Epidemiology of drug resistance and evaluation of possible mechanical transmission of *Trypanosoma congolense* by haematophagous insects in Zambezia Province, Mozambique

By

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A thesis submitted to the Faculty of Veterinary Science, University of Pretoria, South Africa, in fulfillment of the requirements for the degree

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DEDICATION

I dedicate this thesis to my wife Arlete Siquisse and our daughter Shantel Mulandane who have been a source of inspiration and have patiently supported me during this long journey.

DECLARATION

I hereby declare that this thesis is my own work. It is submitted in fulfilment of the degree, Philosophiae Doctor, in the University of Pretoria, South Africa. It has not been submitted before for any degree or examination in any other University.

Fernando Chanisso Mulandane

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THESIS SUMMARY

Epidemiology of drug resistance and evaluation of possible mechanical transmission of *Trypanosoma congolense* by haematophagous insects in Zambezia Province, Mozambique

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African animal trypanosomosis is a debilitating tsetse-transmitted parasitic disease of sub-Saharan Africa. The cyclical vectors of the disease are tsetse flies (*Glossina* spp). However, trypanosomosis can be mechanically transmitted by haematophagous flies from the family Tabanidae (Diptera). Therapeutic and prophylactic drugs used in the control of the disease were introduced more than 50 years ago, and drug resistance is increasingly reported and very few studies regarding the distribution of the disease vectors have been conducted in Mozambique after the country's independence that took place in 1975. The aim of this study was to assess trypanosomosis and trypanocidal drug resistance prevalence in Nicoadala district, Zambezia province, Mozambique, and to investigate the presence of trypanosomosis vectors in three different habitats in the same district, as well as the trypanosome infection rates in these vectors.

To study drug resistance, a cross-sectional study was conducted in five villages (Botao, Mungama, Zalala-Electrosul, Zalala-Madal, and Namitangurine), where 467 cattle were microscopically screened for trypanosomes, followed by a block treatment study, to evaluate treatment efficacy. Trypanosome-positive animals were randomly assigned to two groups; one treated with 0.5 mg/kg b.w. isometamidium (Inomidium®) and the other with 3.5 mg/kg b.w. diminazene (Inomazene®). Cattle were microscopically monitored at days 0, 14 and 28 post-treatment. At day 28, trypanocides were swapped to investigate single or multiple resistance. Microscopically-negative samples from the monitoring days were tested using 18S-PCR-RFLP. At the end of the block treatment experiment, day 42, six animals (9%) in Botao and two animals (9.5%) in Mungama remained positive after drug swap. No relapses occurred in Namitangurine. The presence of single and multi-drug resistance in Nicoadala district, Zambezia province was thus confirmed.

To investigate the composition of Tabanidae and Glossinidae, vectors of trypanosomosis, an entomological survey was conducted. For 365 days, 55 traps (20 NGU, 20 H and 15 Epsilon) were deployed in three grazing areas of Nicoadala district, namely, Namitangurine (25), Zalala (15) and Botao (15). Flies belonging to the above-mentioned families were collected weekly, preserved in 70% ethanol and later identified using identification keys and manuals. Molecular identification of tabanids and tsetse flies, and a phylogeny study of tabanids, were conducted using cytochrome oxidase 1 gene. Additionally, trap efficiency was assessed to compare the performance of the three types of trap in the capture of individuals from both families. Furthermore, seasonal abundance of tabanids was also investigated. As result, individuals belonging to ten tabanid species and three tsetse species were caught. The use of molecular tools confirmed taxonomic identification as well as revealing monophyly in Tabanidae. NGU traps were identified as the most suitable for the capture of both tabanids and glossina. It was also observed that the rainy season was the most appropriate for the capture and study of tabanids. The vector composition lead to the conclusion that the role of tabanids in the transmission of trypanosomes in Nicoadala district may be greater than of tsetse flies.

To assess trypanosome infection rates, DNA was extracted from 480 tabanids and 24 glossina. From the 480 tabanids, 160 were captured in Zalala, 160 in Botao and 160 in Namitangurine. Moreover, the tabanids used belonged to the four most captured species

in the three areas namely *Tabanus par*, *T. taeniola*, *Atylotus agrestis* and *Ancala africana*. The 24 glossina used belonged to the species *Glossina brevipalpis, G. morsitans* and *G. pallidipes*. DNA extracted was submitted to 18S PCR-RFLP and was found to be positive for the presence of *Trypanosoma congolense* and *T. theileri*. The overall infection rates were 70.8% (n=24) for glossina and 13% (n=480) for tabanids. Similarly to what was observed in the block treatment, the only pathogenic trypanosome detected was *T. congolense*, strengthening the idea of this being the only pathogenic *Trypanosoma* species circulating in cattle in the area.

The present study revealed the presence of drug resistance in Nicoadala district. It also demonstrated that there is a small *Glossina* population with an extremely low apparent density surviving in the area as well as a large homogenous population of tabanids cohabiting there, both participating in the epidemiology of trypanosomosis.

Key words: Trypanosomosis, Chemo-resistance, Block treatment, Haematophagous insects, Mechanical transmission.

CHAPTER 1 : General Introduction

1.1 Background

Trypanosomosis is a tsetse-transmitted debilitating parasitic disease affecting domestic and wild vertebrates, including man (Barrett et al. 2003). It is one of the most important diseases in Africa affecting people directly by causing human African trypanosomosis (HAT) and indirectly through animal African trypanosomosis (AAT) (Aksoy 2003).

AAT affects not only the well-being of the population but also efficient food production with 3 million cattle deaths per year attributed (WHO 2002; Angwech et al. 2015). Losses in meat production, milk yield and draught power are estimated to cost approximately US$ 500 million annually and together with losses in crop production and livestock it rise to US$ 5 billion per year (Scoones 2000; Chanie et al. 2013; Bukachi et al. 2017).

The control of the disease relies on three main approaches generally used in parallel: the use of trypanocides for both chemoprophylaxis and chemotherapy (Peregrine 1994), vector control using insecticides and traps (Authié et al. 1993; Vale 2009; Shaw et al. 2013; Pilossof 2016) and the use of trypanotolerant bovines (Murray et al. 1990).

The biological transmission of trypanosomes to vertebrate hosts is done by their cyclical vector, tsetse fly (*Glossina* sp.). However, experimental observations have proven the mechanical transmission of *T. congolense* (Desquesnes and Dia 2003), *T. vivax* (Desquesnes & Dia 2004), *T. brucei* and *T. evansi* to cattle by tabanids and *Stomoxys* spp (Parra-Henao and Alarcón-Pineda 2008; Baldacchino et al. 2013, 2014).

1.2 Thesis Rationale

It has been more than 100 years since the first pathogenic trypanosome was discovered. However, trypanosomosis is still one of the most serious threats to both man and livestock in sub-Saharan Africa. Even with all the efforts to fight the disease, the absence of a successful vaccine and the fact that the therapy and prophylaxis of the disease rely principally on only 3 drugs that have been in use for more than 50 years

allied with the extensive increase of the phenomenon of drug resistance in several countries, constitute a serious weakness to the control of the disease (Delespaux et al. 2008).

The distribution of flies, especially tsetse flies, in the country is known to be discontinuous due to natural barriers and habitat changes resulting from human activities but its definite distribution is still not completely elucidated. The presence of tabanids in Zambezia province have been reported by Dias (1966), however, their role in the transmission of pathogens, especially trypanosomes is still unknown.

Drug resistance has shown to be a serious drawback to the agriculture development in Africa, and have been reported in Zambezia (Jamal et al. 2005), however since then there is no information if it has reduced, increased or even geographically changed. To achieve success in any intervention towards control of trypanosomosis in Zambezia, drug resistance distribution must be carefully assessed. Moreover, reduction or total elimination of tsetse or other insect populations requires an exhaustive analysis of the limits and isolation of the targeted insect population.

All this information suggest the need for surveys on trypanosomosis and distribution of tsetse and other possible trypanosomosis vectors as well as their role in the transmission of trypanosomes for an effective control of this disease in Mozambique.

1.3 Thesis Objectives

In view of the above, the principal objectives of this study were:

1.3.1 General

- Detection of single and multiple drug-resistant *Trypanosoma congolense* populations in Zambezia province and assessment of trypanosomosis vector composition in the area as well as their relative role in the transmission of the disease pathogen.

1.3.2 Specific

- Assess the prevalence of trypanosome infected animals in Nicoadala district, Zambezia province.
- Map the distribution of single/multiple drug resistance in Zambezia province assessed by block treatment approach.
- Identify the pathogenic trypanosome species circulating in the district.
- Collect *Glossina spp.* and tabanids samples from Botao, Zalala and Namitangurine, in Nicoadala district using H, Ngu and epsilon traps.
- Identify Tabanidade and Glossinidae species using morphology and cytochrome oxydase 1 PCR.
- Phylogenetic analysis of Tabanidae and Glossinidae collected using cytochrome oxidase 1 marker.
- Compare the efficiency of traps in the capture of tabanids.
- Determine the seasonal abundance of tabanids.
- Assess trypanosome infection rates in Tabanidade and Glossinidae captured in Botão, Zalala and Namitangurine, in Nicoadala district, using 18S PCR-RFLP.

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CHAPTER 2 : Literature Review

2.1 Introduction

Tsetse-transmitted animal trypanosomosis is currently, one the most important diseases of livestock in Africa, not only due to the thousands of cattle deaths attributed to it but also due to the 4,5 billion USD costs related to control and eradication campaigns, cattle treatment, losses in other livestock dependent activities, research and extension work (Aksoy 2003; Shaw et al. 2015).

The etiological agent of the disease, *Trypanosoma* spp, is biologically and mechanically transmitted to its vertebrate host by haematophagous insects, during feeding. In the vertebrate host, trypanosomes remain and/or multiply in the blood stream, lymphatic vessels and tissues (Shah-Fischer and Say, 1981).During the interaction with the vertebrate host, the parasites cause a large spectrum of symptoms including fever, anemia, weight and production loss, weakness, inflammation of the lymphatic nodes and immunosuppression (Uilenberg 1998).

The transmission of trypanosomes is carried out biologically by tsetse flies and mechanically by other biting insects like tabanids and stomoxys (Baldacchino et al. 2013, 2014; Desquesnes et al. 2009).

In the absence of an effective vaccine due to the antigenic variation of the surface glycoprotein of the parasite and the immunosuppression induced by trypanosome infection (Magez et al. 2008; Tabel et al. 2013) and logistic difficulties associated with the control of the vectors (Aksoy 2000), chemoprophylaxis and chemotherapy with isometamidium chloride and diminazene aceturate, respectively, are currently the main approaches. In the last 50 years, no new trypanocidal drugs have been introduced commercially and drug resistance has been reported in 21 African countries (Lalmanach et al. 2002; Delespaux et al. 2008b; Tsegaye et al. 2015; Solomon and Workineh 2018), including Mozambique (Jamal et al. 2005). However, since Jamal et al. (2005), no update of the drug resistance situation has been provided, thus it is not known if it has withdrawn, progressed or changed geographically. Furthermore, the way by which the

transmission of trypanosomes occurs in the area, as well the vectors involved, has never been addressed since the study by Jamal et al. (2005).

2.2 History and general description of trypanosomes

Trypanosomes are parasitic flagellated protozoa belonging to the class Kinetoplastida. This class comprises organisms that possess an organelle called kinetoplast (hence the name of the class), which contains mitochondrial DNA (Liu et al. 2009; Gluenz et al. 2011). Trypanosomes are long spindle-form protozoa with lengths varying from 8-130 µm (Mulligan 1970).

Trypanosoma was first observed and described in trout blood by Gabriel Valentin, in 1841, working at the Physiological Institute at the University of Bern (Shaih et al. 2013). After that, little progress was made until 1880 when Evans described the first pathogenic trypanosome, later named as *Trypanosoma evansi* and known to cause *surra*, a disease that affects mainly horses and camels, but also other vertebrates like cattle, dogs, buffalo, deer and cats (Röttcher et al. 1987; Desquesnes et al. 2013).

In 1895, working in Zululand, Bruce described a new trypanosome in horse and cattle blood, causing a disease known as *nagana*. This trypanosome was later named *T. brucei*. In 1904 and 1905 progress was made in the study of trypanosomosis, when *T. congolense* and *T. vivax* were found in cattle blood in Congo and Cameroon (Steverding 2008).

In 1910, in Rhodesia (now Zimbabwe), East Africa, a new species of trypanosome was discovered, isolated from human blood. It was named *T. rhodesiense* (Hide 1999). This was after Joseph Dutton named *T. gambiense*, based also on observations of the parasite in human blood along the Gambia River, West Africa (Mulligan 1970). Geographically, the two species occur separately, with *T. gambiense* occurring mainly in western and central Africa and having its transmission linked to the riverine tsetse species *G. palpalis*, whereas T. *rhodesiense* is restricted to South-East Africa and its transmission is more related to the savannah tsetse species *G. morsitans*. There are some overlaps in

the distribution of the two species of trypanosomes in Uganda (Cecchi et al. 2009; Simarro et al. 2010; Franco et al. 2014; Franco et al. 2017).

In 1912, Bruce and coworkers described in Nyasaland, now Malawi, a species of trypanosome that was highly virulent to monkeys and named it *T. simiae.* This was later noticed to be highly virulent to pigs as well (Bruce et al. 1912).

African trypanosomes have a wide range of hosts, and they deserve particular attention from researchers due to their great medical and veterinary importance. They are responsible for human and animal African trypanosomosis. In the case of human African trypanosomosis, *T. brucei rhodesiense* and *T. brucei gambiense* are the etiological agents. Several species have been identified as responsible for animal trypanosomosis, among them *T. brucei sensu latu, T. evansi, T. equiperdum, T. suis, T. simiae, T. congolense* and *T. vivax*. From all the species of the genus *Trypanosoma, T. congolense* and *T. vivax* stand out in Africa for being the species causing the biggest losses to livestock and farmers (Stephen 1986; Rey 1991).

Most of the species of *Trypanosoma* permanently or temporarily inhabit, the gut of haematophagous insects, vectors of the disease to terrestrial animals, like tsetse flies (*Glossina sp.*) (Aksoy 2003), horseflies and some other species of insects belonging to the family Reduvidae, class Hemiptera (*Triatoma infestans, Panstrongylus megistus* and *Rhodnius prolixus*) that transmits American trypanosomes and *T. theileri* (Castro et al. 2010).

2.3 Trypanosome Transmission

To be transmitted to the vertebrate host, the pathogen (often ingested by the vector) must survive and develop inside the vector and be able to be transmitted to another host. The effective transmission of the parasites to their host generally occurs during the blood meal through the saliva, for the salivarian forms and after skin erosion/wound through the feces for the stercoraria form (Kamhawi et al. 2004).

In the stercoraria group, the blood-feeding vector, like Hemiptera-Reduvidae, ingests trypomastigotes along with its blood meal. The trypanosomses develop within the midgut and when the insect feeds and defecates at the same time, the trypanosomes in metacyclic forms, contained in the feces, contaminate the wound. Inside the vertebrate, trypanosomes undergo developmental stages continuing the cycle of transmission (Mulligan 1970).

For salivarian trypanosomes, after being ingested by tsetse flies, from an infected vertebrate host, in their trypomastigote form, the trypanosomes develop and multiply in the midgut of the insect, before migrate to the mouthparts where they develop into metacyclics and are transmitted to a new host, in the course of the next blood meal (Jordan 1985; Malele 2011).

African trypanosomes of medical and veterinary importance are divided into 4 sub-genera and are transmitted by tsetse flies, except *T. equiperdum* which causes Dourine, a venereal disease in horses. According to Noble et al. (1989), trypanosomes reproduce by binary fission and according to the way they are transmitted by the vectors to their final hosts they can be classified into:

I. Salivaria: trypanosomes evolve in the midgut of the vectors from where the metacyclic forms are transmitted by insect bite to the final host after migration to the salivary glands and/or mouth parts. Examples include *T. congolense*, *T. brucei* and *T. vivax*.

II. Stercoraria: trypanosomes evolve in the hindgut of the vectors and are transmitted through the insect feces when it gets in contact with the blood, fluids or wounds of the final host as is the case of *Trypanosoma cruzi* in South America.

III. Trypanosomes transmitted by both ways: *Trypanosoma rangeli*.

In addition to these transmission routes, there is a mechanical transmission, which is known as the process by which haematophagous arthropods physically carry blood

parasites from one host to another, usually via body parts. It is now known and proved that different species of trypanosomes can be transmitted mechanically by haematophagous flies to vertebrate hosts (Baldacchino et al. 2014). Moreover, it is known that the process of mechanical transmission allowed the dispersion of *T. evansi* and *T. vivax* from Africa to South America and more recently to Europe (Jones and Dávila 2001; Desquesnes 2004; Borges et al. 2014; Baptista et al 2018).

Mechanical transmission of parasites, in this particular case, trypanosomes, occurs when an insect meal is interrupted, and it restarts its blood meal in another host injecting saliva before getting the blood, inoculating the parasites present in its mouth parts. Usually, it happens when hosts make defensive movements, which lead the insects to quickly try to get a meal from another site or animal (Müller et al. 2012).

This process can also occur when the insect keeps some blood in its crop and regurgitates it in the next blood meal (Desquesnes 2004). Trypanosomosis and several other diseases like West Nile and Rift Valley fever, Equine infectious anemia, African swine fever and Rickettsia can be transmitted in this way (Foil 1989; Alves et al., 2007; Usaha 2008; Desquesnes et al. 2009; Baldacchino et al. 2014).

2.4 Trypanosomosis vectors

When studying infectious diseases, vector is the name attributed to the agent responsible for transporting the pathogen from one host to another, and usually they are arthropods (Hongoh et al. 2011; Eisen and Eisen 2011). In the case of African trypanosomosis, the primary vector is *Glossina sp*, also known as tsetse fly. Presently, there are 34 species and subspecies of glossina discontinuously distributed in sub-Saharan Africa in an area of approximately 10 million Km^2 (WHO 2004; FAO 2008; Attardo et al. 2014). Their taxonomic classification is based on morphologic characteristics (Krafsur 2010). Tsetse flies are divided into three subgenera or groups namely: Palpalis, Morsitans and Fusca (Vreysen et al. 2013). Out of these groups, only fusca, represented by *Glossina brevipalpis*, and Morsitans, represented by *G. morsitans morsitans*, *G. palidipes* and *G. austeni*, are present in Mozambique (FAO 1987; Sigauque et al. 2000; Wohlford et al. 1999).

Tsetse flies are usually light brown or greyish-brown with inter-species darkness variations and their bodies are divided into head, thorax and abdomen. The abdomen can distend after feeding thanks to the elastic cuticle that covers it (Rogers *et al.,* 1994). When at rest, their wings are completely folded one on top of the other and each wing has a discal medial cell called hatchet cell due to its resemblance to a hatchet. The presence of this cell is an exclusive characteristic of individuals belonging to the genus *Glossina* (FAO 2008).

In addition to tsetse flies, the biological vectors, tabanids can mechanically transmit trypanosomes, except for *T. theileri* which is a stercorarian trypanosome (Rodrigues et al. 2006; Sood et al. 2011). Tabanids are pests of free grazing and confined livestock. They belong to the family Tabanidae (suborder: Brachycera) and are represented by more than 4000 species subdivided into the subfamilies Pangoniinae, Scepsidinae, Tabaninae and Chrysopsinae (Baldacchino et al. 2014). Besides its high biodiversity, the family is distributed worldwide (excluding Antarctica). Species can be differentiated by analysing features such as size, colour, body markings and wing markings (Horváth et al. 2008). Tabanid flies are known as vectors of blood and skin pathogens of livestock and humans (Baldacchino et al. 2013). Not all tabanids cause losses to livestock production; hence not all can be considered major livestock pests. They mechanically transmit more than 35 pathogens and they cause blood loss in livestock (Baldacchino et al. 2014).Furthermore, the primary lesions caused by tabanids can be used for myiasis producing flies to deposit their eggs (Foil and Hogsette 1994).

2.5 Trypanosomosis: pathogenesis, clinical signs and diagnosis

Trypanosomosis in domestic animals is mainly characterized by anaemia (Authié 1994; Stijlemans et al. 2018), weakness and immune depression (Goodwin et al. 1972; Murray et al. 1974; Shah-Fischer and Say 1989; Tabel et al. 2013). Several theories have been used to explain the development of anaemia in trypanosomosis. According to one theory, there is a release of haemolysins by the parasite, which directly damages the red blood cell membrane causing lyses. Another proposed mechanism is that the parasite releases toxic metabolites in the host hematopoietic system, causing a decrease in the

number of circulating red blood cells (Boyt 1991; Mbaya et al. 2010). It has also been postulated that trypanosomes produce neuraminidases that cleave off the sialic acid that is responsible for the protection of erythrocytes. Sialic acid acts as a protective shield for red blood cells and also masks them, preventing the cells from being phagocytosed by macrophages. By destroying the sialic acid in the surface of the erythrocytes, the neuraminidases of trypanosomes make the cells more susceptible to hemolysis by the reticulo-endothelial system (Shehu et al. 2007; Mbaya et al. 2010).

