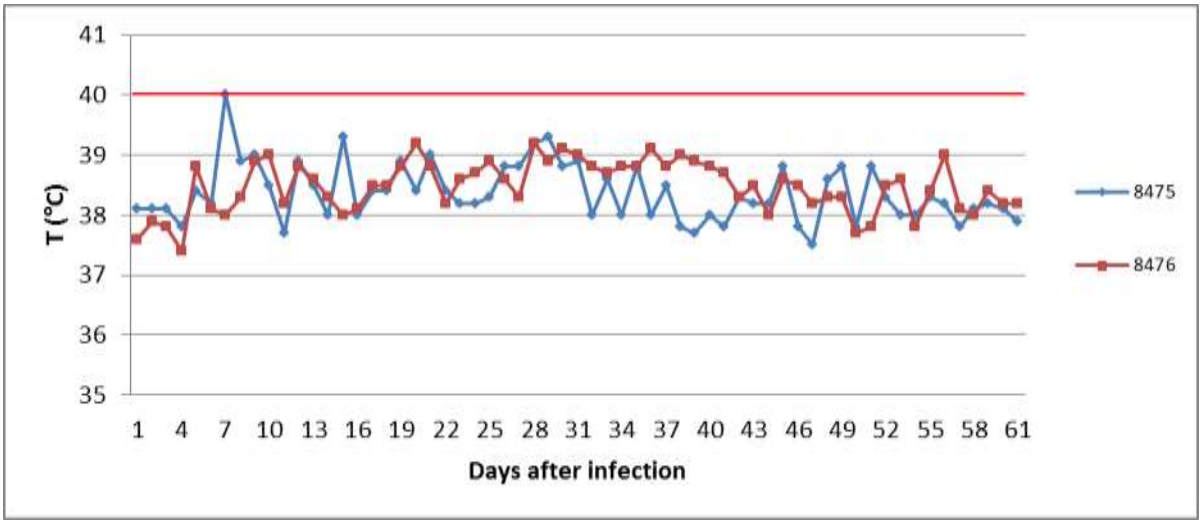


Figure 5.7: Temperature of individual Friesians infected with LVS.

5.3.2 Development of parasitaemia

Figures 5.8 - 5.11 show the trend in parasitaemia scores in the two groups of cattle infected with either HVS or LVS. Generally, all bovines infected with *T. congolense* of high or low virulence developed parasitaemia. The mean parasitaemia for the two Nguni groups are shown in Figure 5.8. In the HVS group, all animals became parasitaemic by day 5 post infection. The average parasitaemia score on this day was 3 which rose to score 4 on the following day. The PPP in the Nguni group infected with LVS varied: only four bovines became parasitaemic by day 5 with an average parasitaemia score of 1; seven were detected positive on day 6 and one on day 7. The average parasitaemia score of this group was 3 by day 6 and 7. The mean parasitaemia for the two Friesian groups are shown in Figure 5.9. All cattle in the HVS and LVS became parasitaemic on day 6 with an average parasitaemia score of 3. All cattle excluding those which received treatment due to severe anaemia, remained parasitaemic throughout the experimental period (61 days).

There were individual variations in the level of parasitaemia in bovines in the HVS group. It was clear that bovine 0742 had kept its parasitaemia levels low (scores 1, 2) from Day 35 until the end of the observation period as compared to the average group parasitaemia (Figure 5.10). This trend in the parasitaemia scores also varied between animals which had not been treated (n = 7) and those which had received treatment (n = 5). Figure 5.11 shows that both groups had experienced similar high parasitaemia scores of 3-5 during the course of infection. However, the animals in the untreated group attained higher frequencies (average number of days for a given parasitaemia score in treated and untreated animals) of scores 1 and 2 compared to those in the treated group.