One important fact about trypanosomosis is that, during the different stages of the disease, the host immune system fails to control and eliminate the infection. This can be explained by the capacity of the parasite to evade the host defences through antigenic variation of the trypanosome surface coat (Borst et al. 1997). When antibodies are produced during the infection, a certain portion of trypanosomes will have already changed the chemical composition of the glycoprotein coat allowing them to escape the antibody wave, multiplying and producing a new peak of parasitaemia. This process can be repeated for months (Vincendeau and Bouteille 2006). Usually, the clinical signs of the disease become apparent between days 7 and 10 post-infection. In ruminants, the main clinical signs are anaemia, oedema, lethargy, decreased fertility, progressive loss of body weight, fever and sporadic loss of appetite during the peaks of parasitaemia (Smyth 1994).

To diagnose trypanosomosis, direct and indirect methods can be used. Direct methods include blood smears and fresh blood drop observation which has the sensitivity of about 10^4 parasites/ml (Paris et al. 1982).

Other direct methods include Woo technique, with a sensitivity of 1000 trypanosomes/ml and that consists in the centrifugation of the blood sample in capillary tubes and examination of the *buffycoat*/plasma junction under the microscope looking for motile trypanosomes (Uilenberg 1998), *buffycoat* technique (450 trypanosomes/ml) and mini-anion-exchange centrifugation technique (mAECT) (50 trypanosomes/ml) (Boyt 1991; Bonnet et al. 2015). The combination of *buffycoat* and mAECT can

increase the sensitivity of detection to 10 trypanosomes/ml (Camara et al. 2010). All the aforementioned techniques are parasitological methods. Further, there are also nucleic acid-based assays or molecular methods such as Polymerase Chain Reaction (PCR) (Dávila et al. 2003; Mugasa et al. 2012) and loop-mediated isothermal amplification (LAMP) of DNA (Notomi et al. 2000; Mitashi et al. 2013), which are very sensitive (1 parasite per ml).

Indirect methods identify the host antibody as a specific response against the presence of the parasite. These methods include indirect immunofluorescence and ELISA which are relatively sensitive but does not differentiate between past and present infections and does not distinguish between species of trypanosomes (Uilenberg 1998) and card Agglutination test for trypanosomosis (CATT): a serological test that is performed in whole blood and uses fixed parasites to agglutinate immunoglobulins and is considered ideal for mass screening in field condition (Magnus et al. 1978; Magnus et al. 2002).

Experimental observations have proven that the *buffycoat* technique offers a good sensitivity to detect trypanosomes in the blood of infected hosts (Paris et al. 1982). It is a relatively easy and fast method that is very useful because it can be used directly in the field (Bailey and Smith 1994). However, in cases of very low parasitaemia the diagnostic results can be affected. This is where highly sensitive techniques like PCR and LAMP, which are capable of detecting the presence of trypanosomes in blood at a level of 1 parasite/ml, are useful. To monitor treatment success or failure, PCR is a fast, clear and easy diagnostic method that allows accurate detection of trypanosomes inhuman and animal species at very low parasitaemia levels (Solano et al. 2002; Dávila et al. 2003; Mugasa et al. 2012).

2.6 Control of Trypanosomosis

Several approaches are used for the control of Trypanosomosis. The use of trypanotolerant cattle in endemic zones, control of tsetse flies, as well as the use of trypanocidal drugs are the most commonly used methods (Murray et al. 1990).

However, none of the mentioned approaches has been shown to be effective and sustainable in the difficult task of trypanosomosis control (McDermott and Coleman 2001). Thus, the recently developed best bet strategies approach aims at maximizing the impact of the three approaches, not only to control trypanosomosis but also to prevent, and in some cases withdraw, drug resistance. This approach consists of the integration of rational drug use, which comprises the treatment of sick animals only after proper diagnosis and using the correct dose, improvement of animal health conditions by deworming and reduction of animal disease risk by controlling the vector (Clausen et al. 2010; Mungube et al. 2012a).

Another strategy that would give good results would be vaccination. However, due to the antigenic variation of the glycoprotein surface of the parasite, the development of a vaccine is hindered. Thus, new alternatives must be developed while vaccination is not available (Huson et al. 2005).

Trypanotolerance is the ability of certain breeds of cattle and some species of wild Bovidae and Suidae to survive and be productive under trypanosome challenge without the need for treatment with trypanocides, where other breeds may die due to the disease (Murray et al. 1990).

The trypanotolerant bovine breed, N´Dama descends from the taurine Hamitic Longhorn, introduced in Africa between 5000 to 2500 BC, while *Bos indicus*, the susceptible one and most prevalent in Africa, was introduced between 200 to 1500 BC. This earlier introduction may have played a role in the co-evolution of N´Dama cattle with the pathogen and tsetse flies, resulting in the development of characteristics that allowed for the survival of the animals even when infected. However, it is worthy to mention that trypanotolerance is hardly inheritable in crossbreeds (Murray et al. 1990).

Trypanotolerant cattle develop less severe anaemia and are able to control the importance and duration of the associated parasitaemia. The use of these animals as a form of trypanosomosis control in Africa is a completely valid and proven approach, yet

only 5 to 8% of the cattle population in tsetse infested countries has this ability and they are confined to West and Central Africa (Kemp and Teale 1998).

Several methods can be used to control tsetse flies and they include: habitat destruction and host removal (Hursey 1985), trapping (Rogers et al. 1994; FAO 2008), use of insecticides (Grant 2001; Pilossof 2016) and sterile insect technique (Vreysen 2001; Vreysen et al. 2013). Additionally, tabanid populations can be controlled using insecticides and traps. These measures can be assisted by vegetation clearing and provision of shelter for the animals (Egri et al. 2013).

The beginning of the effective use of trypanocidal drugs to treat African trypanosomosis can be attributed to the efforts of Paul Erlich who introduced the drug Atoxil around 1905. Since then, several other drugs have been introduced for the treatment of human and animal trypanosomosis, including: suramin and tryparsamide (introduced around 1916-1925), pentamidine (1940-1945) and melarsopol (1949), all four of which are used for the treatment of human trypanosomosis; tartar emetic (1908), homidium bromide (1952), diminazene acetrate (in the 1950´s) and isometamidium chloride (in the 1960´s) for animal trypanosomosis treatment and prophylaxis (Mulligan 1970).

For the control of animal trypanosomosis, several other drugs were introduced. However, due to their toxicity and drug resistance reports, most were abandoned. For example, cattle mortality, due to toxicity, using tartar emetic was approximately 6% (Mulligan 1970). Therefore, isometamidium chloride (ISM), diminazene aceturate (DA) and homidium bromide are currently the most frequently used drugs (Peregrine 1994), with an estimated 35 million doses administered annually (Geerts and Holmes 1998).

In Mozambique, the use of chemotherapy for trypanosomosis control dates to 1912, with the introduction of arsenic acid and later tartar emetic (Rafael 1959; Silva 1959). In 1953, DA was introduced, followed by ISM in the 1960´s. Since the introduction of ISM, both DA and ISM have been used concomitantly for the chemotherapy and chemoprophylaxis of trypanosomosis (Steverding 2008).

Diminazene is an aromatic diamidine. It is an N-acetyl glycine compound chemically known also as 4,4'-(1-Triazene-1,3-diyl)bis(benzenecarboximidamide) (Steverding 2008). It is usually used at a recommended dose of 3.5 - 7.0 mg/Kg of body weight (b.w.) administered intramuscularly and it acts by blocking the replication of trypanome kinetoplast DNA (Aliu et al. 1993; Peregrine & Mamman 1993; Peregrine 1994). It has a short life in blood and is excreted in urine within about 20 days. Diminazene is the most commonly used chemotherapeutic agent against trypanosomosis in Africa (Kellner et al. 1985).

Isometamidium is a phenantridine-aromatic amidine introduced in 1961. It is thought that in T. *congolense*, isometamidium targets mitochondrial electrical potential and inhibits the kDNA topoisomerase, which is involved in the replication of mitochondrial DNA in trypanosomes, although other targets have also been suggested (Sahin et al. 2014). The recommended chemoprophylatic regime is 2 to 4 treatments per annum in average level infested areas and 4 to 6 treatments per annum in heavily infested areas, at a dose of 0.5 - 1.0 mg/Kg b.w. (Kinabo and Bogan 1988).

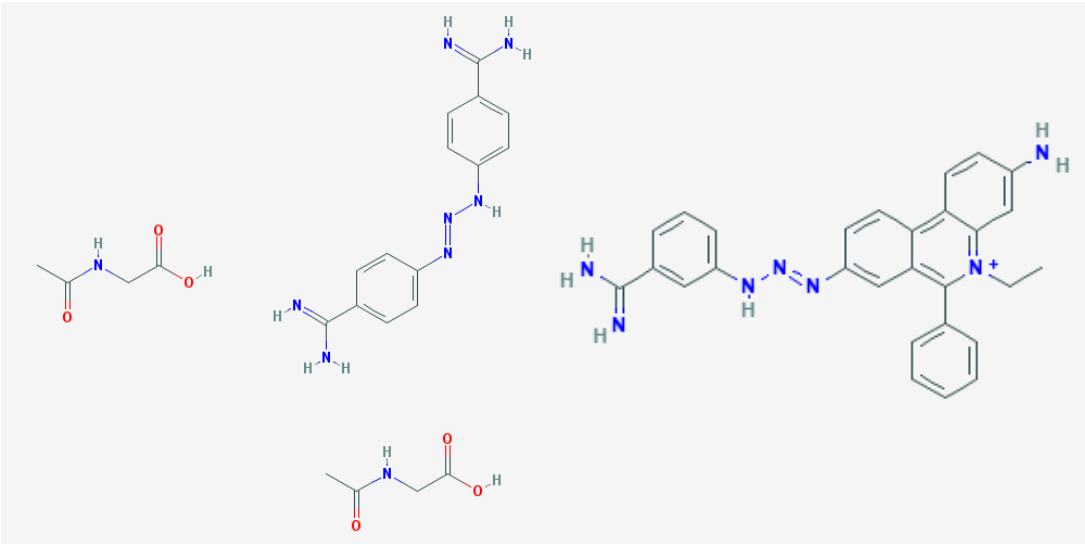

FIGURE 2. 1: Diminazene aceturate (left) and isometamidium chloride (right) 2D structure (Source:https://pubchem.ncbi.nlm.nih.gov/compound/Diminazene_diaceturate and https://pubchem.ncbi.nlm.nih.gov/compound/Isometamidium#section)

2.7 Trypanocidal drug resistance

In Africa, drug resistance (DR) represents a serious threat to the sustainable control of trypanosomosis. Drug resistance has increased mortality and morbidity in livestock populations and consequently reduced the production and productivity of cattle herds. Furthermore, it has amplified the costs of cattle-keeping activities and reduced the available options for trypanosomosis control (Kagira and Maina 2007).

In the last 60 years no new trypanocides have been developed and DR has been reported in 21 countries (Sinyangwe et al. 2004; Delespaux et al. 2008b; Moti et al. 2012; Mungube et al. 2012b; Sow et al. 2012; Tsegaye et al. 2015; Tchamdja et al. 2017; Solomon and Workineh 2018), including Mozambique, where it was experimentally confirmed for both ISM and DA (Jamal et al. 2005).

The intensive and incorrect use of the currently existing trypanocidal drugs are the main reasons for the resistance of trypanosome populations to ISM and DA treatment (Delespaux and de Koning, 2007; Geerts and Holmes 1998). In trypanosomosis, DR is usually the result of the loss of capacity for drug uptake by the parasite, an alteration in drug-target interaction, or even a change in the parasite efflux mechanism (Baker et al. 2013). However, the precise mechanisms underlying DR, for ISM, are still undeciphered.

In the case of DA, it is thought that massive drug accumulation constitutes the stem for the selective trypanocidal action and tests have shown a strong link between resistance and loss or alteration in transport capacity (De Koning et al. 2004). In fact, for *T. brucei*, DA-resistance was found to be associated with a mutation in a P2-type purine transporter, encoded by the *TbAT1* gene, which is responsible for the uptake of the drug by the parasite (Matovu et al. 2003). With the works by Delespaux et al. (2006), it was found that, in *T. congolense*, an orthologue gene of *TbAT1*, the *TcoAT1* gene, seemed to play the same role. Later on, Munday et al. (2013), revealed that for *T. congolense*, *TcoAT1* acts in the transportation of inosine (P1-type purine transporter), as does the syntenic gene TbNT10 in *T. brucei*, and that the observed mutation in the *TcoAT1* gene

does not change the DA sensitivity of the parasite, but is possibly associated to the phenotype as genetic marker.

For ISM, it is assumed that changes in the efflux mechanism in the parasite result in a reduced accumulation of ISM inside the kinetoplast, thus creating resistance (Sutherland et al. 1991; Sutherland and Holmes 1993; Mulugeta et al.1997) and that this process is faster and facilitated in immunologically weak hosts (Tihon et al. 2017).

The phenomenon of drug resistance becomes more worrying when multiple drug resistance enters the equation, drastically decreasing the options for treatment of trypanosomosis. Field and laboratory tests have confirmed the occurrence of single and multiple drug-resistance to ISM and DA and resistant strains of *T. congolense* were isolated in Ethiopia (Afewerk et al. 2000; Tewelde et al. 2004), Mali (Mungube et al. 2012b), Burkina Faso (McDermott et al. 2003) and Togo (Tchamdja et al. 2017).

The delay in producing new trypanocidal drugs together with the absence of an effective vaccine against the disease and the high costs of studies on vector distribution and its control leave no option but to use the 3 currently available compounds sustainably until further developments in the area. Sustainable use means minimal use of trypanocides; strictly following the indicated dose and controlling drug imports and administration by legal authorities (Wilkinson and Kelly 2009; Clausen et al. 2010; Mungube et al. 2012a; Sutcliffe et al. 2014).

In order to provide a sustainable control of trypanosomosis, a good understanding of the DR phenomenon and development of tests for the identification of resistant and sensitive populations of trypanosomes is needed (Delespaux et al. 2008).

To detect DR, field and laboratory tests can be used. Laboratory tests can be performed *in vivo* and *in vitro*. *In vivo* tests include single and multi-dose tests in mice that allow precise determination of the degree of resistance of individual *Trypanosoma* sp and can

include a large number of isolates at relatively low costs. They also include a test in calves that, contrarily to mice tests, is able to accurately predict the curative doses of trypanocidal drugs for sick cattle (Eisler et al. 2001). *In vitro* tests include PCR, which is a very powerful tool, but to date, for *T. congolense*, the mechanism by which the mutation in the genes responsible for DA resistance act has not been completely elucidated (Munday et al. 2013), what makes the availability of PCR based methods for detection of DR, in *T. congolense*, not possible at the moment. For ISM, despite some developments and the identification of genes encoding ATP-binding cassette transporters and drug/metabolite transporters as genes involved in the development of resistance, further studies are needed to provide a powerful molecular tool for DR detection (Tihon et al. 2017). Drug tests in trypanosome isolate cultures also constitute an option for *in vitro* drug resistance determination, yet it can be an expensive approach and is not possible to execute in areas where laboratory facilities are not available (Gray et al. 1993; Coustou et al. 2010; Sahin et al. 2014; Gillingwater et al. 2017).

While molecular tools are not yet completely developed, field tests can be considered as an alternative as they have proven to be reliable, relatively easy and fast to conduct (Eisler et al. 2001; McDermott et al. 2003). In the field, longitudinal block-treatment studies stand out as cheaper and valuable options for areas where laboratory facilities are not available, with the added advantage of not requiring the isolation of trypanosomes. This approach has been used in different countries in Africa (Eisler et al. 2001; Mungube et al. 2012a; Tchamdja et al. 2017).

2.8 Trypanosomosis and its impact in Mozambique

It has been known for a long time that Mozambique is one of the countries that is most affected by tsetse and trypanosomosis. Together with ticks and tick borne diseases, trypanosomosis has been widely recognized as the major constraint to livestock health and production (Sigauque et al. 2000; Jamal et al. 2005; Specht 2008; Machado et al. 2016; Cholleti et al. 2018).

In, Mozambique, 75% of the country is infested by four species of tsetse fly, namely *Glossina austeni*, *G. brevipalpis*, *G. morsitans* and *G. palidipes* (FAO 1987).All the

four species are found in the Zambezia province (Wohlford et al. 1999; Sigauque et al. 2000; Jamal et al. 2005).The first case of trypanosomosis in the country was reported in 1908 in Matutuine district. At the time, the disease was thought to occur as a result of mechanical transmission and this remained so until other cases were observed in the years that followed and tsetse flies were captured in the district in 1921 (Sigauque et al. 2000). In 1937, *Missão de Combate às Tripanossomíases* (Mission for Control of Trypanosomiasis) was created to survey and control tsetse and trypanosomosis in Mozambique (MCT 1960). Along the years, several other regions were confirmed to be infested with tsetse flies and some strategies such as vegetation clearing and elimination of wildlife were adopted to fight the disease and the vector (DNP 1999).With approximately 43000 heads of cattle (SPPZ 2012),the Zambezia province represents one of the areas most affected by trypanosomosis. During the civil war, that lasted 16 years (1976-1992), most of the cattle died. Attempts to restore the cattle population have been taking place since 1995. However, high mortality due to trypanosomosis is considered one of the major reasons for the reduced success in the approach (DNSV 1995).

Surveys conducted in 2006in the central area of the country, Zambezia province included, showed a great increase in the number of cases of animal trypanosomosis from 1989 to 2005. The resettlement of people in tsetse-infested areas after the war and livestock husbandry conducted without awareness of basic animal health management rules are considered to be the main causes for this increase (Specht 2008).

Furthermore, a lack of control of the movement of both cattle and people in addition to poor tsetse and trypanosomosis control are leading to alterations in cattle distribution in the country, consequently contributing to the spread of the disease (Specht 2008). In 2005, drug resistance was identified in Chinde, Nicoadala and Maganja da Costa districts, in Zambezia province (Jamal et al. 2005).

2.9 Aim

An effective control of trypanosomosis depends on the availability of updated information, not only about the disease prevalence but also about the distribution of vectors and their role in the epidemiology of the disease. Hence, the aim of the present

study was to provide an update on the distribution of trypanosome infections and drug resistance in Nicoadala district, Zambezia province, and evaluate the relative role of haematophagous insects in the transmission of trypanosomes.

2.10 Thesis overview

In view of the above aim, the following is the overview of this thesis:

Chapter 3: To study the distribution of drug resistance in Nicoadala district, Zambezia province, trypanosome positive animals were treated with diminazene aceturate and isometamidium chloride and monitored for relapses. With this experiment single and multiple drug resistance was assessed. Treated animals were monitored using the *buffycoat* and PCR techniques.

Chapter 4: In this chapter is described how tsetse flies and tabanids were captured in three distinct habitats in Nicoadala district and identified using the taxonomy and the Cytochrome Oxidase 1 molecular marker. The seasonal abundance of biting flies and the efficiency of the traps used were also verified

Chapter 5: This chapter is focused on the detection of *Trypanosoma* spp in tsetse and tabanids. This assessment was conducted through the use of 18s-PCR-RFLP, which is a pan-trypanosoma PCR. The species of *Trypanosoma* circulating in Glossinidae and Tabanidade was, thus, assessed. With this approach additional conclusions can conjectured regarding the maintenance/diffusion of drug resitant trypanosomes by trypanosomosis vectors, especially tabanids.

Chapter 6: In this chapter, the results of the thesis are discussed, and a general conclusion is formulated. The path to accomplish the task of providing updated information on trypanososmosis and trypanocidaldrug resistance is evaluated and recommendations on future prospects are summarized. Moreover, the challenges faced during the development of the drug resistance and entomological surveys are analyzed.

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CHAPTER 3 : Resistance to trypanocidal drugs in cattle populations of Zambezia Province, Mozambique

3.1 Introduction

African animal trypanosomosis (AAT) is a tsetse-transmitted, debilitating parasitic disease affecting both domestic and wild vertebrates (Barret et al. 2003). This disease is caused by protozoa, belonging to the genus *Trypanosoma* (Shah-Fischer and Say 1981). In domestic animals, trypanosomosis is a disease with a great economic impact, affecting not only the well-being of the livestock population, but also efficient food production in crop-livestock production systems (Davila et al. 2003 and Shaw et al. 2014). Among all African pathogenic trypanosome species, *Trypanosoma congolense* is arguably the one causing major losses in southern Africa (Sigauque et al.2000, Sinyangwe et al. 2004 and Specht 2008).The human form of the disease, known as sleeping sickness, is also sporadically detected in Mozambique, although disease surveillance is weak and underdetection and underreporting are possible (Büscher et al. 2017; Franco et al. 2017).

The treatment and prevention of trypanosomosis in cattle is carried out using three drugs, namely isometamidium chloride (ISM), diminazene aceturate (DA) and homidium bromide. In the last 50 years, no new trypanocides have been made available in cattle and drug resistance has been reported in several countries (Delespaux et al. 2008). In trypanosomosis, drug resistance is usually the result of the loss of capacity for drug uptake by the parasite, an alteration in drug-target interaction, or even a change in the efflux mechanism (Baker et al. 2013), as it is suggested for ISM (Sutherland et al. 1992).

Drug resistance is often associated to areas with high drug usage (Delespaux and De Koning 2007). This is compounded by the lack of new drugs and the incorrect utilization of existing ones (Geerts and Holmes 1998). Precise mechanisms of drug resistance are still undeciphered. However, in *T. brucei*, DA-resistance was found to be associated with a mutation in a P2-type purine transporter, encoded by the *TbAT1* gene, which is responsible for the uptake of the drug by the parasite (Matovu et al.2003 and De Koning et al.2004). In *T. congolense*, an orthologue gene of *TbAT1*, the *TcoAT1* gene, seemed to play the same role (Delespaux et al. 2006). However, Munday et al.

(2013) found that for this particular species, *TcoAT1* acts in the transportation of inosine (P1-type purine transporter), as does the syntenic gene TbNT10 in *T. brucei*. The observed mutation in the *TcoAT1* gene does not change the DA sensitivity of the parasite but is associated to the phenotype as genetic marker. The possible link between those two mutations remains unclear. Isometamidium kills protozoa by blocking the synthesis of nucleic acids. It intercalates between DNA base pairs and blocks the RNA and DNA polymerases. In sensitive strains, ISM is transported into and accumulated in the kinetoplast. It is assumed that changes in the efflux mechanism in the parasite result in a reduced accumulation of ISM inside the kinetoplast, thus creating resistance (Sutherland et al. 1991, Sutherland and Holmes 1993 and Mulugeta et al.1997).

In Mozambique, the use of chemotherapy for trypanosomosis control dates back to 1912 with the introduction of arsenic acid and later tartar emetic. Nevertheless, reports of failure of these compounds and, in some cases, the development of drug resistance were reported. In 1953, DA was introduced (Rafael 1959 and Silva 1959). In the 1960´s, ISM was introduced, and since then, it has been used, together with DA, as a chemoprophylactic and chemotherapeutic drug against trypanosomosis. Resistance to anti-trypanocidal drugs has been reported in at least 21 countries in Africa (Chitanga et al. 2011), including Mozambique, where it was experimentally confirmed for both ISM and DA (Jamal et al. 2005 and Macucule 2014).

Despite the above mentioned explanations, the mechanism of resistance has not been fully elucidated for either drug. The lack of knowledge about the exact gene(s) that are responsible for the resistance profile in the livestock pathogenic species considered, constrains the development of diagnostic molecular tools. In the absence of progress in this area, field tests (e.g. block treatment) can be considered as the alternative, as they have proven to be reliable, relatively easy and fast to conduct. Furthermore, they do not require the isolation of trypanosomes (Eisler et al. 2001 and McDermott et al. 2013). These advantages constitute the rationale of the approach used in the present study.

The semi-nested 18S PCR-RFLP, chosed for monitoring the animals during the post-treatment period, is a pan-trypanosome PCR that can detect trypanosome infections caused by different species including *T. vivax*, *T. brucei* and *T. congolense*. The restriction analysis allows not only for a clear distinction between the three mentioned species but also the distinction between the subgroups within the *T. congolense* group (Geysen et al. 2003).

Drug resistance has been shown to be an important drawback to agricultural development in Africa in general, and in Mozambique in particular, where it has caused thousands of cattle deaths and a drastic reduction in the cattle population of Zambezia province. Despite this, local veterinary services have no structured program for trypanosomosis control and for regulating the use of trypanocidal drugs that is left to the initiative of the owner and/or animal health technicians. The present study provides an update on trypanocidal drug resistance in Nicoadala district and specifically evaluates the proportion of resistant strains in previously described hotspots. In addition to information on drug resistance, AAT epizootiology should be further investigated, and the role of biological and mechanical transmission should be urgently revisited. Studies on vector species composition and distribution are in progress in order to shed light on the respective roles of tsetse flies and other biting flies in the transmission of trypanosomes.

3.2 Material and methods

3.2.1 Study area

The study was conducted in five villages in Nicoadala district, Zambezia province (Figure 1). The villages were chosen according to trypanosome prevalence obtained from a previous surveys conducted in Zambezia province between 2009 and 2013. The district has a history of high usage of trypanocides and has been previously identified as a drug resistance hot spot (Jamal et al. 2005).

3.2.2 Cross sectional study and sampling framework

Cattle blood samples for both parasitological and molecular analysis were collected in May-July 2014. Sample size was estimated according to Cannon and Roe (1982), where

expected prevalence was 50% with a 95% confidence interval and a 5% desired absolute precision. In each of the villages, all animals were screened for trypanosomes using the buffy-coat technique (BCT) (Murray et al. 1977). Four ml of blood were collected using vacutainer tubes containing di-sodium salt of ethylenediaminetetraacetic acid (EDTA) from the coccygeal vein of each animal. Two capillary tubes were then filled and sealed with crystaseal (Hawksley, Lancing, United Kingdom) and the blood was centrifuged at 1500 rpm for 5 minutes in a microhaematocrit centrifuge (Hawksley®, Lancing, United Kingdom). One of the capillary tubes was used for packed cell volume (PCV) reading. The other was used for BCT and the preparation was examined for the presence of motile trypanosomes under a light microscope (Leica Mycrosystems, Wetzlar, Germany) at a magnification of 400X.In total, 467 bovines from five villages were screened for trypanosomes.

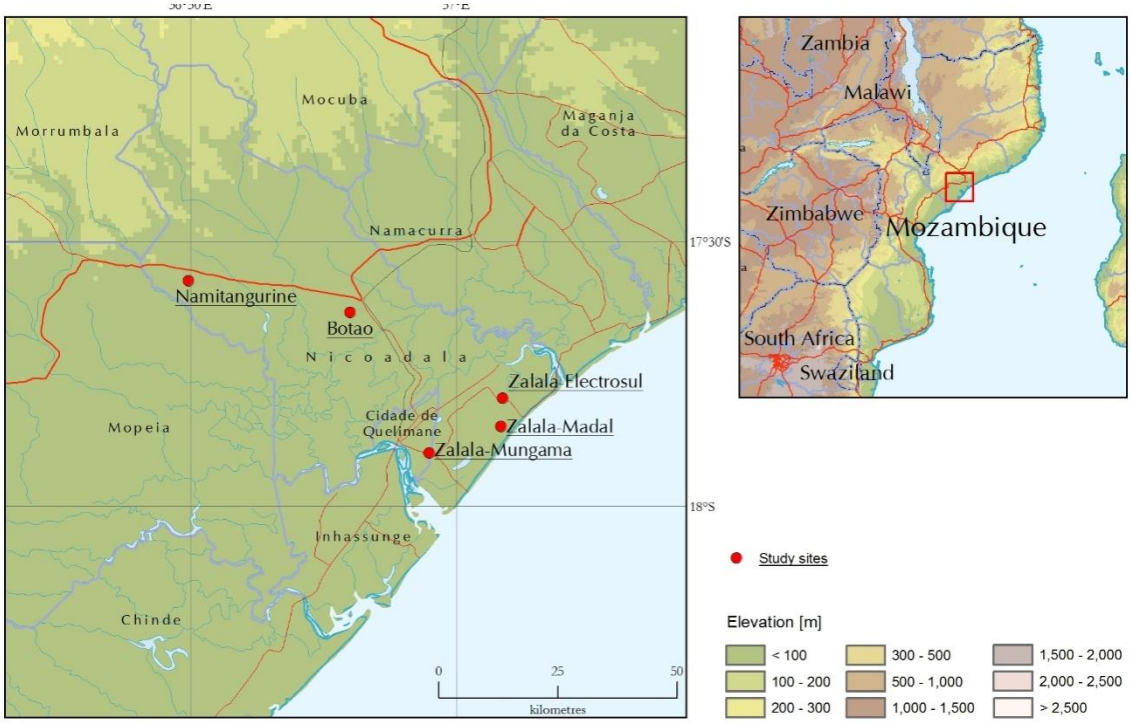

FIGURE 3. 1: The five study villages in Nicoadala district, central province of Zambezia. The study area is identified by the red square in the map of Mozambique.

3.3 Drug Resistance Study Test

The trypanosome-infected animals from each village were randomly divided into two groups with a minimum of 9 animals assigned to each group, for drug-sensitivity tests of DA (Inomazene®, INOUKO Generics, Paris, France) and ISM ((Inomidium®,

INOUKO Generics, Paris, France). The number of animals per group was estimated according to the formula from Cannon and Roe (1982) to detect the presence of at least one resistant isolate, using an infinite population size and an expected proportion of trypanosome resistant isolates of 30%. The first group was treated with 3.5 mg/kg of body weight (b.w.) DA and the second one with 0.5 mg/kg b.w. ISM (2% solution), as described by Mungube et al. (2012a). The animals were monitored for relapses on days 14 and 28 post-treatment, using the BCT method. At day 28, the trypanocidal drugs were switched to expose the strains previously treated with DA to ISM and vice versa, and to verify if there was resistance to only one drug (single-) or to both drugs (multi-resistance). Animals were monitored for 14 more days. At the end of the experiment all animals that remained positive were treated with DA at 7.0 mg/kg b.w. All positive animals were ear-tagged. Animal ID, owner´s name, location, GPS coordinates, age (calf, young, adult), sex, breed (zebu, taurine, crossbred), PCV, body condition (cachectic, lean, good) and infection type (trypanosome species) were recorded.

On monitoring days, for all animals, 1 vacutainer tube was filled with approximately 2ml of blood per animal for DNA extraction and molecular tests (semi-nested 18S rRNA PCR). Blood was kept at -20ºC until analysis. Protocols and interpretation of the molecular analysis results followed the method described by Geysen et al. (2003).

3.4 DNA extraction and semi-nested 18S rRNA PCR

Genomic DNA was extracted from blood samples collected from the animals using a Qiagen® QIAamp DNA extraction kit (Qiagen, Hilden, Germany), following manufacturer instructions. For the molecular detection of trypanosomes, 18S semi-nested rRNA PCRs were run, targeting a fragment of the 18S ribosomal RNA gene, on an Eppendorf Mastercycler® gradient thermocycler (Eppendorf AG, Hamburg, Germany), under the following conditions: 10 s at 98ºC; 40 cycles of 98ºC for 1 s, 58ºC for 5s, 72ºC for 15s and a final step of 72ºC for 1 min. For this, two reactions were carried out in a final volume of 25µl containing 1X Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Sweden), 200µM of each dNTP, 20pmol of each primer, and 5µl of eluted DNA for the 1st reaction and 2.5µl of PCR product from the 1st reaction for the nested reaction. Water was added to obtain a final volume of 25 µl. The primers usedwere18ST nF2 5'-CAA CGA TGA CAC CCA TGA ATT GGG GA-3' and 18ST nR3 5'-TGC GCG ACC AAT AAT TGC AAT AC-3' for the 1st reaction and

18ST nF2 and 18ST nR2 5'-GTG TCT TGT TCT CAC TGA CAT TGT AGT G-3', for the nested reaction. For PCR product visualization, the samples were run in a 2% agarose gel where 2 µl of loading dye were mixed with 5 µl of nested PCR product and loaded onto the gel. A 1000bp DNA ladder was also loaded (4 µl) for fragment size determination, and the gel run for 45 min at 120 volts. The gel was stained with GelRed (Biotium, Inc., Fremont, CA, USA) at 4 µl per 100 ml of gel directly added to the gel before polymerization.

3.5 Restriction Fragment Length Polymorphism (RFLP)

All the nested PCR products (positive samples in agarose gel) were digested with *MspI* and *Eco571* (Acul) enzymes at 37º for 60 min, in a final volume of 15 µl containing 1X Enzyme buffer, 0.5 µl *MspI*, 0.5 µl *Eco571*, 8.5µldeionized water and 4.0µl of PCR product. The samples were then run in a 3% agarose gel at 80 volts for 120 minutes, where 2 µl of loading dye was mixed with 4 µl of digested PCR product and loaded onto the gel together with a 50 bp DNA ladder. The gel was stained by adding GelRed (Biotium, Inc., Fremont, CA, USA) at 4 µl per 100 ml of gel before polymerization.

3.6 Results

3.6.1 Cross sectional study: trypanosome prevalence

Out of the 467 animals screened using BCT, 107 were found to be positive for *Trypanosoma congolense* infection in three of the five villages. No positive animals were diagnosed in both Zalala-Electrosul and Zalala-Madal villages. Only *T. congolense* infections were detected during the screening. Detailed description of the total prevalence per village can be found in Table 1. In the group of trypanosome-positive animals, the mean PCV (%) was 27%, significantly different from the negative sample group, i.e. 31% (P-value <0.0001, t-test). The lowest PCV (12%) was found in one animal Botao.

TABLE 3. 1: Prevalence (by BCT), and mean PCV (%) off all sampled animals, in the 5 study villages in Nicoadala district (Mozambique), before treatment, May 2014.

Village	Animals screened	Positive animals	Prevalence (%)	Mean PCV (%)
Botao	149	66	44.3	30
Zalala-Mungama	50	21	42.0	31
Namitangurine	120	20	16.7	28
Zalala-Madal	110	0	0	32
Zalala-Electrosul	38	0	0	31
Total/ Mean	467	107	22.9	31

3.6.2 Treatment response to isometamidium chloride

On day 14 after treatment, three animals that had been treated with ISM were still positive in Botao, while in Mungama four animals treated with ISM were positive. On day 28, the day on which the drug swap was carried out, four animals from the ISM group, were positive in Botao. In Mungama and Namitangurine however, no animals from the ISM group were found to be positive (Table 2).

3.6.3 Treatment response to diminazene aceturate

On day 14 after treatment with DA, the number of positive animals was six and three in Botao and Mungama, respectively. On day 28, four animals in Botao and four animals in Mungama were still positive after treatment with DA (Table 2). As was observed in the ISM group, no relapses were recorded in Namitangurine.

3.6.4 Multiple drug resistance

After swapping the drug, microscopic analysis on day 42 revealed six positive animals (9%; n = 66) in Botao and two positive animals (9.5%, n = 21) in Mungama, thus pointing to the presence of multiple drug resistance.

TABLE 3. 2: Treatment response results, indicated as the number of animals remaining trypanosome positive after treatment, verified by BCT and if negative checked by PCR-RFLP, in Zambezia Province in 2014. To the positives found by PCR-RFLP were added the positives already detected by BCT.

Village/Method	Day 14		Day 28		Day 42	
	DA	ISM	DA	ISM	DA/ISM	ISM/DA
Botao/BCT	6/31 (19.4%)	3/35 (8.6%)	4/31 (12.9%)	4/35 (11.4%)	2/31 (6.5%)	4/35 (11.4%)
Mungama/BCT	3/10 (30.0%)	4/11 (36.4%)	4/10 (4.0%)	0/11 (0%)	2/10 (20.0%)	0/11 (0%)
Namitangurine/BCT	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
Botao/BCT + PCR-RFLP	7/31 (22.6%)	5/35 (14.3%)	8/31 (25.8%)	5/35 (14.3%)	4/31 (12.9%)	4/35 (11.4%)
Mungama/ BCT + PCR-RFLP	5/10 (50.0%)	4/11 (36.4%)	5/10 (50%)	2/11 (18.2%)	3/10 (30.0%)	1/11 (9.0%)
Namitangurine/ BCT + PCR-RFLP	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)

3.6.5 Molecular test by 18S PCR-RFLP

All the negative results from monitoring days 14, 28 and 42 were verified using semi-nested 18S PCR-RFLP. The 18S PCR allowed for the detection of positive cases where the BCT-method detected negatives as shown in Table 2. A gel figure showing *T. congolense* positive results detected by 18S PCR-RFLP can be found in figure 2.

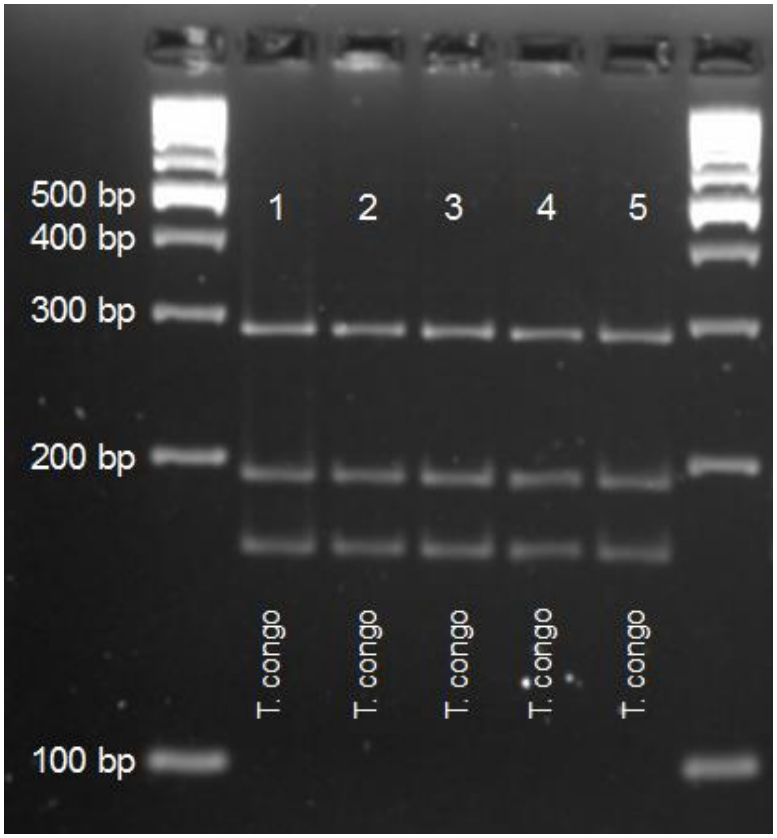

FIGURE 3. 2: RFLP restriction enzyme analysis using MspI and Eco571 digestion of 18 Ssu-rDNA from Trypanosoma congolense isolates in agarose gel. 100 bp DNA ladder was included in both sides on the gel.

3.7 Discussion

The Mozambican province of Zambezia is considered to be within an area infested by tsetse flies with medium to high trypanosome challenge, the "common fly belt" (RTTCP 2000). This is supported by the findings of the present study, where trypanosomes were detected in cattle in three out of five study villages. There may be several reasons for the absence of positive cases in Zalala-Madal and Zalala-Electrosul. One such reason could be the use of trypanocides, as it is known that most of the cattle in this area belong to the commercial sector and treatment with trypanocides is frequent (Specht 2008). However, the observed absence of positive cases could also be attributed to anthropogenic activities in the area (urbanization, clearing of vegetation for agriculture and new settlements), which may have altered the distribution of tsetse flies in the area or at least influenced their density, although this was not verified against other data in the present study. According to Malele et al. (2011), tsetse distribution can be affected by different factors such as changes in the land use and increases in human

population and activities. These activities usually result in changes in the ecology of the areas previously occupied by tsetse flies, mainly through the elimination of breeding sites and hosts.

Specht (2008), working in Zambezia province in 2004, found a 15% prevalence of *T. congolense*, making it the dominant trypanosome species in the area. In the present study, only *T. congolense* infections were detected. Similar results were found by Jamal et al. (2005), when studying the susceptibility of *T. congolense* isolates in Zambezia province. In several studies conducted in sub-Saharan Africa, *T. congolense* has been found to be the most prevalent trypanosome species in cattle, especially in southern Africa (Sigaúque et al. 2000, Van Den Bossche 2001, Simukoko et al. 2007, Laohasinnarong et al. 2011, Mwandiringana et al. 2012; Simo et al. 2015), while in other areas *T. vivax* is the dominant species (Ahmed et al. 2016; Cecchi et al. 2014).

One of the clinical signs of trypanosomosis in cattle, especially in cases where it is caused by *T. congolense* infections, is low PCV (Mbewe et al. 2015 and Marcotty et al. 2008). When comparing the mean PCV between trypanosome-negative and -positive animals, a significant difference ($P<0.0001$) was observed, with lower mean PCV's in trypanosome-positive animals.