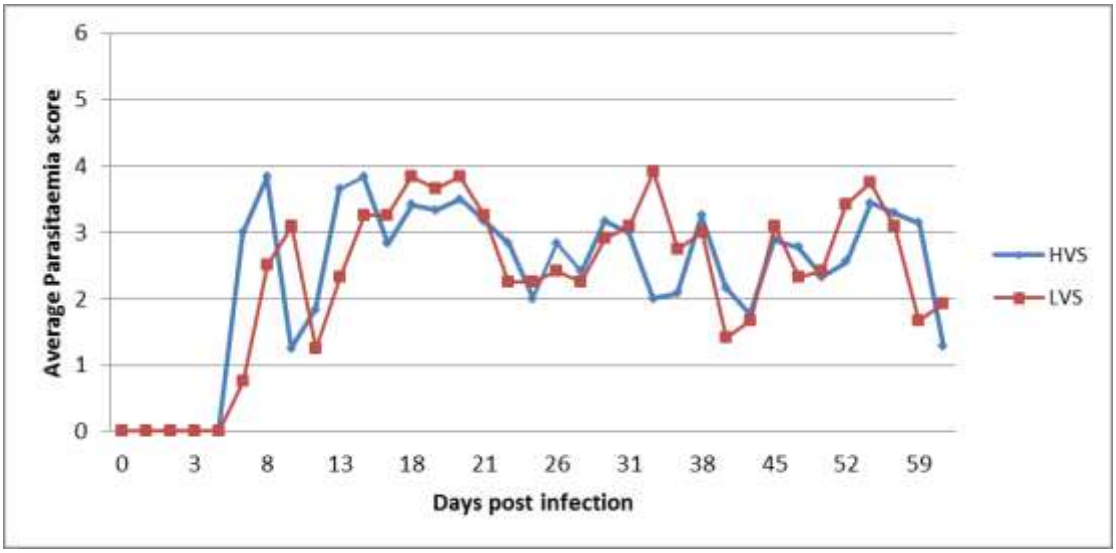


Figure 5.8: Mean parasitaemia scores for Nguni infected with HVS and LVS.

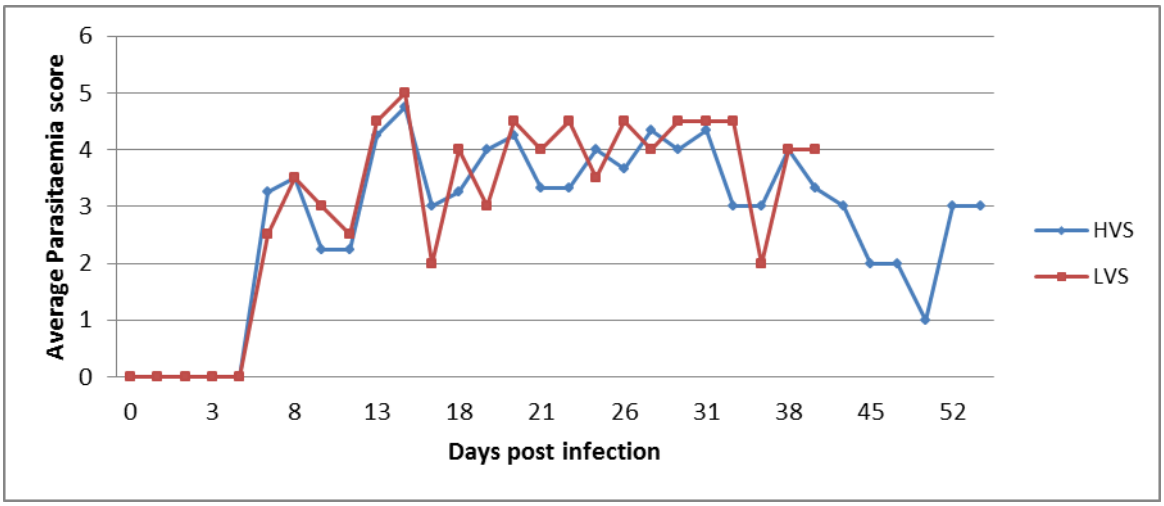


Figure 5.9: Mean parasitaemia scores for Friesians infected with HVS and LVS.

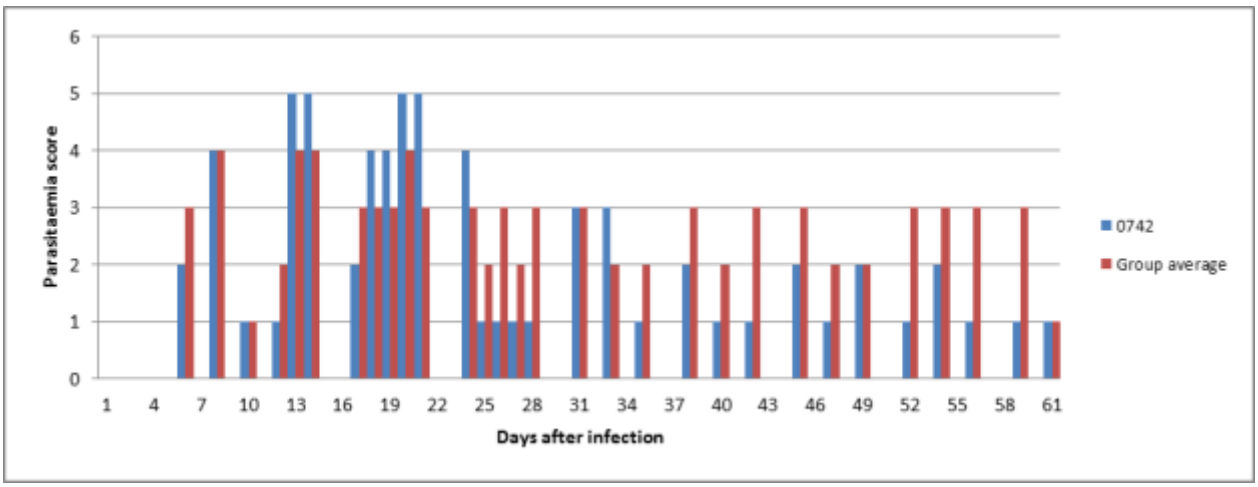


Figure 5.10: Parasitaemia score of 0742 vs group average in HVS.

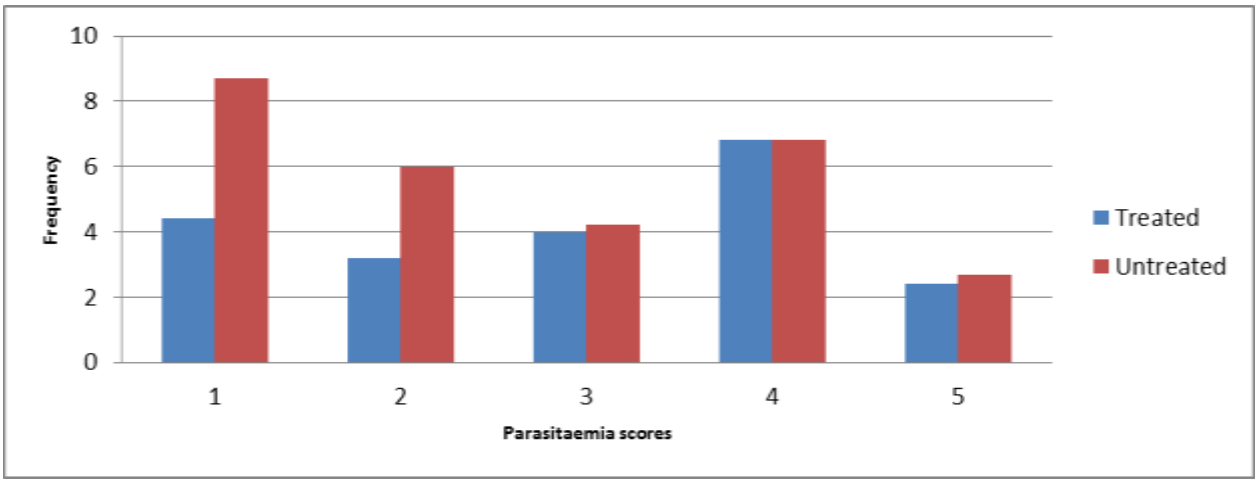


Figure 5.11: Comparison of parasitaemia score frequencies (average number of days for a given parasitaemia score in trated and untreated animals) between treated and untreated Nguni infected with HVS.

5.3.3 Development of anaemia

Figures 5.12 - 5.15 show the PCV values in different treatment groups during the course of the infection. Overall, all Friesians in the two groups and 42% of Nguni (5/12) infected with the HVS had low PCV (≤ 19%) for three consecutive days and received treatment. In the Friesian group, the first animal was treated 20 days following infection with the HVS compared to the Nguni HVS group, in which the first animal was treated 40 days post infection. None of the Nguni cattle infected with the LVS and the negative control group received treatment during the course of the experiment.

There were clear individual differences in the PCV values during the course of the disease between animals within the two Nguni groups (Figures 5.12, 5.13). The mean PCV values of animals in the HVS were kept lower than in the LVS group (Figure 5.14). There were no differences in the mean PCV values in Friesian cattle in the two groups (Figure 5.15).