Drug resistance (DR) to trypanocides is a dynamic process and it is spreading and increasing in Africa. The detection of DR and identification of resistant *Trypanosoma* sp. populations remains a challenge. In the present study, a block treatment test was applied to assess single and multiple drug resistance to ISM and DA (Mungube et al. 2012a). Polymerase chain reaction (PCR) and BCT, which are both methods that are typically employed to estimate the prevalence of trypanosome infection, were used to detect ISM and DA treatment failure in the current study. Although BCT is a commonly used method for field assessment of trypanosomosis and for individual animal treatment monitoring, it is limited by the levels of parasitaemia in the infected animals (Paris et al. 1982). The visualization of parasites in body fluids is currently the most widely used method for the diagnosis of animal trypanosomosis in endemic regions (Cecchi et al.

2014). However, according to Van den Bossche (2001), parasitaemia can fluctuate below the levels of detection by microscopy. Nevertheless, with the advent of PCR, this limitation was surpassed, as it allows for the detection of trypanosomes in samples with very low parasitaemia (Clausen et al. 1998; Desquenes and Davila 2002). Nonetheless, due to its high cost, PCR is not the technique of choice to support decisions on treatment options for individually diagnosed animals (Cox et al. 2010). In the present study, using BCT, trypanosome-positive animals were diagnosed, treated and monitored for relapses in the field. However, PCR detected evidence of trypanosome infection in samples that appeared negative using microscopic examinations during the monitoring phase on days 14, 28 and 42 post-treatment.

Using the BCT method to check for trypanosomes 28 days post-treatment, the treatment failure rate for ISM was 6% and 0%, while the one for DA it was 6% and 19%, in Botao and Mungama, respectively. These results revealed the difficulty that exists for farmers to be successful in the treatment of trypanosomosis. After the drug swap, conducted to detect multi-resistant strains, a total of eight isolates that were resistant to both drugs were identified on day 42. The present results confirmed those obtained by Jamal et al. (2005), which indicated that resistance to both, ISM and DA is present in Zambezia province. Multi-resistant cases have also been detected e.g. in Burkina Faso (Clausen et al. 1992), Zambia (Sinyangwe et al. 2004), Ethiopia (Miruk et al. 2008), Mali (Mungube et al. 2012a) and Togo (Tchamdja et al. 2017). In the present study, contrary to what was reported by Geerts et al. (2001), Mungube et al. (2012a) and Tchamdja et al. (2017), treatment failure was higher for DA than for ISM. Multiple drug resistance can be a serious problem to cattle keepers, especially in this specific case, where the tested drugs are considered to be the most reliable ones presently available on the market.

Most of the cattle in Zambezia province belong to commercial farmers and this is usually associated with intensive use of trypanocides. Although it cannot be assumed that the results from the present study are a reflection of this, it is a conjecture that cannot be disregarded.

The results confirmed the existence of single and multi-drug resistance in Nicoadala district, Zambezia province. Furthermore, based on the previous reports of resistance in the area, it is worth noting that the current results demonstrated that the geographical location of trypanocides-resistant hot spots have remained unchanged for at least the past 12 years. This information is fundamental when considering the control of trypanosomosis in the area. A better understanding of the drug resistance in the area allows the definition of evidence-based, adapted measures for the progressive control of AAT (Diall et al. 2017).

The use of best bet strategies (Mungube et al. 2012b; Tchamdja et al. 2017) for the control of the disease in the area could be a valuable approach as it can be seen that the sanative pair of drugs can, at any moment, no longer be an option. The use of strategies involving rational drug use, which consists in the treatment of sick animals only and after proper diagnosis and using the correct dose should be urgently implemented by the local veterinary services. Moreover, general improvement of animal health conditions by deworming and reduction of animal disease risk, and vector control as described by Clausen et al. (2010) can also be of great impact. The development of molecular tools to allow for a faster assessment of the status of drug resistance is also advisable and should be encouraged.

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CHAPTER 4：CHARACTERIZATION OF HAEMATOPHAGOUS FLIES IN THE TRYPANOCIDE RESISTANCE FOCI OF NICOADALA DISTRICT, MOZAMBIQUE

4.1 Introduction

The distribution of tsetse flies in Mozambique is known to be discontinuous due to natural barriers and habitat changes resulting from human activities. The presence of tabanids is particularly notable in Zambezia province, particularly in the Nicoadala district that has been previously identified as a trypanocidal resistance area (Mulandane *et al.* 2018). However, their role in the transmission of pathogens remains unknown.

Understanding the level of interaction between haematophagous insects and their hosts can, in a certain way, help understand the epidemiology of the diseases they transmit (Gondwe et al. 2009). However, little has been done to update the knowledge on the composition of haematophagous fly populations in the country since the work of Dias (1966).

Several different methods can be employed for the study of tsetse and tabanids biodiversity, with standardized trapping often employed. The use of trapping for haematophagous insects species distribution studies have been extensively described in scientific literature (Herczeg *et al.*, 2014; Krčmar *et al.*, 2014; Brightwell & Dransfield, 2014; Nthiwa *et al.*, 2015; Wondimu *et al.*, 2017; Lendezele *et al.*, 2017).

There is a plethora of available trap-types, all described as effective for different species in different habitats. From the literature, the H-trap and NGU trap are arguably the most effective in south-east Africa as they were designed for the four main species present in the area (Kappmeier, 2000; Brightwell *et al.* 1987). In the present study, H trap, developed in South Africa for *G. brevipalpis* and *G. austeni* (Kappmeier 2000), Epsilon trap, developed in Zimbabwe as an alternative trap for savanna species such as *G. pallidipes* and *G. morsitans* (Esena 2013) and NGU trap, developed in Kenya for catching savannah flies such as *G. pallidipes* and also effective for tabanids (Brightwell *et al.*, 1987), were selected given the species currently identified as present in the country.

The Chelex100 protocol for DNA extraction is a quick and simple method that allows for the obtantion of good genetic material for analysis. It does not require the use of proteinase K and phenol-chloroform however it is as efficient as the methods that use them. During the extraction process Chelex binds to the cations that act as cofactors for DNases, protecting, this way, the DNA from degradation by DNases (Walsh et al 1991). It has been proven effective for extraction of DNA from insects in some publications (Solano et al 2000; Ravel et al 2007).

The lack of the information regarding tsetse and tabanids distribution together with constant reports of high trypanosomosis prevalence in Nicoadala district urge for an accurate monitoring study targeting the haematophagous vectors of trypanosomosis. This would allow their species composition to be elucidated with the ultimate goal of gaining a better understanding of their role in maintaining and spreading trypanosomes in the trypanocidal drug resistance foci of Nicoadala district.

4.2 Material and Methods

4.2.1 Study Area

Three grazing areas, Botao, Namitangurine and Zalala, all located in the Nicoadala district (17.608ºE; 36.820º N), were used for the survey. The region has a rainy tropical savannah climate according to the Köppen-Geiger system (Köppen and Geiger 1928), with two seasons: rainy and dry. The summers are characterized by elevated rain levels. The average temperature in Nicoadala is 25.6 °C with about 1428 mm annual rainfall. In Zalala, the vegetation is mostly composed of coconut trees, grasses and small shrubs covering approximately 420 ha. Botao (±1300 ha) is an open forest with large grassland areas. Namitangurine (±1600ha) is a slightly closed forest with a high diversity of trees and shrubs. The dominant tree species are *Pterocarpus angolensis, Swatzia madagascariensis, Afzelia quanzensis, Milletiastuhlmannii, Khayanyasica, Pericopsis angolensis, Combretum imberbe, Brachystegia spiciformis* and *Pteliopses myrtifolia.* Both Botao and Namitangurine are areas with favorable pasture and water availability with some wild animals present (MAE, 2005).

4.2.2 Sampling

For 12 months, a total of 5 H-traps, 5 epsilon traps and 5 NGU traps were deployed in Botao. Similar approach was adopted in in Zalala. On the other hand, in Namitangurine, 10 H-traps, 5 epsilon traps and 10 NGU traps were deployed. Trapping sites were geo-referenced using a Garmin (GPSMAP®76) global positioning system (GPS). The traps were left at the deployment sites for the duration of the study (Barclay & Hargrove, 2005; Bouyer et al., 2010). All traps were placed at least 200 m apart to prevent interference (Mihok, 2002; Lendzele *et al.*, 2017). Each trap was baited with 4 polyethylene sachets (150µm thick, surface area of 30 cm^2) containing 4 ml of 1-octen-3-ol and one 15ml plastic bottle with a 5 mm hole on top containing acetone (Vale *et al.* 1980). Flies were collected weekly and those captured were placed in 1,5 ml tubes containing 1 ml of 70% ethanol until further analysis was conducted (Sigauque *et al.*, 2000 and Vale and Hall, 1985). All the bottles containing trapped flies were labeled with location, trap number, date and time and then replaced with empty ones.

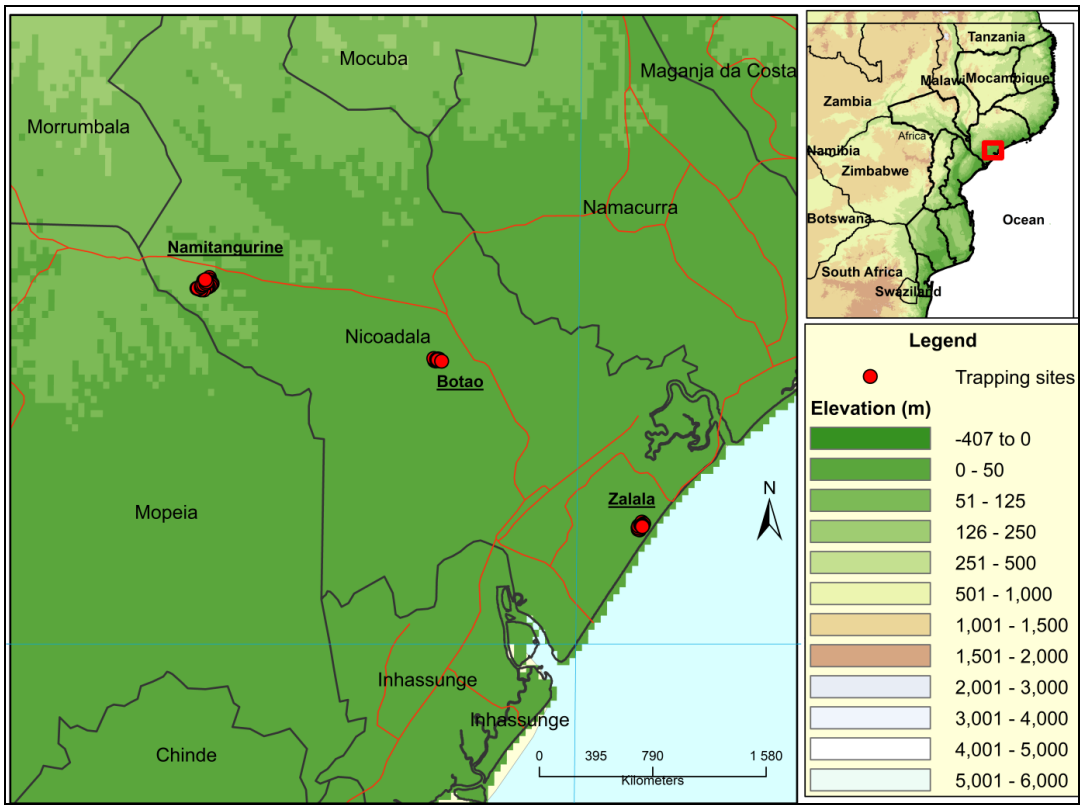

FIGURE 4. 1: Trapping sites in the three grazing areas in Nicoadala district, central province of Zambezia. The study area is identified by the red square in the map of Mozambique**.**

4.2.3 Identification of haematophagous insects and statistical analysis

Captured insects were dry mounted using entomological pins before identification. All tsetse flies captured were identified using tsetse control personnel training manual key for *Glossina* species identification (Pollock 1991 and FAO 2008). Tabanids were identified to genus and species level, using the morphological keys of Oldroyd (1952, 1954, 1957) supplemented by Dias (1966) and Taioe et al. (2017). This was done both in Mozambique at the Biotechnology Center – Eduardo Mondlane University and in South Africa at the Department of Veterinary Tropical Diseases – University of Pretoria. Additionally, to access species richness and evenness from and between the grazing areas, alpha (α) and beta (β_w) diversity were calculated using the Shannon index (Shannon & Weaver, 1949) and the Whittaker index (Whittaker, 1972), respectively. The Shannon index allows for the estimation of species richness (number of species) and evenness in a certain habitat. Shannon index values ranges from 1.5 to 3.5 and the lower the value, the smaller number of species it has and the less evenly the species are distributed in the referred habitat (Shannon & Weaver, 1949). The Whittaker index refers to the total numbers of species that are unique to each habitat. It allows for the comparison between two or more habitats presenting similarities or differences between them. It can also be described as the probability of finding two individuals belonging to the same species in two different habitats. Whittaker index values range from 0 (no similarity) to 1 (total similarity) (Whittaker, 1972).A comparison of trap efficiency was performed considering both the total number of individuals and the number of species captured by each type of trap. Seasonal abundance were assessed and compared. For all statistical analysis, the frequency of each tabanid or glossina species was presented in percentages and summarized in tables and charts. To determine if there is significant variance in average numbers of tabanids collected from different locations, tabanids captured per trap and per season, Tukey´s honest significance test for one-way ANOVA was used in Statistica version 13.0 (TIBCO Software Inc., 2017.http://statistica.io).

4.2.4 DNA Extraction for Molecular Identification

Three legs from each individual fly were removed and placed into a 1.5 ml tube to which 200µL of 5% Chelex® 100 chelating resin (under stirring) was added. Legs were crushed using a pestle and incubated for1hour at 56ºC followed by another incubation at 95ºC for 30 min. Then the sample was centrifuged for 3 min at 12000 x g and frozen until the PCR step. Before the PCR, the samples were centrifuged to allow good

separation of the phases. Transfer of any Chelex beads was avoided due to their ability to inhibit PCR (Walsh, 1991 and Ravel et al. 2007).

4.2.5 Amplification of tabanids DNA by CO1 PCR

To conduct the identification of tabanids, to genus and species level, using molecular tools, PCR targeting the Cytochrome Oxidase 1 (CO1) gene was performed using an Applied Biosystems® thermo cycler (Thermo Fisher Scientific, Sweden). In a total volume of 20 µl, 10 µl of 2X Phusion Flash Master Mix, 0.5 µM of each primer and 6.5 µl of double distilled water (ddH2O) were added. PCR conditions were: 98ºC for 10s, 30 cycles of 98ºC for 1s, 50ºC for 5 s and 72ºC for 15 s, followed by a final elongation step of 72ºC for 1 minute. Genomic DNA from *Glossina brevipalpis* and *G. austeni* from Biotechnology Center-Eduardo Mondlane University reference collection were used as positive control and ddH2O as negative control. The primers used were LCO GGTCAACAAATCATAAAGATATTGG and HCO2198 TAAACTTCAGGGTGACCAAAAAATCA and the expected size of the fragment was approximately 653 bp (Folmer *et al.*, 1994; Taioe *et al.*, 2017). For PCR product visualization, the samples were run in a 2% agarose gel where 2 µl of loading dye were mixed with 5 µl of PCR product and loaded onto the gel. A 1000bp DNA ladder was also loaded (4 µl) for fragment size determination, and the gel run for 45 min at 100 volts. The gel was stained with GelRed (Biotium, Inc., Fremont, CA, USA) at 4 µl per 100 ml of agarose solution directly added before polymerization.

4.2.6 Sequencing and Phylogenetic Analysis

Four replicates of the PCR amplicons from the CO1 PCR amplification were produced and pooled. After confirmation in agarose gel, 50µl of the product was sent for sequencing at INQABA BIOTECH™ (Pretoria, South Africa).

All sequences were viewed and assembled using BioEdit version 7.0.9 (Hall 1999). Assembled sequences from both Tabanidae and Glossinidae species were separately aligned with various sequences from GenBank using the online version of Mafft with default parameters (https://mafft.cbrc.jp/alignment/server/). The aligned matrices were viewed, edited an truncated in Mega 7 (Kumar *et al.* 2016) andused for all analysis. Data-display networks (DDN) were constructed in Splitstree version 4 (Huson and

Bryant 2006), from uncorrected p-distances using both parsimony informative uninformative characters. Bootstrap (bs) support for the DDN was calculated from 1000 replicates. jModelTest version 2.1.10, via the Cipres Science gateway (Miller *et al.*, 2010), was used for model estimation. A maximum likelihood (ML) analysis was performed in RaXML version 8 using the estimated models (GTR+G+I) with the autoMRE function invoked for the calculation of bootstrap support (Stamatakis 2014). A Bayesian approach (MrB) for phylogenetic inference was conducted in MrBayes version 3 (Ronquist and Huelsenbeck 2003). Four simultaneous cold Monte-Carlo Markov Chains searched for 10 million generations, with each 1000th tree sampled. In both cases, TIM1+G+I was employed as the substitution model as estimated by jModelTest. Before tree construction, the first 15% of the trees were discarded as burn-in for the calculation of the posterior probabilities (pp). Effective sample size were calculated and viewed using Tracer version 1.6 (Rambaut *et al.*, 2014).

4.3 Results

During a period of 12 months, using 55 traps, a total of 4379 tabanids and 24 tsetse flies were captured in three areas of Nicoadala district, from which 35.88% of the tabanids where captured in Zalala, 33.41% in Namitangurine and 30.71% in Botao.

Using morphological characterization, it was found that the tabanids caught are classified into four genera, *Tabanus*, *Ancala*, *Atylotus* and *Haematopota*. The *Tabanus* genus, with 3207 individuals captured, accounted for 73.24% of the total capture, followed by the genus *Atylotus* 1017 (23.22%), *Ancala* 149 (2.4%) and *Hematopota* 6 (0.14%).

Tabanus par with 66.8% and *Atylotus agrestis* with 23.2% were the most frequently caught species. Only six individuals of *Hematopota* spp were trapped and are the most infrequently caught species in the study. Six from the 10 species of tabanids captured accounted for less than 1% of total capture (Figure 4.2). *Tabanus par*, *T. taeniola*, *Atylotus agrestis* and *Ancala Africana* were captured in all the three habitats (Table 4.1). Out of the 4379 tabanids captured, 16 *Tabanus* specimens were damaged and/or

discolored by alcohol, thus morphological identification to species level was not possible.

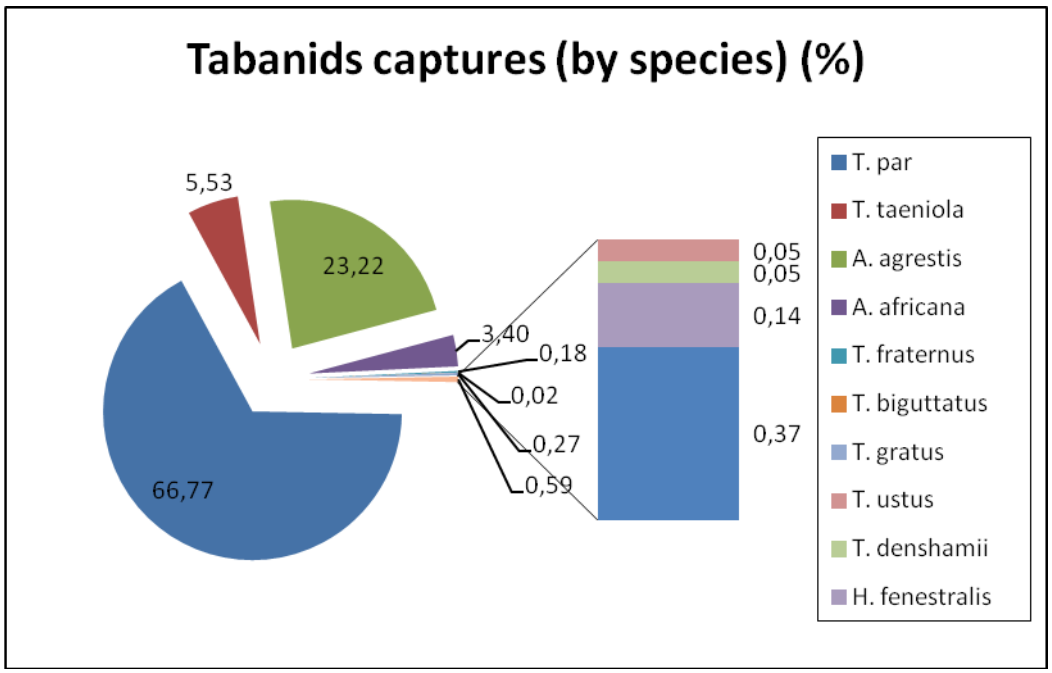

FIGURE 4. 2: Pie chart showing the percentage of each tabanids species caught in Nicoadala district. The captured species' names can be seen, in full, in table 4.2**.**

Three of the four *Glossina* species present in Mozambique were captured. These species were *G. brevipalpis, G. pallidipes* and *G. morsitans*. Only one *Glossina,* a *G. brevipalpis,* was found on the coastal beach of Zalala. The area of Namitangurine, with dense forest and possible higher presence of wild hosts contributed with more than 50% of the total captured *Glossina*. Additionally, it was the only area where all three *Glossina* species were captured (Table 4.2).

For the molecular analysis, the dimensions of the final alignment matrices were 113 taxa and 658 characters and 18 taxa and 659 characters for Tabanidae and Glossinidae, respectively. Identical substitution models, GTR +G +I (AIC criteria) and TIM1+G+I (BIC criteria) were recovered for both datasets. Congruent topologies across all methods were recovered (Figures 4.3 to 4.6).

TABLE 4. 1: Tabanid flies captured in all three grazing areas, in Nicoadala district.

Tabanid fly species	Grazing Area			Total flies captured
	Zalala	Botao	Namitangurine	
Tabanus par	894	1130	900	2924
Atylotus agrestis	482	89	446	1017
Tabanus taeniola	79	71	92	242
Ancala Africana	96	47	6	149
Tabanus gratus	5	0	7	12
Tabanus biguttatus	0	0	1	1
Tabanus fraternus	0	2	6	8
Tabanus denshamii	1	0	1	2
Haematopota spp	3	3	0	6
Tabanus ustus	2	0	0	2
Undetermined	9	3	4	16
Total	1571	1345	1463	4379

TABLE 4. 2: Glossina spp capturedin all three grazing areas, in Nicoadala district.

Glossina fly species	Grazing Area			Total flies captured
	Zalala	Botao	Namitangurine	
Glossina brevipalpis	1	6	11	18
Glossina morsitans	0	0	4	4
Glossina pallidipes	0	0	2	2
Total	1	6	17	24

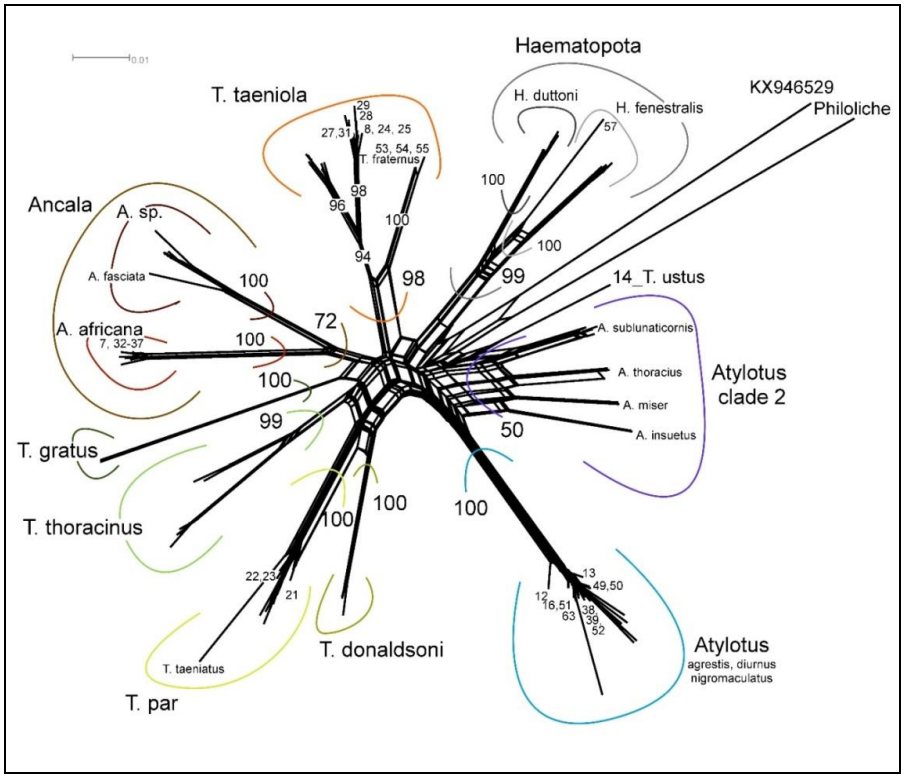

FIGURE 4. 3: An uncorrected p-distance data-display network, using all characters recovered from SplitsTree using the Tabanidae COI data. Bootstrap (bs) support calculated from 1000 replicates are indicated for the various groupings (only bs. values >50 was considered). The double-digit numbers on the tips of the network represent the sequences from this study.

Three specimens could confidently be identified as *Ancala africana* (7, 32, 37: DDN bs=100, ML bs=98, MrB pp=1) (Figures 4.3 & 4.4). Two *Atylotus* clades were recovered across all methods. All ten specimens sequenced for this study fell within a collapsed node representing *A. agrestis*, *A. diurnus* and *A. nigromaculatus* (DDN bs=100, ML bs=99, MrB pp=1) (Figures2&3). Three specimens (21-23) could confidently be identified as *Tabanus par* (DDN bs=100, ML bs=100, MrB pp=1). A sequence uploaded by Mugasa *et al.* (2018), apparently representing *T. taeniatus* consistently fell within the *T. par* group. Seven specimens (8, 24, 25, 27-29) were confidently recovered as *T. taeniola* (DDN bs=94, ML bs=98, MrB pp=0.99), with four specimens possibly identified as *T. fraternus* (18, 53-55) (DDN bs=100, ML bs=100, MrB pp=1). Together, these tabanids groupings formed a closely related and well-supported monophyletic clade (DDN bs=98, ML bs=97, MrB pp=1) (Figures 4.3 & 4.4).

A single specimen (57), formed a well-supported clade with *Haematopota fenestralis* (DDN bs=100, ML bs=95, MrB pp=1). The specimen morphologically identified as *T. ustus* (14) could not be verified as belonging to any species-group due to a lack of reference sequences on GenBank.

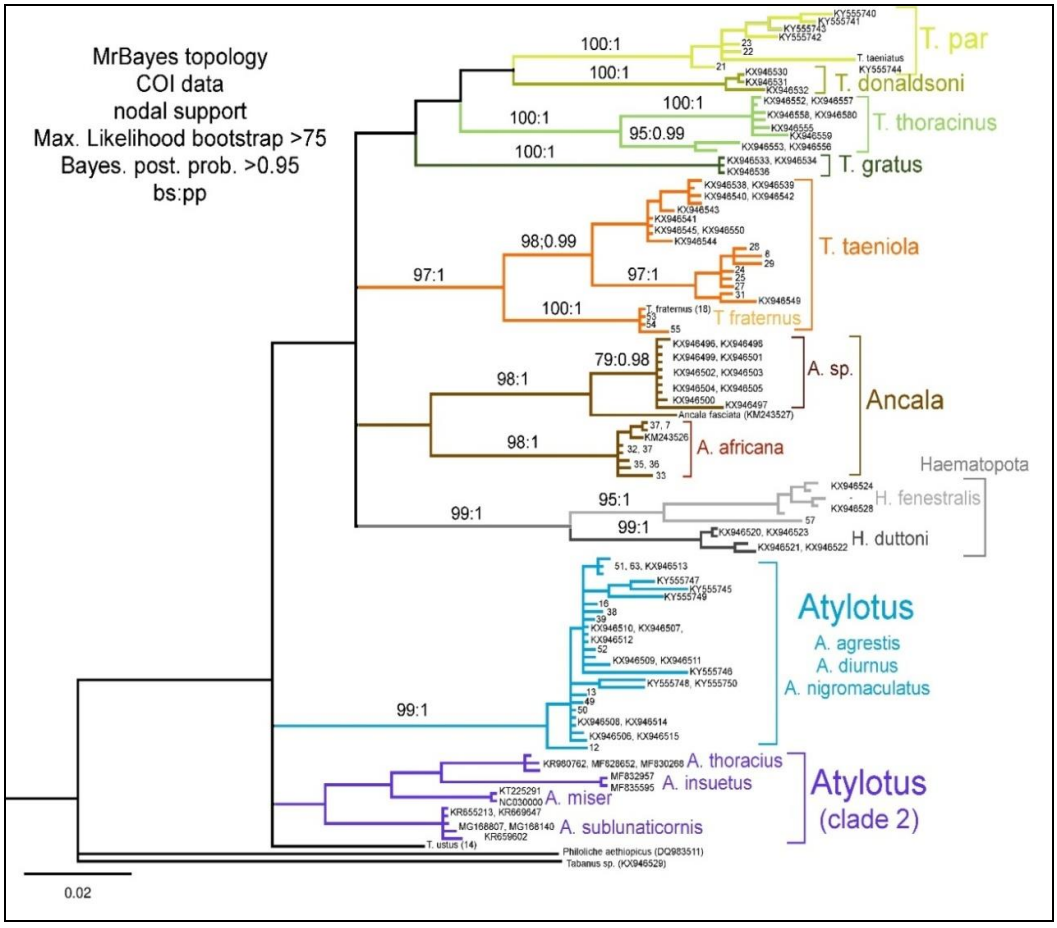

FIGURE 4. 4: A phylogram recovered from a MrBayes analysis using the Tabanidae CO1 data. Nodal support presented on the branches arebootstraps (bs) calculated from maximum likelihood analysis in a RaXML analysis (autoMRE function) and posterior probabilities (pp) calculated from a MrBayes analysis. Only bs values >75 ad pp values > 0.95 were considered significant.

Congruent topologies, analyzing the Glossinidae CO1 data, from all three analysis were recovered (Figures 4.5 & 4.6). In all analyses, the Fusca and Palpalis groups were well-supported monophyletic clades (DDN bs=99, ML bs=100, MrB pp=1; DDN bs=100, ML bs=100, MrB pp=1 respectively). Two well-supported monophyletic clades

representing the Morsitans group were consistently found across all analyses (Clade 1: DDN bs=94, ML bs=87, MrB pp=1; Clade 2: DDN bs=100, ML bs=86, MrB pp=0.99). All four specimens could confidently be identified. Three specimens (40, 42, 43) were molecularly identified as *Glossina morsitans* (DDN bs=100, ML bs=99, MrB pp=1) and one specimen as *G. brevipalpis* (DDN bs=100, ML bs=96, MrB pp=1).

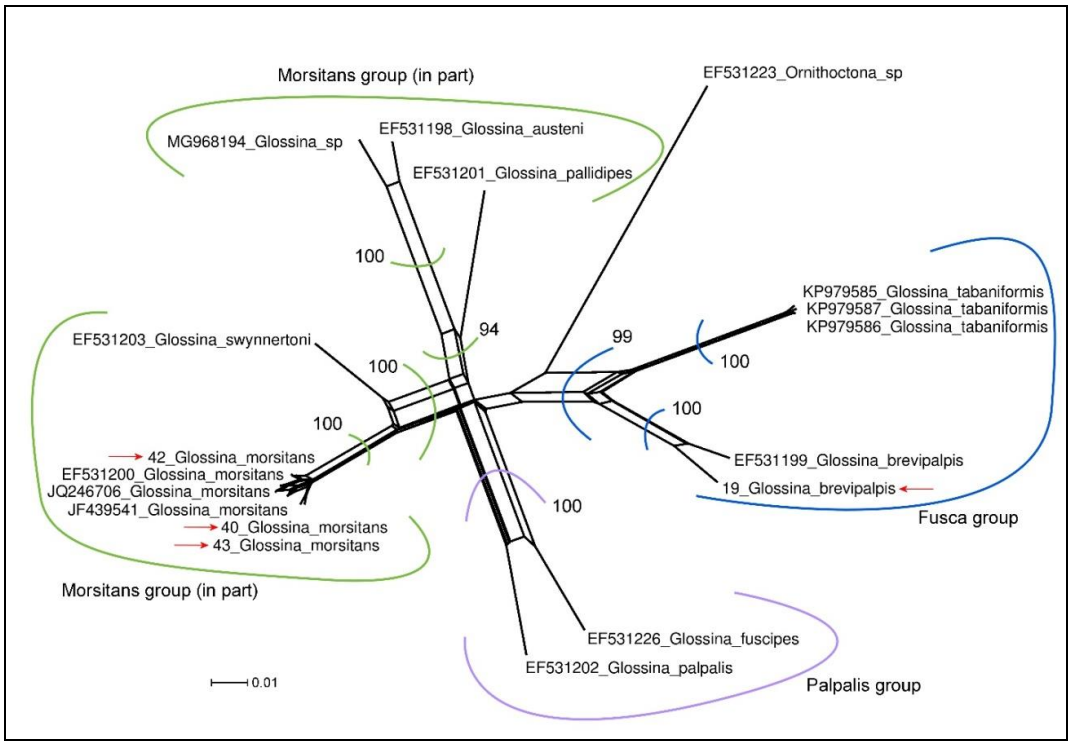

FIGURE 4. 5: An uncorrected p-distance data-display network, using all characters recovered from SplitsTree using the Glossinidae COI data. Bootstrap (bs) support calculated from 1000 replicates are indicated for the various groupings (only bs. values >50 was considered). The double-digit numbers on the tips of the network represent the sequences from this study. Red arrows indicate the specimen sequences from this study.

Alpha diversity analysis revealed a slightly higher tabanid species richness and evenness in Zalala (Shannon index of 1.08) and Namitangurine (0.98), with 9 species of tabanids captured in each area. Botao with a Shannon index of 0.64, revealed a lower diversity and evenness in the area. Moreover, species abundance was higher in Zalala and Namitangurine (Table 2). The comparison of Zalala and Namitangurine diversity gave lower β diversity (β_w=0,222) than the comparison between Zalala and Botao

(β_w=0,250). Moreover, the comparison between Namitangurine and Botao gave a lower β diversity index (also β_w=0,250). This reveals a smaller difference in tabanid species composition between Zalala and Namitangurine.

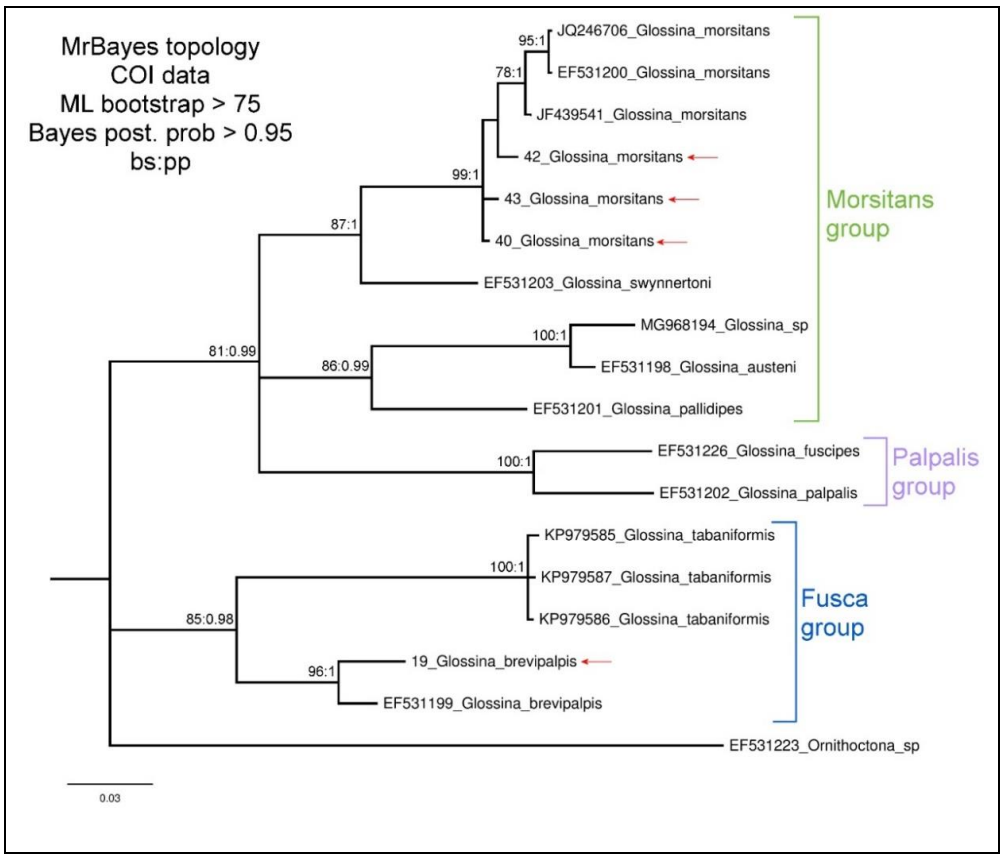

FIGURE 4. 6: A phylogram recovered from a MrBayes analysis using the Glossinidae CO1 data. Nodal support presented on the branches are bootstraps (bs) calculated from maximum likelihood analysis in a RaXML analysis (autoMRE function) and posterior probabilities (pp) calculated from a MrBayes analysis. Only bs values >75 and pp values > 0.95 were considered significant. Red arrows indicate the specimen sequences from this study.

Analyzing the trap efficiency, NGU (1735 tabanids and nine tsetse captured) demonstrated better performance in general. It also managed to successfully capture all three species of *Glossina*. The H traps only captured *G. brevipalpis* (Figure 4.7). There was a significant difference when comparing the H trap and NGU trap at p = 0.000036 (Tukey´s honest significance test), using the total number of tabanids captured.

Furthermore, there was also significant difference, using the same parameter, when comparing H trap and Epsilon trap at p = 0.00022 (Tukey´s honest significance test). Nevertheless, there was no significant difference when comparing Epsilon trap and NGU trap at p = 0.351 (Tukey´s honest significance test).

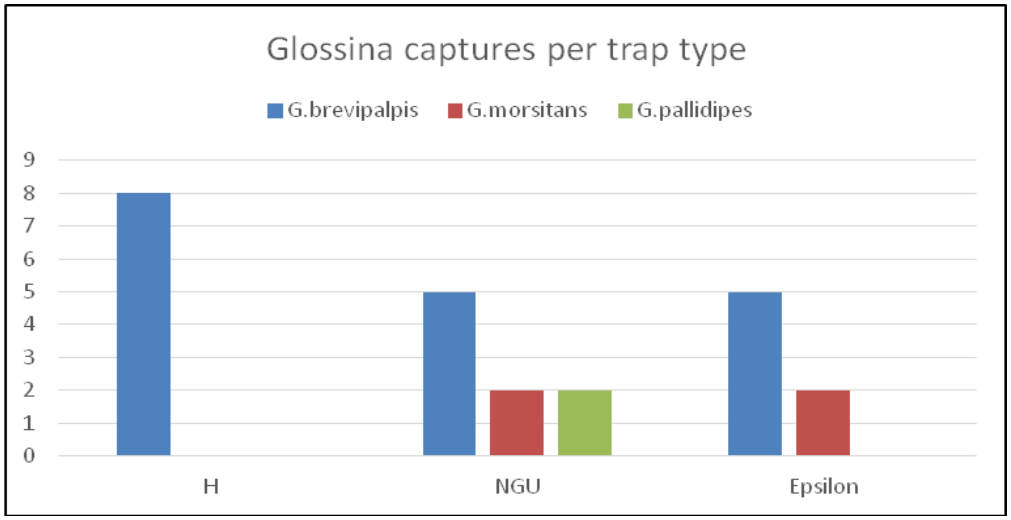

FIGURE 4. 7: Bar graph showing the total Glossina spp captured by each type of trap.

There was significant difference at p = 0.00009 between the captures in dry and rainy season, with the latter accounting for 70.5% of the total captures (Figure 4.8). The target taxa had a high activity peak in March (755 tabanids captured) juxtaposed with July (106 tabanids), during which the lowest activity was recorded (Figure 4.9).

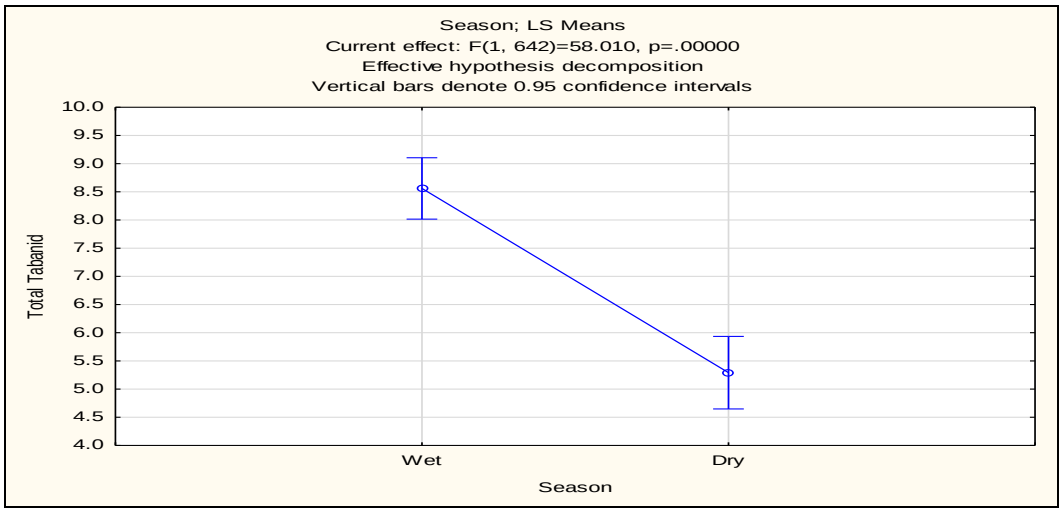

FIGURE 4. 8: Comparison of the seasonal occurrence oftabanids caught analyzed in Statistica version 13.0 (TIBCO Software Inc., 2017. http://statistica.io).