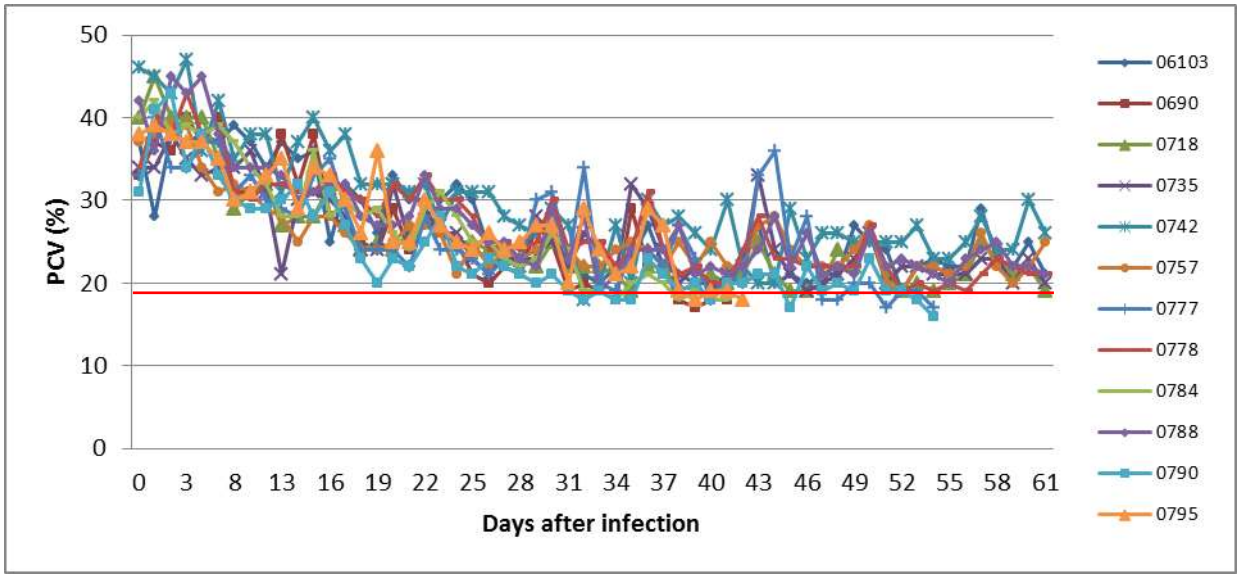


Figure 5.12: PCV of individual Nguni infected with the HVS.

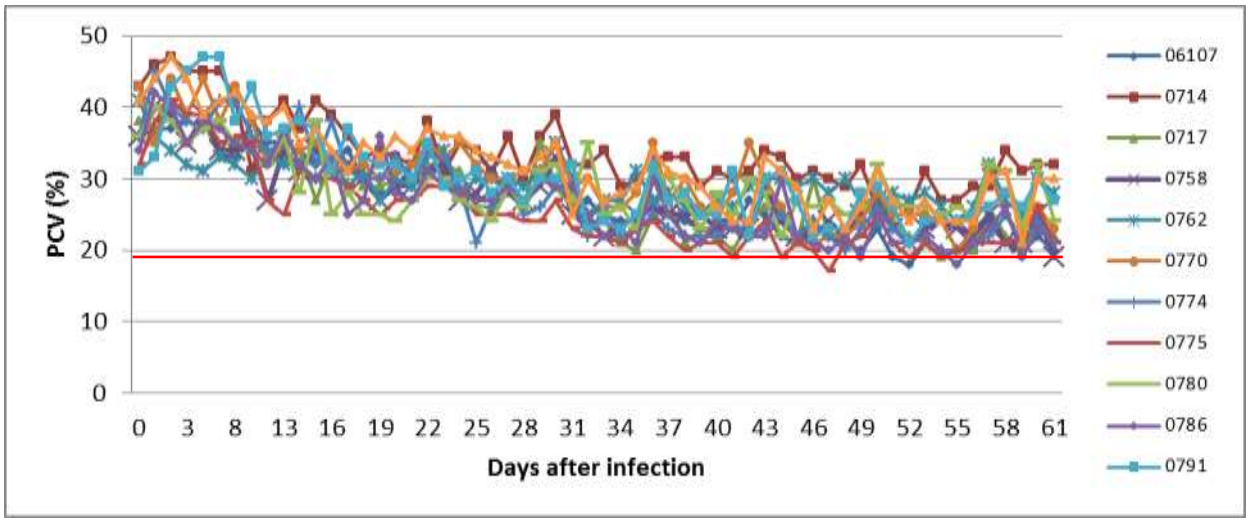


Figure 5.13: PCV of individual Nguni infected with the LVS.