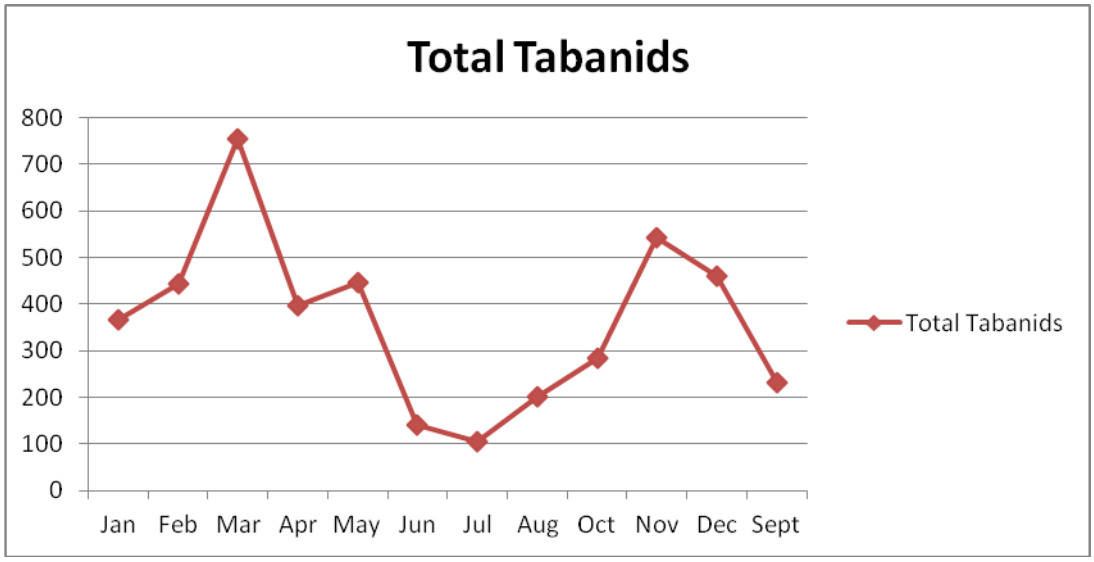

FIGURE 4. 9: Total tabanids caught during the twelve months of trapping.

4.4 Discussion

Considering the important role of tabanids and tsetse flies in the transmission of pathogens, alarmingly little research attention has been devoted to these taxa in Mozambique. Little basic information such as their classification and distribution patterns are available, with the last major work focused on elucidating these aspects that of Dias (1966), having been conducted more than half a century ago. Few surveys aiming at the distribution (Sigauque *et al.*, 2000), genetic characterization (Wohlford *et al.*, 1999) or infection of tsetse flies (Rodrigues *et al.*, 2017; Garcia *et al.*, 2017) were previously conducted.

According to Dias (1966) and RTTCP (2000), the Nicoadala district falls within an important geographic area for both Glossinidae and Tabanidae. While the first author studied the diversity and distribution of tabanids in the country and in the province where the district is located, RTTCP aimed at the conception of tsetse distribution maps and development of better approaches for tsetse capture in the region comprising Mozambique, Zambia, Zimbabwe and Malawi.

In the district, three distinct areas (Namitangurine, Botao and Zalala) were surveyed. The absence of significant differences when comparing Botao and Zalala could be

attributed to the fact that both Zalala and Botao are more open areas with more human interference (cattle grazing, habitation and agriculture). However, tabanids did not present significant differences in the number of species captured in the three habitats. It is known that tabanids are cosmopolite insects that can survive in different environmental conditions (Baldacchino et al. 2014). However, it is difficult, at this moment, to state any clear hypotheses on what the cause of this non-significant difference in the distribution of tabanids in the district may be.

The results showed differences in the number of tsetse captured in each area. However, the reasons for these differences are not totally clear. They could be due to the vegetation type and cover, but certainly, the presence of meal sources other than cattle, which vary across the areas, might also play a role. Studies have shown that host preference can have a great influence on the distribution of tsetse flies (Van den Bossche & Staak, 1997; Simukoko *et al*., 2007; Gondwe *et al*., 2009). Moreover, *G. morsitans* is considered to be one of the most sensitive species and its density can start decreasing quickly at a minimum sign of human population settlements in the habitat (Van Den Bossche *et al.* 2010).

Only one tsetse fly was captured in Zalala area. This can be attributed to the lack of conditions for tsetse survival, mainly due to the type of vegetation cover, which consists of very dispersed and small pockets of shrubs and trees. These conditions may be insufficient to provide adequate shelter and shade for the flies. Moreover, the fact that cattle are the only food source available in the area can be considered a limiting factor to the expansion of the tsetse fly population. Finally, the dense human settlements that are rapidly expanding and changing the surrounding environment will negatively impact fly survival (Kilemwa 1999; Van den Bossche *et al.*, 2010; Malele *et al.*, 2011;Torres *et al.*, 2016).

The results from this study are similar to those of Ahmed *et al.* (2005) in Nigeria, Lendzele *et al*. (2017) in Cameroon and Taioe *et al.* (2017) in South Africa and Zambia, regarding the high abundance and diversity of *Tabanus* versus other tabanid genera,

which were caught in lower numbers. *Tabanus par* and *Atylotus agrestis* were the dominant tabanid species in all three areas. A similar result was also found in the study by Taioe *et al*., (2017). Although with some variations, the results of these studies indicate the predominance of these two species in the Southern African region. Contrastingly, Lendzele *et al*., 2017, working in Cameroon, found *T. par,* one of the least abundant species of tabanids, possibly indicating that the eastern part of the continent is more favorable for the species. Moreover, contrarily to what was found in Cameroon (Lendzele et al. 2017), where *T. gratus* showed higher frequency, in the present study, it was one of the least frequent, with only 12 individuals captured (0,3%). Similarly to the findings of Ahmed *et al.* (2005) and Taioe *et al*. (2017), the present study also suggests a low abundance and diversity of *Hematopota* in the region.

All specimens collected were subsequently placed in 70% ethanol for long-term storage, which dissolved the pigments of the flies, making morphological identification difficult. Additionally, there was a general lack of CO1 data from species collected in this study on GenBank at the time of writing, making molecular identification difficult. The collapsed group represented by the three *Atylotus* species, *A. agrestis*, *A. diurnus* and *A. nigromaculatus* posed a problem for the confident identification of the *Atylotus* specimens, morphologically identified as *A. agrestis*. This is probably due to incorrectly identified specimens uploaded to GenBank, or the inability of CO1 to distinguish between *Atylotus* species or fuzzy genetic boundaries between these closely related *Atylotus* species. *H. fenestralis* could only be compared to highly similar sequences from a single study making confident identification difficult due to a lack of genetic variability. All specimens within the Fraternus group as described by Oldroyd (1954) were identified as *T. taeniola*, despite four specimens grouping separately (18, 53-55), with one specimen identified as *T. fraternus*. Due to a lack of *T. fraternus*reference sequences available and the subtle morphological difference, especially in specimens stored in 70% ethanol (*T. fraternus* has slight additional pigment on the wings, lacking in *T. taeniola*), confident identification was impossible. Currently, no biological differences have been described between the species, making separation difficult. The species boundaries between *T. par* and *T. thoracinus* remains unclear and was not treated in the most recent work by Mugasa *et al.* (2018), who recovered two “genetic

variants" from Uganda in their study, but did not include *T. par* specimens and could not find morphological differences between the genetic variants.

The low numbers of Shannon index obtained in the present study were mainly due to the high frequency of *T. par* (66.8%) and *Atylotus agrestis* (23.2%), showing the existence of clear dominant species and a very low evenness of species. Despite the differences in vegetation composition and tabanid hosts present, Zalala and Namitangurine presented low β_w, meaning that the probability of finding two individuals from the same species in both areas is high. The more species two communities share, the lower is the β_w. The use of diversity indexes allows for the expression of a pattern of species distribution (Melo *et al.* 2008). In fact, for the present study, the identification of similarities or differences in tabanid distribution in the grazing areas can contribute to the development of strategies for the control of the flies, considering their role in pathogens transmission, more specifically, in the mechanical transmission of trypanosomes.

Epsilon and NGU traps captured *G. pallidipes* and *G. morsitans.* Since both traps were designed for trapping savannah species in Kenya and Zimbabwe, respectively, this result was expected (Brightwell *et al*., 1987; Esena, 2013). The H trap demonstrated better performance in the capture of *G. brevipalpis*, with 8 individuals captured, when compared to NGU and Epsilon that captured 5 *G. brevipalpis* flies each. This contrasts the findings of Malele et al. (2016) in Tanzania, where the H traps did not capture any *G. brevipalpis*. However, several other studies have demonstrated good performance of H traps in the capture of *G. brevipalpis* (Kappmeier 2000; Mamabolo *et al.*, 2009; Beer *et al*., 2016) in South Africa and (Sigauque *et al.*, 2000) in Mozambique. In fact, the H trap was specifically developed and optimized for the capture of *G. brevipalpis* (Kappmeier 2000)

In a number of studies, the results have shown higher numbers of captured tabanids during the hot and rainy season (Lendzele *et al*., 2017; Ahmed *et al.* 2005; Herczeg *et al.* 2014; Parra-Henao & Alarcón-Pineda, 2008). The results of our survey are in

complete agreement with these previous observations, with the rainy season accounting for two folds the total capture number of the dry season. Additionally, the identification of tabanid activity peaks is extremely important to understand the pathogen transmission dynamics and hence to the development of appropriate control strategies for both pathogens and vectors.

In the present study, after a tapping period of 365 days, covering three important grazing areas in Nicoadala district and involving 55 traps in total, only 24 *Glossina* were captured. Considering the trapping effort, this represents a very low number of tsetse flies collected. However, the study results also shows that they may still play an important role as cyclical vectors of *Trypanosoma* spp in the trypanocide resistance foci of Nicoadala district. However, the strong presence of tabanids, including species that have previously been identified as trypanosomosis vectors, suggests their participation as mechanical vectors and a contribution to the high prevalence of trypanosomosis in the district as reported by Jamal et al. (2005), Specht (2008) and Mulandane et al. (2018). These conclusions can be supported by the findings from Desquesnes & Dia (2003, 2004), where mechanical transmission of *T. congolense* and *T. vivax* by *Atylotus agrestis* and *A. fuscipes* were experimentally proven. They are also supported by Abebe and Jobre (1996), in which high *T. vivax* and *T. congolense* infection where detected in tsetse free zones. Furthermore, some authors defend that mechanical transmission of trypanosomes, can, in certain circumstances be as efficient as biological transmission (Foil, 1989; Desquesnes *et al*., 2009; Baldacchino *et al.*, 2014), which raises the question: what if, in a drug resistance focus like Nicoadala dictrict, even in complete absence of tsetse flies, trypanosomes can still circulate in vertebrates as a result of a combination of resistance trypanosome populations and efficient mechanical vectors? Therefore, it is safe to say that the epidemiology of trypanosomosis in Nicoadala district may be strongly influenced by the presence of tabanids and the flies remain a menace to cattle-keeping activities in the area. Thus, any intervention regarding trypanosomosis control in the district should take this into account.

4.5 References

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CHAPTER 5 : Detection of *Trypanosoma* infections in haematophagous insects using 18S PCR-RFLP n the drug resistance focus of Nicoadala District, Mozambique

5.1 Introduction

Tsetse transmitted animal trypanosomosis is one the most important diseases in sub-Saharan Africa. Trypanosomosis causes the death of thousands of heads of cattle and reduces the availability of meat, milk and other cattle-derived products (Elkarib 1961; Jordan 1985; Cecchi *et al.* 2014; Ahmed *et al.* 2016).

Studies involving detection of trypanosomes in vectors like glossina and tabanids are of extreme importance as they help understand the epidemiology of trypanosomosis in a certain area (Isaac *et al*. 2016). Such results are of paramount importance and can be used for real-world applications such as regional vector control/management or policy making. The present study was conducted to investigate tsetse and tabanid infection rates and the trypanosome species circulating in these vectors, in Nicoadala district.

Classically, trypanosome detection in flies is quantified by dissecting flies and looking for the parasite under a light microscope. However, in tsetse flies this method requires fresh flies and is dependent of the parasite density. In Tabanidae, where the parasite does not undergo development, but remains viable for very short time, before the blood meal is digested by the fly, this method is only reliable if dissecting Tabanidae mouthparts directly subsequent to feeding. These limitations were surpassed by the advent of molecular methods such as Polymerase Chain Reaction (PCR) which allows not only for the detection of the parasite in the fly (fresh or preserved), but also has the advantage of detecting mixed infections as well as the identification of the parasites to subspecies level (Morlais *et al.* 1998). This constitute the rationale for the use of 18S PCR-RFLP in the assessment of the infection rates in the two families.

5.2 Material and Methods

5.2.1 Flies collection and selection

A total of 480 tabanids and 24 glossina preserved in 70% ethanol were used in the study. From the 480 tabanids, 160 were captured in Zalala, 160 in Botao and 160 in Namitangurine. To estimate the sample size and get a statistically representative number

of 160 individuals to be sampled per area, the formula of Cannon and Roe (1982) was used, where the expected prevalence was 10% with a 5% confidence interval and a 5% desired absolute precision. It gave a sample size of 138 tabanids per area but to increase the probability of detecting a positive sample, 160 tabanids were used. These were selected equally into the four most captured species in the three areas namely *Tabanus par, T. taeniola, Atylotus agrestis* and *Ancala africana*. The flies where collected using horizontal trap (H-trap) (Kappmeier 2000), Epsilon-trap (Esena 2013) and NGU trap (Brightwell *et al.*, 1987) as described in chapter 4 of the present thesis. In total, 4379 tabanid flies were collected.

From the 24 glossinas used in the study, 17 (11 *G. brevipalpis, four G. morsitans* and two *G. pallidipes*) were collected in Namitangurine, six (all *G. brevipalpis*) in Botao and one *G. brevipalpis* in Zalala as described in chapter 4.

5.2.2 DNA Extraction

DNA was extracted from individual flies using an ammonium acetate precipitation protocol modified from Bruford *et al.* (1998) as follows: the flies were pressed in tissue paper to eliminate ethanol, placed in a clean 1,5 ml tube and 300 µl of lysis buffer plus 5 µl of Proteinase K (20 mg/ ml) added. Then, they were grinded with a hand pestle and incubated at 37º C overnight. After incubation at 37º C, the samples were placed at room temperature for 5 minutes to bring down the temperature, followed by the addition of 400 µl of 4M ammonium acetate, vortex (mixed with a vortex) for 15 seconds and incubated on ice for 30 minutes. Then, the samples were centrifuged for 20 min at 13000 rpm at 4º C and the supernatant transferred to a new 1.5 ml tubes. 600 µl of absolute ethanol was added and the tubes were gently inverted to mix the contents, followed by incubation at -80º C for 1 hour and a posterior centrifugation for 10 minutes at 13000 rpm at 4º C. The supernatant was discarded and 600 µl of 70% ethanol added and the tubes were centrifuged for 20 min at 13000 rpm at 4º C. Ethanol was carefully discarded, and the pellet put to dry by inverting the tube facing down on tissue paper at room temperature. The DNA was suspended in 50 µl TE Buffer and left overnight at room temperature to dissolve.

5.2.3 Semi-nested 18S rRNA PCR and restriction fragment length polymorphism (RFLP)

For the molecular detection of trypanosomes in the flies, 18S semi-nested rRNA PCRs were run, targeting a fragment of the 18S ribosomal RNA gene, on an Eppendorf Mastercycler® gradient thermocycler (Eppendorf AG, Hamburg, Germany), under the following conditions: 10 s at 98ºC; 40 cycles of 98ºC for 1 s, 58ºC for 5s, 72ºC for 15s and a final step of 72ºC for 1 min. For this, two reactions were carried out in a final volume of 25µl containing 1X Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Sweden), 20pmol of each primer, and 5µl of eluted DNA for the 1st reaction and 2.5µl of PCR product from the 1st reaction for the nested reaction. Water was added to obtain a final volume of 25 µl. The primers used were18ST nF2 5'-CAA CGA TGA CAC CCA TGA ATT GGG GA-3' and 18ST nR3 5'-TGC GCG ACC AAT AAT TGC AAT AC-3' for the 1st reaction and 18ST nF2 and 18ST nR2 5'-GTG TCT TGT TCT CAC TGA CAT TGT AGT G-3', for the nested reaction. PCR products were visualized on a 10% agarose gel, where 2 µl of loading dye were mixed with 5 µl of nested PCR product and loaded onto the gel. A 100bp DNA ladder was loaded (4 µl) for fragment size determination, and the gel run for 45 min at 120 volts. The gel was stained with GelRed (Biotium, Inc., Fremont, CA, USA) at 4 µl per 100 ml of gel directly added to the gel before polymerization.

Then, aliquots of all the nested products detected positive in agarose gel were digested with *MspI* and *Eco571* (AcuI) enzymes at 37º for 60 min, in a final volume of 15 µl containing 1X Enzyme buffer, 0.5 µl *MspI*, 0.5 µl *Eco571*, 8.5 µl deionized water and 4.0 µl of PCR product. The samples were then run in a 10% polyacrylamide gel at 100 volts for 90 minutes, where 2 µl of loading dye was mixed with 4 µl of digested PCR product and loaded onto the gel together with a 100 bp DNA ladder. The gel was stained with 10 000X SYBER Green I® (Molecular Probes Inc., Eugene, USA) in accordance with the manufacturer´s instructions. All suspected *T. theileri* were submitted to *Mbo*II digestion for confirmation and the digestion products run in a 10% polyacrylamide as described above.

5.2.4 Statistical Analysis

Data related to trypanosome detection in tabanids and tsetse flies were transferred to an Excel file. Mean infection rate per species, per species/area and per total tabanids/area were calculated. To determine whether observed differences in tsetse and tabanids infection rates, infection rates per area and per species were statistically significant, Tukey´s honest significance test for one-way ANOVA was used in Statistica version 13.0 (TIBCO Software Inc., 2017. http://statistica.io).

5.3 Results

From the 480 tabanids screened, 13.0% (66/480) tested positive for the presence of *Trypanosoma*. The overall trypanosome infection rate for tsetse flies was 75.0% (18/24).

Only *T. congolense* infections were detected in *Glossina* spp and only in *G. brevipalpis* and *G. morsitans* (figure 5.1). The low sample number does not allow for good statistical comparisons.

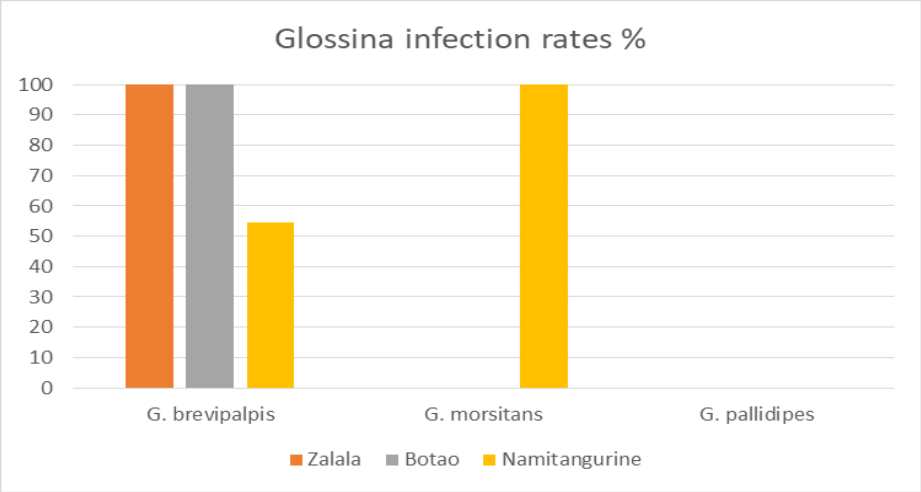

FIGURE 5. 1: Percentage of infected tsetse flies (total n=24) per sampling area detected using 18S PCR-RFLP**.**

Namitangurine with 18.8% (30/160) presented the highest tabanid infection rate followed by Botao with 13.1% (21/160) and Zalala with 9.4% (15/160) (figure 5.2). There were no significant differences when comparing the infection rates between all

the three sites at p = 0.732616 (Zalal vs. Botao), p = 0.980165 (Zalala vs. Namitangurine) and p = 0.837178 (Botao vs. Namitangurine) (Tukey´s honest significance test).

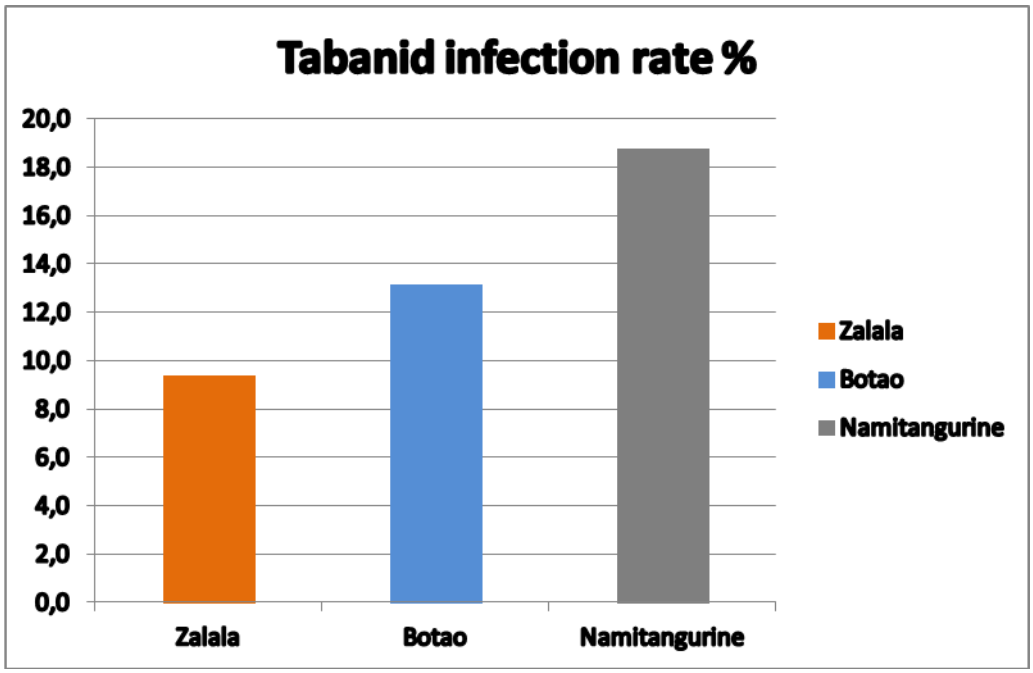

FIGURE 5. 2: Percentage of infected tabanid flies per sampling area detected using 18S PCR-RFLP, Zalala (n=160), Botao (n=160) and Namitangurine (n=160).

Species comparisons revealed that *A. agrestis*, with 25.0% (30/480), had the highest infection rate, followed by *T. par* and *T. taeniola*, both yielding 3.8% of infected flies (18/480). No *A. africana* specimen tested positive for trypanosome infection (Figure 5.3). There was no significant difference in infection rate per species between the 3 species where *Trypanosoma* infections were detected at p = 0.998436 (*T. par* vs. *T. taeniola*), p = 0.188029 (*T. par* vs. *A. agrestis*) and p = 0.233271 (*A. agrestis* vs. *T. taeniola*) (Tukey´s honest significance test).

Comparing the infection rate per species/area, *A. agrestis* remained the species with the highest infection rate in both Namitangurine and Botao. In Zalala, *T. par* had a higher infection rate (Figure 5.4).

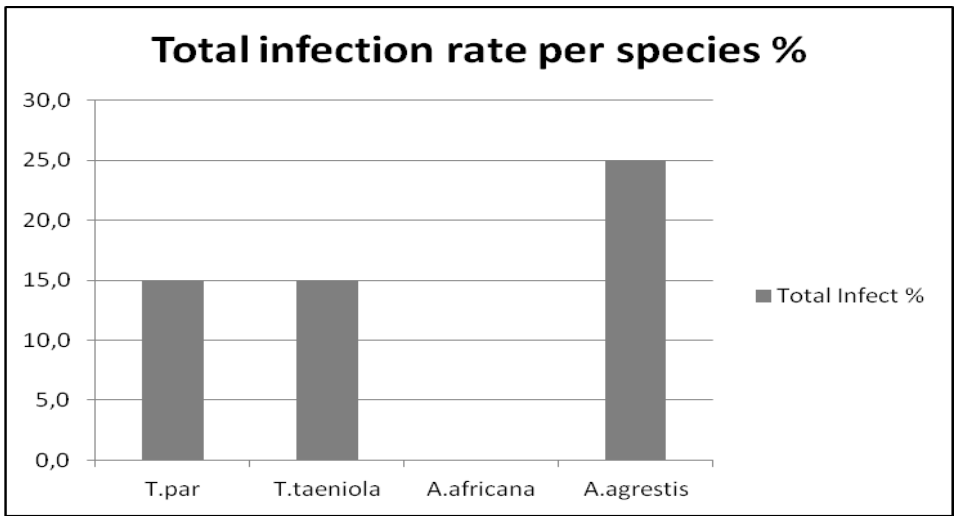

FIGURE 5. 3: Percentage of infected tabanid flies per species, T.par = *Tabanus par* (n=160), T.taeniola = *Tabanus taeniola* (n=160), A.africana = *Ancala africana* (n=160), A.agrestis = *Atylotus agrestis* (n=160), as detected by 18S PCR-RFLP.

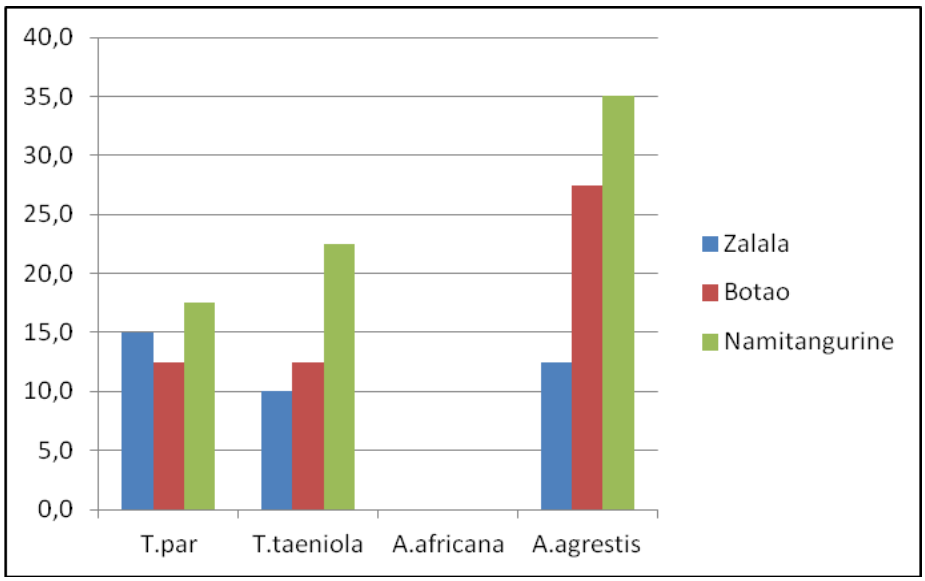

FIGURE 5. 4: Percentage of infected tabanid flies per species/area, T.par = *Tabanus par* (n=160), T. taeniola = *Tabanus taeniola* (n=160), A. africana = *Ancala africana* (n=160), A. agrestis = *Atylotus agrestis* (n=160), as detected by 18S PCR-RFLP.

The trypanosome DNA detected in tabanids belonged to *T. congolense*. Additionally, *T. theileri* was detected (figures 5.5 and 5.6). Tabanids from Namitangurine presented the

highest *T. congolense* infection rate (15.6%), followed by Botao (12.5%) and Zalala (5.6%).

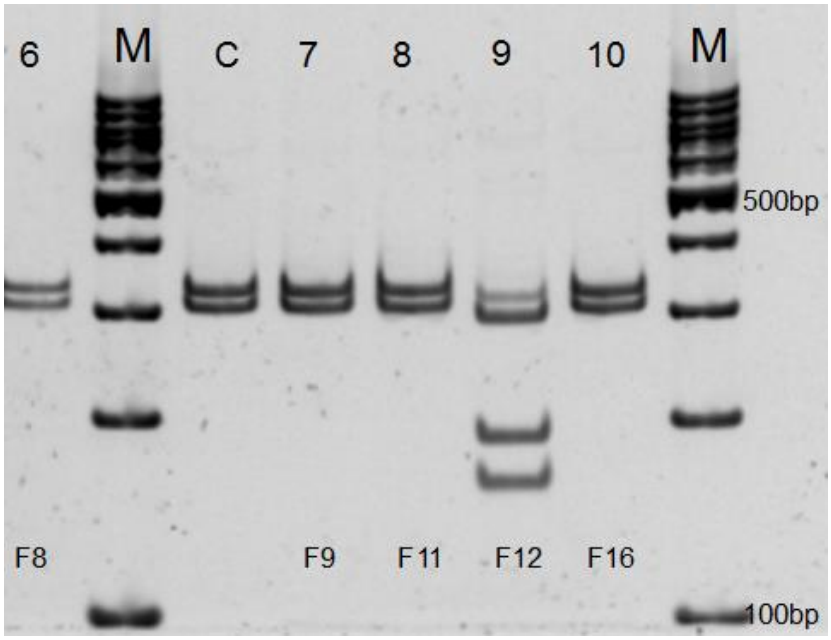

Figure 5. 5: RFLP restriction enzyme analysis using *MspI* and *Eco571* digestion of 18 Ssu-rDNA from *Trypanosoma congolense* (lane 9) and *T. theileri* (lanes 6, 7, 8, and 10) in polyacrilamide gel. Positive control for *T. theileri* (lane C) and 100 bp DNA ladder (M) were included on the gel.

TABLE 5. 1: Tukey´s honest significance test for *T. Congolense* infections per site.

	Tukey HSD test; variable **T.co** (Infection_data_Tco_Tth in Tabanids_data.stw) Approximate Probabilities for Post Hoc Tests Error: Between MS = 20.722, df = 9.0000			
Cell No.	Area	{1} 2.2500	{2} 4.7500	{3} 5.5000
1	Zalala		0.725948	0.589591
2	Botao	0.725948		0.970690
3	Namitangurine	0.589591	0.970690	

For the suspected *T. theileri*, the opposite was found, with specimens from Zalala (3.8%) yielding the highest infection rate, followed by Namitangurine (3.1%), which in turn was followed by Botao (0.6%) (Figure 5.7). However, there were no significant differences in terms of total *T. congolense* infections (Table 5.1), *T. theileri* infections (Table 5.2) or both infections (Table 5.3), between the sites (Tukey´s honest significance test).

TABLE 5. 2: Tukey´s honest significance test for *T. theileri* infections per site.

	Tukey HSD test; variable **T.th** (Infection_data_Tco_Tth in Tabanids_data.stw) Approximate Probabilities for Post Hoc Tests Error: Between MS = 3.4167, df = 9.0000			
Cell No.	Area	{1} 1.5000	{2} .50000	{3} 1.2500
1	Zalala		0.732616	0.980165
2	Botao	0.732616		0.837178
3	Namitangurine	0.980165	0.837178	

TABLE 5. 3: Tukey´s honest significance test for total *Trypanosoma* infections per site.

	Tukey HSD test; variable **total** (Infection_data_Tco_Tth in Tabanids_data.stw) Approximate Probabilities for Post Hoc Tests Error: Between MS = 20.472, df = 9.0000			
Cell No.	Area	{1} 3.7500	{2} 5.2500	{3} 6.7500
1	Zalala		0.887423	0.631693
2	Botao	0.887423		0.887423
3	Namitangurine	0.631693	0.887423	

In *A. grestis* only *T. congolense* infection was detected and was the highest from all the species 25.0% (30/120). *T. par* and *T. taeniola* had similar *T. congolense* infection rates

8.3% (10/120) but the second species presented a slightly high *T. theileri* infection rate (Figure 5.8).

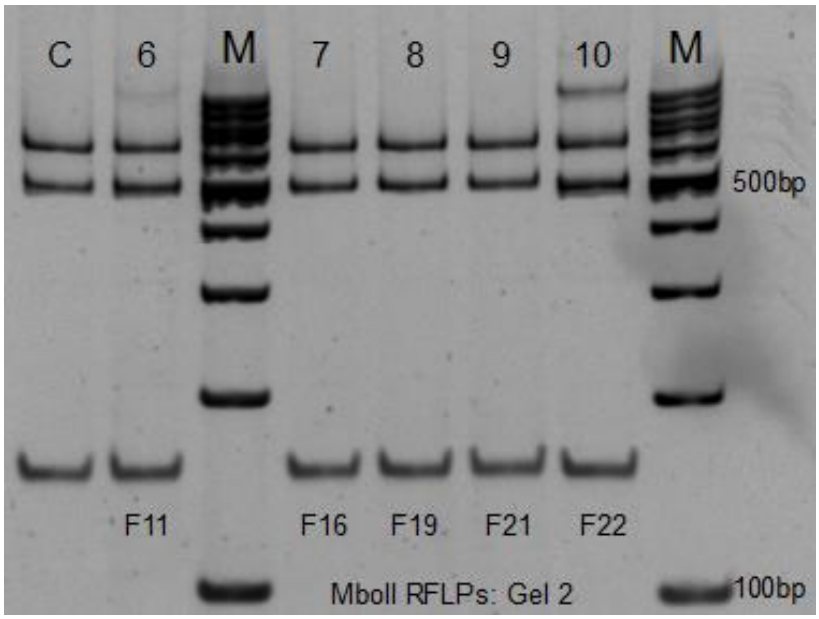

FIGURE 5. 6: RFLP restriction enzyme analysis using MboII digestion of 18 Ssu-rDNA from *T. theileri* (lanes 6, 7, 8, 9, 10) in polyacrilamide gel. Positive control for *T. theileri* (lane C) and 100 bp DNA ladder (M) were included on the gel.

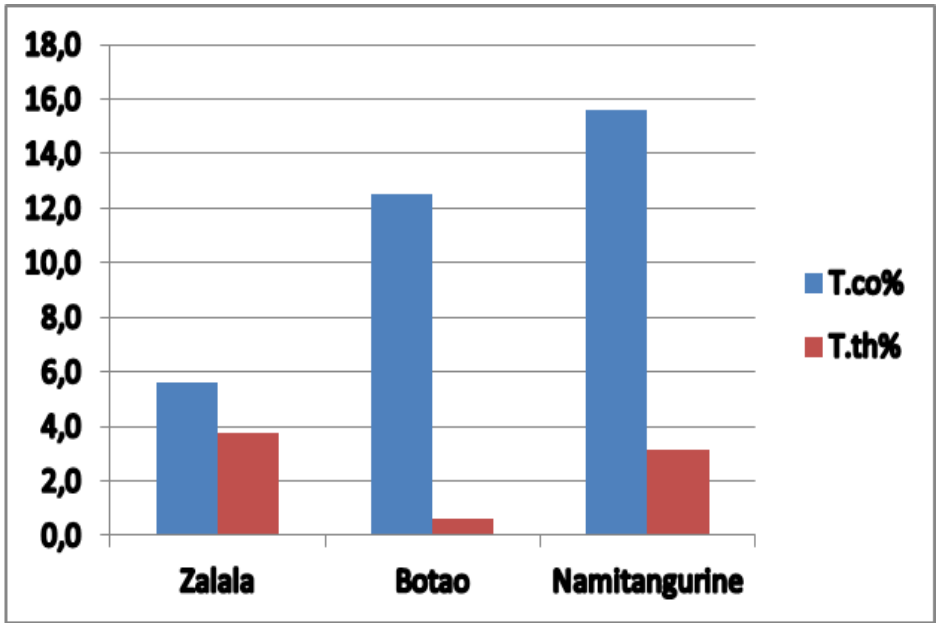

FIGURE 5. 7: Percentage of infected tabanid flies per species of *Trypanosoma*/area, Zalala (n=160), Botao (n=160), Namitangurine (n=160), as detected by 18S PCR-RFLP.

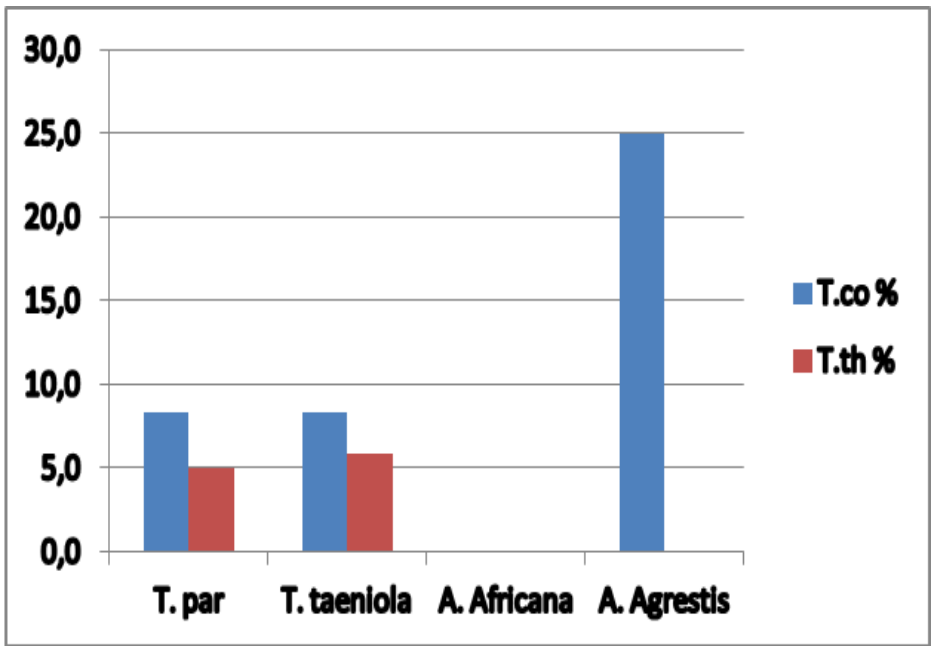

FIGURE 5. 8: Percentage of infected tabanid flies per species of *Trypanosoma* in each species of tabanid, T. par = *Tabanus par* (n=160), T. taeniola = *Tabanus taeniola* (n=160), A. africana = *Ancala africana* (n=160), A. agrestis = *Atylotus agrestis* (n=160), as detected by 18S PCR-RFLP.

5.4 Discussion

Our results revealed a high prevalence of *Trypanosoma congolense* found in the 24 tsetse analyzed. By dissection, the infection rate varies between 1 and 6% as reported by Malele *et al.* (2011); Shereni *et al.* (2016). With PCR, clearly documented as a more sensitive technique, the results of infection rate studies are slightly higher, varying from 14 to 20%, as revealed by (Mwandiringana *et al.* 2012; Simo *et al.* 2012, 2015; Isaac *et al.* 2016). Furthermore, the study that detected an infection rate of 6% with dissection, using PCR, (Malele *et al.* 2011) detected an infection rate of up to 93% in the same flies.

G. brevipalpis appears to be the most important vector due to the high infection rates detected (72.2%, n=18). This is reinforced by the fact that it was the most collected *Glossina* species, compared to the other two species, as described in chapter 4. Motloang *et al.* (2012), on their experimental study in South Africa, concluded that, despite the higher abundance, the role of *G. brevipalpis* in the transmission of *T.*

congolense was negligible. However, on the view of the results from the present study, it is important to reassess the role of *G. brevipalpis* in the transmission of trypanosomes not only due to the high infection rates found but also due to the fact that in one year of trapping, in the trypanosomosis foci of Zalala, only *G. brevipalpis* was captured (details in chapter 4).

No infected *G. pallidipes* flies were found, however, the results still yielded a very high overall infection rate (70.8%, n=24), confirming the importance these flies play in the circulation of *Trypanosoma* species. Similar to this study, surveys on cattle in Nicoadala district by Jamal *et al.* (2005); Specht (2008) and Mulandane *et al.* (2018) also indicated a high prevalence of trypanosomosis.

Detection of trypanosomes in these tsetse flies is common and has been reported in a large number of studies (Mihok *et al.* 1992; Mamabolo *et al*. 2009; Malele *et al.* 2011; Mwandiringana *et al.* 2012; Salekwa *et al.* 2014; Simo *et al.* 2012; 2015; Shereni *et al.* 2016).