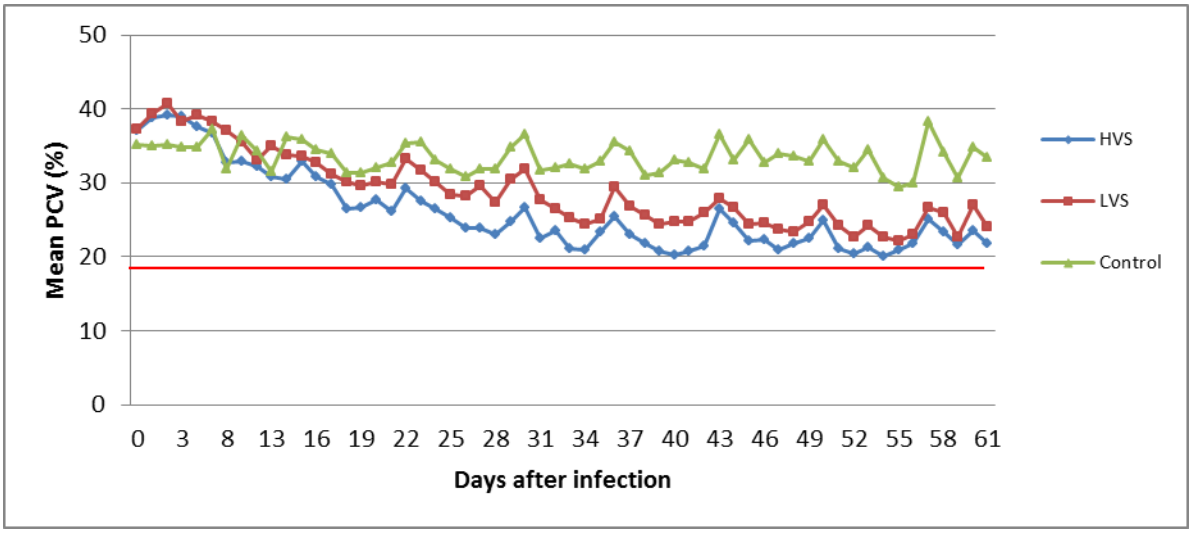


Figure 5.14: Mean PCV of Nguni infected with HVS, LVS and the control group.

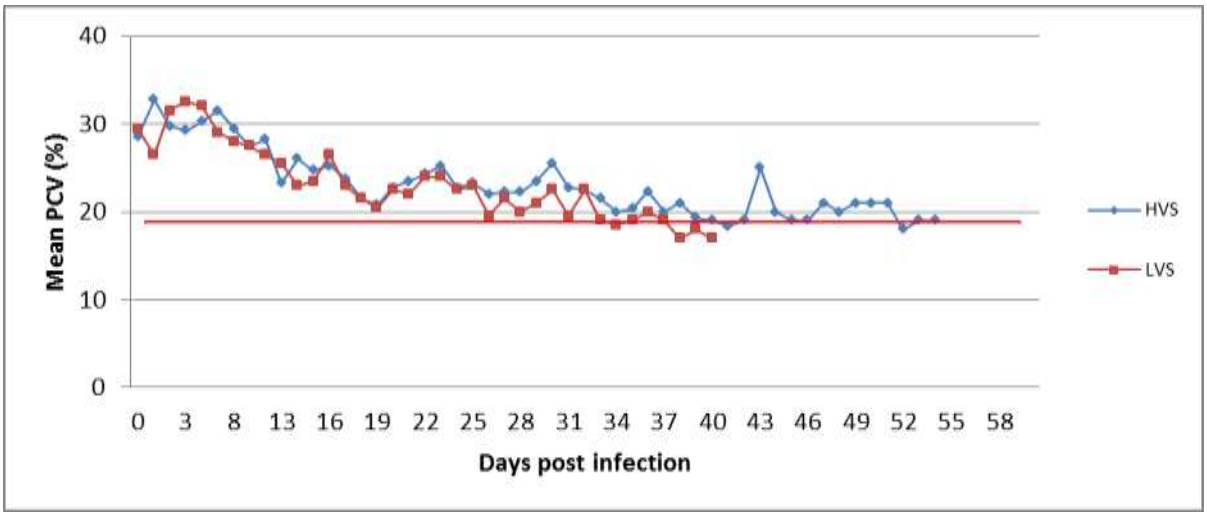


Figure 5.15: Mean PCV of Friesians infected with HVS and LVS.

5.4. Discussion

All of the Nguni and Holstein Friesian cattle reacted to the intra-venous (i.v.) inoculation of either *T. congolense* HVS or LVS with a range in pre-patent period of 5 - 7 days. Similar PPP was reported by Dwinger *et al*. (1992) after inoculation of *T. congolense* using the intradermal route: average of 5.5 days in N'Dama and 6.3 in Zebus. Other workers also reported a PPP of up to 10 days using i.v. route (Sekoni *et al*., 1990) and 9.4 $\pm$ 0.9 days after subcutaneous inoculation (Bengaly *et al*., 2002a). Following artificial challenge with a defined number of flies, the PPP in primary infections are similar in taurine (trypanotolerant) and Zebu (susceptible) suggesting that infections establish efficiently in both groups (Authié, 1994).

From previous literature, pyrexia is said to be a common clinical feature of animal trypanosomosis characterized by intermittent fever, which is prominent during the early phase of the disease (Magona *et al*., 2009). These authors reported that the body temperature in *T. congolense* infections rose up to 39.4 - 40 °C, however, they did not mention the duration of fever. Bengaly *et al.* (2002b) who infected cattle with *T. congolense* Savannah-type also mentioned that pyrexia was observed as earlier symptoms but no further details of the duration of fever were mentioned in the results. Dwinger *et al*. (1992) recorded body temperature for the first two weeks during experimental *T. congolense* infection of N'Dama and Zebu cattle. They found no difference between the two breeds and some of the Zebu cattle did not show a rise in temperature. Greig and McIntyre (1979) reported that routine recording of rectal temperatures in trypanosomosis infections is "unrewarding" because of large differences between animals. In the current study, infections with HVS and LVS in both breeds of cattle did not produce high temperature in all animals while few reacted for one day. Sekoni *et al*. (1990) also found that pyrexia in experimental infections with *T. congolense* and *T. vivax* was intermittent. It can be concluded that pyrexia is not associated with *T. congolense* infections and should not be considered as a factor in selection of trypanotolerence in cattle. This conclusion is supported by the results obtained from the infections of the susceptible Friesians in the present study where only 2/6 had experienced high temperature and for only one day in both animals.

Paling *et al*. (1991) and Achukwi *et al*. (1997) treated their experimental cattle that attained a PCV of 15% or less as a threshold and treated animals were eliminated from the study. In the study of Achukwi *et al*. (1997) two animals died of the infection despite treatment. Dwinger *et al*. (1992) mentioned that without chemotherapeutic interventions the susceptible Zebus would have died (PCV between 12 - 14%) during the experimental period. Bengaly *et al*. (2002b) infected Zebu cattle using *T. congolense* savannah-type which produced severe reactions resulting in death of experimental animals (PCV reached 12.5%). The current study took these into consideration and for ethical issues a threshold of PCV of 19% or less for three days required treatment. This prevented unnecessary suffering of animals and all treated ones recovered.

Results of this study indicated that the two strains previously characterized in mice as of high virulence (MVU10) and of low virulence properties (BmrngK2) produced similar disease in Nguni cattle to that shown in mice. On the other hand, the HVS and LVS produced severe disease in the Friesians with no differences demonstrated during the course of infection. This confirmed the difference in susceptibility of the two breeds of cattle to Nagana as previously reported (Dwinger *et al*., 1992; Moloo *et al*., 1999) and to a number of other diseases (Bayer *et al*., 1999). The Nguni cattle on the other hand, have been shown to adapt well to low-quality feed, and can be used for other purposes, e.g., as droaght animals (Bester *et al*., 2003).

The present study showed that Nguni cattle have a certain degree of trypanotolerance (innate) to infection with a high virulent strain of *T. congolense*. It is clear that there were individual variations in parasitaemia and PCV values within the Nguni cattle such that 42% (5/12) of animals had to be treated. Nguni animal number 0742 demonstrated a low parasitaemia trend from day 35 to the end of the study period (score 1 and 2), which is in line with what had been reported in the trypanotolerant N'Dama and Baoule cattle (Pinder *et al*., 1988; Paling *et al*., 1991). The within-breed variations have also been reported in N'Dama where a large random variation between individuals was detected in PCV values during infection (Dwinger *et al*., 1992). In an attempt to identify immune responses to African trypanosomosis, Pinder *et al*. (1988) infected 11 trypanotolerant Baoule and five susceptible Zebu cattle by feeding of *T. congolense*-infected flies. They found that four (36.4%) of the Baoule were only mildly susceptible (controlled parasitaemia and anaemia), five experienced intermediate susceptibility and two

underwent severe reactions similar to two Zebus. These studies demonstrated the presence of a spectrum of susceptibility among the trypanotolerant breeds.

In conclusion, it has been shown that there were major differences in disease susceptibility between Nguni and Friesian cattle with respect to the degree of anaemia and parasitaemia. Thus, the LVS Nguni group all resisted the infection and controlled anaemia compared to LVS Friesians group which had to be treated. The Nguni cattle showed a considerable level of trypanotolerance to infection with HVS demonstrated by 58.3% (7/12) of animals that were able to control anaemia. The innate resistance shown by the Nguni cattle and manifested by the capacity to resist severe anaemia to *T. congolense* in this study can be an important factor in the epidemiology of Nagana and animal production in northern KZN as was demonstrated in other tsetse-infested areas in Africa (Roberts and Gray, 1973; Dwinger *et al*., 1994). Trypanotorlerance is a genetic trait, heritable and with individual variations within the breed (Murray *et al*., 1982). It is, therefore, possible to select and breed for higher trypanotolerance in Nguni cattle using those which showed higher levels of innate resistance. Unfortunately, the pure Nguni breed of cattle does not exist in high numbers in northern KZN due to unplanned crossing with European and other types, which were considered to be of high productivity by the communities. This has resulted in “diluting the Nguni trait” (Bester et al., 2003).

CHAPTER 6

6.1 General discussion and conclusions

Although South Africa has not experienced human African trypanosomosis, animal African trypanosomosis (Nagana) has been prevalent in northern KZN since the 18th century (Bruce, 1895). Since the association of tsetse flies with trypanosomosis, efforts have been made to eliminate these flies in South Africa. Thus, one successful attempt was the elimination of *G. pallidipes* by aerial spraying of breeding grounds (du Tiot, 1954). At present, tsetse flies exist in an estimated area of 18,000 sq km particularly in nature protected areas and game reserves, which makes it more difficult to control due to conservational and biodiversity issues (Armstrong, 2003). As a result, more research is being conducted to study the ecology and biology of the flies and epidemiology of the disease in order to come up with information relevant to control or eradication.