The *G. morsitans* sample size is small, thus no statistical questions can be addressed to the results found, however the infection rate observed in the current study should be interpreted as an indication of the important role this species plays as vector of trypanosomosis. In fact Vreysen *et al.* (2013) described *G. morsitans* as one of the most important species in the morsitans group and the major vector of AAT in Eastern and Southern Africa. However, this study had an insufficient number of flies to support the statement statistically. Not surprisingly, all *G. morsitans* flies were captured in Namitangurine, which is the least disturbed out of the three study areas and thus there was possibly a higher probability of interaction with a wider range of susceptible mammals that are considered to be reservoirs of trypanosomes. A similar result was found by Salekwa *et al.*(2014) in Tanzania and Shereni *et al.*(2016) in Zimbabwe, where *G. morsitans* were captured in less disturbed areas or conservation areas and tested positive for *Trypanosoma*, though the infection rates differed from those found in this study. Moreover, Vreysen *et al.*(2013) described *G. morsitans* as a savannah species

and that their distribution often correspond with that of wild animals and thus less disturbed environments.

Due to the very small sample size, no accurate discussion can be presented on the infection rate of *G. pallidipes*. However, in other studies, *Trypanosoma* infection were detected in this species (Malele *et al.* 2011; Nthiwa *et al.* 2015), which shows that they can actively harbor and transmit trypanosomes. The absence of infection in *G. pallidipes* and the difference in infection rates in the three species analyzed, can be due to feeding preferences (Bitew *et al.* 2011), genetic differences, the availability of reservoirs, the parasitaemia of the vertebrate host (Jordan 1986; Leak 1999) and the nutritional status of the flies (Kubi *et al.* 2006; Akoda *et al.*, 2009). Taking into account the study area it is reasonable to infer that the main host, at least in Zalala and Botao, is cattle.

In the present study, tabanids were found to be positive for *Trypanosoma* infections. The overall infection rate for tabanids was 13%. This result strongly suggests a role for tabanids in the transmission of trypanosomes to vertebrate hosts as was suggested by Hall (2001); Desquesnes & Dia (2003) and Desquesnes *et al.* (2009).

One important fact is that, regardless of the site of collection, tabanids were detected positive to *Trypanosoma* infection. In fact, the three tabanids species that showed positive results for *Trypanosoma* infection, namely *Tabanus par*, *T. taeniola* and *Atylotus agrestis* were captured in the three trapping sites in relatively high numbers as described in Chapter 4.

The detection of trypanosomes in tabanids is common and has been described in quite a few studies (Hall 2001; Taioe *et al.* 2017) due to the fact that these vectors are involved in the transmission of trypanosomes to vertebrate hosts and that their participation in the transmission of the pathogens is becoming more and more important with the ecological transformations (encroachment) that are progressively affecting the tsetse densities and

distribution in Africa (Desquesnes & Dia 2003; Vale & Hargrove 2015; Moore *et al.* 2012).

Atylotus agrestis showed the highest infection rates out of all the tabanids species. This should be considered and the fact that it was the second most frequent species captured, in the three habitats, as described in the entomological survey (described in Chapter 4) draws the attention to this particular species. Moreover, the competency of *A. agrestis* in the mechanical transmission of *T. congolense* has been experimentally proven by Desquesnes & Dia (2003) in a fly-proof corral in Lahirasso (Burkina Faso), where two of eight animals exposed to trypanosome infected animals in the presence of *A. agrestis* tested positive for *T. congolense* after 20 days.

Tabanids habitat preference varies from species to species. It can go from large open areas to forested habitats. Additionally, feeding patterns also vary from species to species and it is known that their host preferences are generally quite diverse (Foil and Hogsette 1994; Baldacchino *et al.* 2014), the highest infection rate was observed in Namitangurine, followed by Botao and Zalala. This can be explained by the type of area itself. Namitangurine is characterized by dense thickets and is a less disturbed area with records of a presence of wild animals like sable antelopes, kudus, reedbucks, duiker and bush pig (MAE, 2014) that serve as food sources for tsetse and tabanids and can also act as reservoirs of trypanosomes. According to Hodo & Hamer (2017), wildlife species not only serve as food sources for the insect vectors, but also carry an extensive diversity of pathogens, including trypanosomes.

Salivarian trypanosomes can be found in a wide variety of hosts such as ruminants, carnivores, rodents and reptiles (Mulla & Rickman 1988; Njiokou *et al.* 2006; Anderson *et al.* 2011; Auty *et al.* 2012;), which proves the crucial role of these animals in the circulation of trypanosomes in a certain habitat. Moreover, both presently and in the past, control measures directed to trypanosomes reservoirs have shown to be successful in the reduction of trypanosomosis prevalence, though they have proven not to be environmentally sustainable (MCT 1960; WHO 2013; Wiel 2016).

When comparing Botao and Zalala, the latter is a more disturbed area, close to the sea and with more human settlements. This was evident in the overall infection rate as it was the lowest from the three. It is therefore plausible to argue that human activity has a significant influence on the abundance and distribution of tsetse flies. Moreover, the human presence may have resulted in a considerable reduction or probably elimination of wildlife (trypanosome reservoirs) from the area. Torres *et al.*, (2016) specifies that habitat destruction or degradation due to human activities is the number one cause for reduction or extinction of wildlife populations. Furthermore, according to Van den Bossche *et al.*, (2010), demographic pressure can cause deep modifications in tsetse habitats, which may result in alterations in tsetse densities and distribution.

The potential of tabanids to mechanically transmit trypanosomes is high, especially for *A. agrestis* that, according to Oldroyd (1954), is a tabanid species dispersed across a large area, being found from Senegal to South Africa. Moreover, studies on detection of *Trypanosoma* in tabanids and understanding the possible participation of these haematophagous insects as mechanical vectors of trypanosomosis have been reported by Desquesnes *et al.* (2009); Muzari (2010) and Taioe *et al.* 2017. Furthermore, *T. taeniola*, one of the species detected positive for *Trypanosoma* infection in the present study, has also been found to be infected by *T. congolense* in Burkina Faso as reported by Solano & Amsler-Delafosse (1995).

The only pathogenic trypanosome found in the current study was *T. congolense*. This is well in accordance with Mulandane *et al.* (2018) that detected, in 2014, *T. congolense* as the only circulating *Trypanosoma* species in cattle in Nicoadala district. Moreover, the high infection rates with *T. congolense* in interface areas like Botao could be explained, as stated earlier in this discussion, by the presence of wildlife in the vicinity.

Additional to *T. congolense*, *T. theileri* was also found in tabanids. *T. theileri* is a large stercorarrian non-pathogenic trypanosome transmitted by tabanids to cattle and highly

prevalent around the globe (Schlafer 1979; Rodrigues *et al.* 2006; Martins *et al.* 2008; Sood *et al.* 2011).

This is the first study to document trypanosome infection rates in haematophagous insects in Nicoadala district, an area identified as a drug resistance hotspot. So, the question raised is: what if in a drug resistance focus like Nicoadala dictrict, even in complete absence of tsetse flies, trypanosomes can still circulate in vertebrates as a result of a combination of resistance trypanosome populations and efficient mechanical vectors? When associating the present results to the ones found by Mulandane *et al.* (2018) on the prevalence of *T. congolense* in Nicoadala district, one becomes compelled to strongly suggest that the infection of cattle with trypanosomes in the district by tsetse flies is actively amplified by the participation of tabanids as mechanical vectors, which contributes substantially to the high prevalence detected in different studies. In fact, in different trypanosomosis foci, mechanical transmission of trypanosomes by tabanids has been wrongly viewed as negligible and this may be contributing to the failure of some control strategies targeting the decrease of the disease prevalence. Moreover, mechanical transmission of trypanosomes, depending on the circumstances and conditions, may be as efficient as biological transmission, although its potential impact has never been estimated (Foil 1989; Desquesnes *et al*. 2009; Baldacchino *et al.* 2014). Therefore, it also raises another very important question: would the complete eradication of the tsetse eradicate the disease in the area? Are tabanids just an amplification factor or could the cycle be maintained by tabanids alone in the presence of resistant trypanosome isolates in the case of failure in elimination of the same resistance isolates through chemotherapy or other possible methods? The present study also calls attention to the need for the development of strategies aimed to control trypanosomosis, including not only the control of tsetse flies as cyclical vectors of the trypanosomes, but also of tabanids, which are capable of transmitting trypanosomes.

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CHAPTER 6 : General Discussion and Conclusion

6.1 Introduction

The present study was aimed at (i) the detection of trypanosomes and trypanocidal drugs resistance in Nicoadala district, (ii) the detection of potential haematophagous vectors of trypanosomosis in the district and (iii) the understanding of the relative role of these vectors in the transmission of trypanosomes. These three objectives were addressed by conducting three major experiments: a trypanosomosis survey and block treatment for drug resistance detection; trapping and identification using morphology and cytochrome oxidase 1 PCR for vector molecular identification; and using 18S PCR-RFLP for the detection of trypanosomes in haematophagous flies.

6.2 Assessment of trypanocidal drugs resistance in T. congolense population

Nicoadala district was identified in 2005 as a DR hotspot (Jamal *et al.* 2005). However, since then, no further information regarding the dynamics of the phenomenon was made available. Thus, the current study not only constitutes the first update on DR in the region in the last 12 years, but also provides updated information on the prevalence of trypanosomosis as well as the species composition of circulating trypanosomes in Nicoadala district. Additional to Nicoadala district, Jamal et al. 2005 also worked and detected DR in Chinde and Maganja da Costa districts. Therefore, for the Nicoadala district, it is now known that DR has not changed geographically since 2005. It is also known that *T. congolense* may be the only pathogenic *Trypanosoma* species circulating in the area.

The block treatment (BT) test was used to evaluate single and multiple drug resistance in *T. congolense* populations in Nicoadala district. The BT has been described in the literature and has been presented as tool for drug resistance assessment while molecular tools, that would have been faster, are not yet adequately validated. However, due to the residual presence of ISM in the blood of the cattle, caution is recommended when interpreting the outcomes results in cases where DA is administered after ISM failure (McDermott et al. 2003; Mungube *et al.* 2012). The success of the BT in the detection of resistant isolates in Nicoadala district allows for the extrapolation of the test, ensuring its replication in other trypanosomosis hotspots. The BT test can be considered as a

good model for *in vivo* testing because it allows for the evaluation of the exact curative dose. This extrapolation is not possible with the test in mice. Another advantage is that it includes all *Trypanosoma* species that infect cattle, including *T. vivax* that has no or a very poor infectivity for mice (Eisler *et al.* 2001). Furthermore, the BT allows for the use of the affected cattle breed in the affected area, with no major changes to the normal routine of the farm and the animals (McDermott et al. 2003). For the use of BT in field assessment of DR is recommended that the survey takes place at the end of the rainy season in order to maximize the probability of having large numbers of trypanosome infected cattle as the end of rainy season is considered a period of high risk of infection of cattle with trypasonosomes (Mungube et al. 2012).

To detect treatment failure in the BT test, both Polymerase chain reaction (PCR) and the *buffycoat* technique (BCT) were used. Both methods are typically used to estimate the prevalence of trypanosome infections and have enough sensitivity to be used for the purpose of trypanosome detection, though PCR has consistently proved to be 2 to 7 times more sensitive than the BCT for the diagnosis of trypanosome infections (Paris *et al.* 1982; Clausen *et al.* 1998). The use of PCR in the present study can be considered an improvement in the protocol if compared to the approach followed by Mungube et al. 2012 where only microscopy was used for the follow up of treated animals.

Information on trypanosomosis prevalence, and particularly DR, is important for the development of control strategies. In the case of Nicoadala district, where multiple DR was detected, the adoption of the best bet strategies (BBT) would be the best option in the current absence of newly developed trypanocides. In the present case, BBT include (i) rational drug use i.e. reducing the drug use to only clinical cases administered properly with the right dose, (ii) reducing the drug use by preventing other parasitic diseases (Clausen et al. 2010) and (iii) accurate monitoring of treated animals to detect any drug failure (Eisler et al. 2001). The use of trypanocidal drugs of verified quality is recommended especially in cases where the farmers purchase the drugs themselves. Though allegedly all the drugs present in the country are imported through the Ministry of Agriculture and Food security, farmers can obtain trypanocides from a variety of sources. Additionally, stimulation of tsetse and other insects' control through the use of

insecticides and netting is recommended. As a matter of fact, it is demonstrated in studies that tsetse control reduces drug expenditure by 50% and cattle mortality by 71% (Clausen et al. 2010; Kamuanga et al. 2001).

6.3 Taxonomic and molecular characterization of Tabanidae and Glossinidae and trap efficiency comparison

To analyse the vectors capable of biologically and mechanically transmit trypanosomosis, an entomological survey was conducted. Only 24 *Glossina*, belonging to three of the four species present in the country were captured. Additionally, 4729 tabanids subdivided into 10 species were also captured. An entomological survey was carried out for 365 consecutive days, using 55 traps. This sampling effort contributes to the robustness of the collected data and strongly indicates (i) the presence of one *Glossina* population, of extremely low apparent density, (ii) a large homogenous population of tabanids (few species) cohabiting there.

No significant difference was found in the total capture of tabanids in the three selected grazing areas, what contributed to a low Whitaker index when comparing the diversity between the habitats. The lack of habitat specificity, especially for the most representative species, *Tabanus par, Atylotus agrestis, T. taeniola* and *Ancala africana* may have contributed to this finding since these species were captured in the three different grazing areas namely Zalala (open area), Botao (interface between closed and open area) and Namitangurine (closed thicket area). Additionally, the clear dominance of the four above mentioned species contributed to a low Shannon index in the tree habitats, revealing a low diversity if taken into account that Tabanidae counts more than 4000 species (Baldacchino *et al.* 2014). Across all studied regions, *Tabanus* was the dominant genus, and this result was similar to other studies conducted in Zambia and South Africa (Taioe *et al*. 2017) it can be conjectured that this is the dominant genus in South-East Africa.

The results from the cytochrome oxidase 1 (CO1) analysis support monophyly in the family Tabanidae as suggested in several other studies (Wiegmann et al. 2000; Yeates et al. 2007; Morita et al. 2008; Morita et al. 2016; Taioe et al. 2017; Shin et al. 2018). CO1

data generated in the present study allowed for the confirmation of the identified tabanid species, which strengthened the possibility of using this tool for further tabanid diversity studies, especially in cases where morphological identification is difficult.

The better performance of the NGU traps in capturing all tsetse species makes it a well-designed tool for studies in South-East Africa. In addition to the efficient tsetse capture, this trap also had showed a better performance in the capture of tabanids, compared to H and Epsilon traps. We recommend thus this trap for entomological surveys across South-East Africa. Furthermore, a combination of NGU and H traps can provide consistent results in the capture of the *Glossina* and tabanid species which occur in Mozambique.

Seasonal differences were observed. The rainy season is the best for the capture and study of tabanids. The total number of tabanids collected in the rainy season was twice the total collected in the dry season. Furthermore, in the rainy season, not only more tabanids were captured, but also their diversity was higher. This is in agreement with the observations from Barros (2001); Parra-Henao and Alarcón-Pineda (2008); Herczeg *et al.* (2014) and Lendzele *et al*. (2017). Additionally, the peak of tabanids in March, the end of the rainy season, may explain the high prevalence of trypanosomosis reported by Mulandane *et al*. (2018). However, further studies should be conducted to confirm this pattern.

No seasonal succession was observed within the most abundant species. This lack of variation may contribute to an increased interspecific competition between tabanids and increased pressure on cattle during the competition for feeding. Consequently, both the levels of stress on the animal and the probability of acquiring different infections derived from tabanids via mechanical transmission are likely to have increased. This result differed from Barros (2001), where a clear seasonal succession was verified in the most abundant species captured. Studies of the seasonal abundance of tabanids are crucial to understand their participation in the transmission of pathogens as described by Foil (1989).

6.4 Molecular detection of Trypanosoma infections in flies

To further investigate animal trypanosomosis epidemiology, in addition to information on drug resistance and vector composition, tsetse and tabanid infection rates were assessed. With the current approach it was possible to confirm *T. congolense* as the predominant pathogenic *Trypanosoma* species, and probably the only one circulating in cattle. The implication of *G. brevipalpis* as the main trypanosomosis vector in the area contrasts with the results of Motloang *et al*. (2012), assessing the role of the same species as a vector of trypanosomes in Kwazulu-Natal, South Africa . Therefore, further investigation on the vector competency of *G. brevipalpis* should be conducted.

The presence of pathogenic trypanosomes in tabanids strongly suggests that the participation of tabanids in the mechanical transmission of these parasites may be playing a very important role in the epidemiology of trypanosomosis and that these insects are possibly involved in the amplification of trypanosome infection in Nicoadala district, contributing to the higher prevalence verified in the different surveys.

The relative widespread distribution of *Atylotus agrestis* across Africa (Oldroyd, 1954), which is in accordance with what was found in all three grazing areas, associated with its capacity to transmit trypanosomosis as described in different studies (Solano and Amsler-Delafosse, 1995; Desquesnes and Dia 2003), suggests that this species could be the most important mechanical vector in the trypanosome transmission cycle in the area. However, *T. par* and *T. taeniola* can also be implicated. The fact that no trypanosomes were detected in *Ancala africana* does not exclude this species from participation in transmission of trypanosomes.

6.5 Conclusion and recommendations

With the present results, the conclusion is that trypanosomosis, probably caused exclusively by *T. congolense*, and drug resistance are still present in Nicoadala district and that drug resistance has not changed geographically for at least, the last 10 years. A very small population of tsetse flies responsible for the biological transmission of trypanosomes is present in the district. Despite the high abundance, there is a small diversity of Tabanidae in Nicoadala district, and they seem to play an important role in

the mechanical transmission of trypanosomes. Additionally, considering the performance of the traps, both NGU and H traps are recommendable for Tabanidae and Glossinidae capture and study in Mozambique given the tsetse species present and it is recommended that the study of these two families take place in the rainy season.

Addditional to the above conclusions, it can be also concluded that block treatment is an adequate method for the field assessment of drug resistance. The *buffycoat* and the 18S PCR-RFLP are appropriate techniques for the detection of trypanosome infections in cattle, with the second one being more sensitive for the task. Furthermore, the 18S PCR-RFLP is also adequate for the detection of trypanosomes in haematophagous insects. The cytochrome oxidase 1 is an appropriate genetic marker for the identification of tabanid species and for tabanids phylogenetic studies. This is the first study conducted in Nicoadala district, involving the characterization of Tabanidae and Glossinidae and the detection of the trypanosomes they harbor. As a pioneer study, it constitutes a starting point for future work on the subject. However, further studies with longer periods of trapping should be conducted to have a better picture of seasonal variation of tabanid abundance and diversity. The inclusion of accurate descriptions of the type of vegetation associated to the studied *Glossina* and Tabanidae species would provide additional elements for analysis and better understanding on the insect's habitat preference in the area. The two biggest questions that remain are: (i) can tabanids maintain the circulation of resistant trypanosome populations in the absence of tsetse flies, especially in highly fragmented environments like Zalala where tsetse populations can soon be completely eliminated due to human pressure? and (ii) would a complete tsetse elimination in the drug resistance foci of Nicoadala district eradicate trypanosomosis?

6.6 References

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ETHICAL APPROVAL

UNIVERSIDADE EDUARDO MONDLANE

Centro de Biotecnologia

CB • UEM

CBUEM/COMETH_0014/2014

Research Protocol Approval Letter

To whom it my concern

This document is aimed to inform that the research protocol titled Epiedemiology of Drug Resistance and Evaluation of Possible Mechanical Transmission of *Trypanosoma congolense* by Hematophagous Insects in Zambezia Province" has been reviewed and approved by Scientific Board of the Biotechnology Centre, Eduardo Mondlane University, Maputo, Mozambique. It complies with the International Epizooties Organization, national and local requirements concerning animal experimentation, care and welfare. The handling of animals will be carried by trained and approved stuff. The experimental procedures will be based on trypanosomosis infection in cattle in Zambezia province, from May to July, 2016.

Date 7. 03. 2014

Signature (Head of the Scientific Board)

UNIVERSIDADE EDUARDO MONDLANE CENTRO DE BIOTECNOLOGIA

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

Ref: V014-18

28 August 2018

Prof L Neves
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
(Luis.neves@up.ac.za)

Dear Prof Neves

Project V014-18
Epidemiology of Drug Resistance and Evaluation of Possible Mechanical Transmission of *Trypanosomosa congolense* by Hematophagous Insects in Zambezia Province, Mozambique

The application was evaluated by the Animal Ethics Committee of the University of Pretoria. We have no ethical concern regarding your study.

It is noted that this project will be completed in a facility outside of South Africa. Since the AEC has not inspected the facility, please note that we cannot comment on the quality of the facility other than what was provided in the study questionnaire.

If you have any questions, please feel free to contact the committee.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee
Copy F Mulandane (Researcher)

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