In the present study, comparison between the ability of *G. brevipalpis* and *G. austeni* to acquire and transmit *T. congolense* revealed higher vectorial competence in *G. austeni*. It was reported that *T. congolense* does mature to infective metacyclic forms in *G. brevipalpis* (Wilson *et al*., 1972), however at very low infection rates. The inoculation of the infected proboscises as determined by DNA collected from these flies did not produce patent infection in mice (Mamabolo *et al*., 2009), which demonstrated the failure of *T. congolense* to mature in the proboscis. In the present study, both *G. austeni* and *G. brevipalpis* colony flies were fed a day after emergence and both were able to establish infections in the midgut successfully. However, development to maturity occurred only in *G. austeni*. These results are indicative that notwithstanding the higher population densities in areas close to the Hluhluwe-Umfolozi Game Park (Kaapmeier *et al*., 1998; Esterhuizen *et al*., 2005) where high infection prevalence in cattle had been reported (Van den Bossche *et al*. 2006; Ntantiso, 2012), *G. brevipalpis* remains a poor vector of trypanosomes. However, the fact that mechanical transmission of trypanosomes by tsetse flies can't be ruled out, this needs to be demonstrated in the case of *G. brevipalpis* using sentinel cattle. The relatively low numbers of *G. austeni* collected in this study may indicate that the efficiency of the H-trap is poor or the species abundance is low. During the development of the H-Trap, it was evident that selected odour (Kappmeier and Nevill, 1999) was more efficient in attracting

G. brevipalpis than *G. austeni*. Based on the assumption that the low catches may be associated with either the trap or population densities, it is therefore recommended that the current trap be improved based on the biology and ecology of *G. austeni*. This will assist to determine the actual densities of this species.

The present data do not support results from two recent studies on the trypanosome infection rates in tsetse flies conducted in the same area using only polymerase chain reactions (PCR) analysis (Gillingwater *et al*., 2010; Mamabolo *et al*., 2009). Mamabolo *et al*. (2009) detected trypanosome DNA in 89% of the flies examined but the results did not specify the fly species identity. Furthermore, this molecular test does not distinguish between mature and immature infections. In the study by Mamabolo *et al.* (2009), the injection of a suspension of macerated fly proboscis did not produce any viable infections in mice that were monitored for the development of trypanosome parasitaemia. Similarly, Gillingwater *et al*. (2010) reported that a higher percentage (20%) of flies (species not identified) tested positive for trypanosome DNA in the midgut, with only 1.6% of mature infections. The higher infection rates detected by the PCR analysis can most probably be related to recent feeding of the flies on infected animals although these trypanosomes would not necessarily develop successfully in the midgut or the proboscis.

Results of the virulence indicated that there is variation in the profiles of *T. congolense* strains circulating in northern KZN. This was evident from the range of clinical outcomes within *T. congolense* savannah sub-type expressing mild clinical to severe reactions in mice. The characteristics of the disease were similar to those obtained by Masumu *et al*. (2006). Though the number of isolates of the *T. congolense* Kilifi-type was few in the present study, the results obtained confirmed previous report by Bengaly *et al*. (2002a) that this strain is non-virulent.

The prevalence of highly virulent strains collected from dip tanks in the vicinity of game parks (Hluhluwe and Ndumo) and inside the game parks was high. At Boomerang commercial farm, moderate and low virulent strains were more prevalent than the high virulent ones. The only strain isolated from Nhlanzana, which is farther away from game parks, produced mild disease in mice. It is believed that the proportion of highly virulent strains remains significantly higher in the sylvatic transmission cycle (Van den Bossche

et al., 2011). Results of this study agree with that statement based on the observation made inside, near and away from the game parks. At game parks, wild animals constitute the main reservoir of trypanosomes for tsetse flies while farther away cattle act as reservoir hosts. Thus, trypanosomes at Boomerang and Nhlanzana dip tank may have lost their virulence due to frequent circulation in host cattle.

The host-parasite interactions in relation to trypanotolerance or innate resistance in Nguni cattle to *T. congolense* in this study is the first of its kind in South Africa and requires further elaboration. This phenomenon is an important factor in the epidemiology of Nagana and animal production in tsetse-infested areas in Africa (Roberts and Gray, 1973; Dwinger *et al*., 1994). The two most studied trypanotolerant breeds of cattle are the N'dama and Baoule, sub-types of *Bos Taurus* in West Africa (Leak, 1999). Genetic resistance to trypanosomosis also occurs in some wild animal species, goats and some strains of laboratory mice (Murray *et al*., 1984; Mutayoba *et al*., 1989).

The characteristics of trypanotolerance are that cattle tolerate the presence of parasites, are able to control parasitaemia and have the capacity to resist severe anaemia and production loss during infections (Murray *et al*, 1984; Murray and Black, 1985; Leak, 1999). In contrast, the susceptible breeds of cattle lack such traits (Kemp and Teale, 1998) as it was shown that trypanotolerance has major genetic components (Murray *et al*., 1982). The ability of Nguni cattle to control severe anaemia in this study is demonstrated in two ways. First, the performance of Nguni cattle was compared to the Friesians and, secondly, by using high and low virulent strains of *T. congolense*. It is concluded that Nguni cattle have demonstrated a significant level of anaemia control to challenges with HVS (58.3% of cattle) and LVS (100%). It should be stated that both the HVS and the LVS produced severe disease in all Friesians, which had to be treated. Thus, the innate resistance reported here in Nguni cattle is similar to that shown by the N'Dama cattle which were raised and challenged in a tsetse-free area (Kemp and Teale, 1998). Paling *et al*. (1991) provided evidence that trypanotolerance is expressed during primary infections with *T. congolense* in naïve cattle. The low percentage of cattle requiring treatment in the group infected with the HVS is not unusual as trypanotolerance is a "relative rather than an absolute trait" (Leak, 1999). It was found that analysis of PCV levels in N'Dama cattle during primary infection with *T. congolense*

showed considerable variation between individuals during the early stages of the disease (Dwinger *et al*, 1992). Thus, trypanotolerant breeds can be severely affected under high-challenge situations (Authié, 1994; Leak, 1999). Repeated trypanosome infections enhance acquired immunity in innately resistant cattle and contribute to greater resistance to the disease compared to susceptible cattle (Authié, 1994; Leak, 1999). It would have been of interest if this observation was also demonstrated in Nguni cattle used in the present study by re-challenge and monitoring them over an extended period of time. However, the innate mechanisms in trypanotolerant cattle are more efficient in controlling disease, making them less reliant on humoral responses (Naessens *et al*., 2002).

N'Dama cattle are also more resistant to tick infestations and have a lower *Anaplasma* prevalence than the susceptible Gora Zebu cattle (Mattioli and Cassama, 1995). These qualities of tick resistance were previously reported in Nguni cattle (Spickett *et al*., 1989; Scholtz *et al*., 1991). The tick resistance trait in Nguni is an added value to resistance to trypanosome infection. Nguni performed well under optimal conditions while the exotics performed poorly under prevailing management practices of communal systems (Scholtz, 1988). The profile of the Nguni shows that it emerged by a process of natural selection in a highly stressful environment and it has the genetic potential to perform better under suboptimal production environment (Bester *et al*., 2003).

6.2 The study and the socioeconomics aspect of Nagana in northern KZN

The KZN Province has one of the highest levels of poverty in the country with rural areas most affected (http://www.nda.org.za). About 60 % of its population live in the rural areas and are dependent on agriculture with livestock farmers usually having few animals. The loss of an individual animal has proportional greater significance (Perry *et al*., 2005). It is essential to identify the animal-health constraints to resource-poor livestock keepers to enable the authorities to provide services directed towards alleviating these constraints. For over 22 years, information on Nagana causing morbidity, mortality and production losses have been rudimentary. It is only very recently that one study emerged highlighting the epidemiology of the disease and possible impact in the livelihood of resource-poor farmers in the northern parts of the province (Ntantiso, 2012). Nagana is chronic in nature, generally a debilitating factor which kills slowly. It is a disease of poverty in resource-poor settings due to

inaccessibility of treatment and lack of efficient control policies. The present study has contributed to the identification of factors relating to trypanosome vectors, virulence of the transmitted parasites, the risk of Nagana at the livestock-wildlife interface and the possible role Nguni cattle as “trypanotolerant cattle” may play as productive animals in the Nagana-endemic areas.

6.3 Recommendations

The last intervention by the Government to curb Nagana outbreaks in northern KZN was in 1990 (Kappmeier *et al*., 1998). More than 100,000 cattle were treated using ethidium bromide and pyrethroid dips were introduced to reduce tsetse challenge. Van den Bossche *et al*. (2006) confirmed that Nagana was still prevalent and recommended that further research is required to develop appropriate control measures. The current study has demonstrated that cattle kept at the game-livestock interface were exposed to more virulent *T. congolense* strains and that these strains produced severe disease in cattle under controlled condition. Ntantiso (2012) has also shown that cattle of three rural communities in Nagana-endemic areas suffered significant anaemia compared to cattle subjected to lowere tsetse challenge. The South African Veterinary Authorities have to take cognisance of the present study and that of Ntantiso (2012) and start implementing a policy directed towards tsetse and trypanosomosis control or eradication which would result in improvement of animal productivity and in poverty alleviation. More studies are required to further investigate the trypanotolerance trait in Nguni which also requires monitoring the production performance of cattle under high tsetse challenge.

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