UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Elucidating the molecular epidemiology of trypanosomes for evidence-based tsetse-vector control in the Shimba Hills human-wildlife-livestock interface, Kenya

Faith Isikhuemen Ebhodaghe

Submitted in partial fulfilment of the requirements for the degree

Doctor of Philosophy

(Entomology)

in the

Department of Zoology & Entomology

Faculty of Natural & Agricultural Sciences

University of Pretoria

South Africa

July 2022

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Elucidating the molecular epidemiology of trypanosomes for evidence-based tsetse-vector control in the Shimba Hills human-wildlife-livestock interface, Kenya

Faith Isikhuemen Ebhodaghe

Supervisors: Prof. Armanda D. S. Bastos

Head, Department of Zoology & Entomology

University of Pretoria

Private Bag 20 Hatfield 0028, South Africa

Email: adbastos@zoology.up.ac.za

Dr Michael N. Okal

Postdoctoral Research Fellow, Animal Health Theme

International Centre of Insect Physiology & Ecology

P. O. Box 30772-00100, Nairobi, Kenya

Email: mnyanganga@icipe.org

Dr Daniel K. Masiga

Principal Scientist & Head, Animal Health Theme

International Centre of Insect Physiology & Ecology

P. O. Box 30772-00100, Nairobi, Kenya

Email: dmasiga@icipe.org

Declaration

I declare that this thesis/dissertation, which I hereby submit for the degree PhD Entomology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Faith Isikhuemen Ebhodaghe

July 2022

Disclaimer

This thesis consists of a series of chapters that have been prepared as stand-alone papers already published, accepted for publication or manuscripts intended for publication. Consequently, unavoidable overlaps and/or repetitions may occur, and the reference style and format may differ between chapters.

General Abstract

Field studies that systematically evaluate the transmission dynamics, reservoir hosts, and genotype diversity of trypanosome parasites are scanty in sylvatic ecologies in sub-Saharan Africa. Such studies are important for providing insights into infection hotspots and strategising targeted control of human sleeping sickness and cattle *nagana* disease caused by tsetse-borne trypanosomes. This thesis characterised the epidemiology of tsetse-borne trypanosomes in Shimba Hills —a wildlife area in southeast Kenya where *nagana* is endemic but understudied. Specifically, the thesis assessed the entomological inoculation rates of cattle trypanosome infection and also, unraveled wildlife sources of livestock infection and profiled the genotype diversity of the main trypanosome parasite circulating in populations of tsetse flies and reportedly affecting animal health in Shimba Hills. Using conventional-PCR and amplicon sequencing analyses targeting the Internal Transcribed Spacer-1 (ITS1) gene, 8.62% (60/696) (95% CI: 6.53 — 10.71) of wild-caught tsetse flies collected in biconical traps in Shimba Hills (November 2018 to September 2019) were confirmed positive for DNA of seven species and subspecies of trypanosomes. *Trypanosoma vivax* was the most prevalent trypanosome in tsetse flies. The other trypanosomes detected in tsetse flies in order of decreasing prevalence were *T. congolense* Kilifi, *T. congolense* Savannah, *T. simiae* Tsavo, and *T. godfreyi* and similar prevalence for *T. brucei sl* and *T. simiae*. *Glossina pallidipes* were the most widely distributed and abundant among the three species of tsetse flies collected in biconical traps and were more likely to infect cattle. Entomological inoculation rates were homogenous across landscapes but increased significantly towards the wildlife reserve in Shimba Hills. Bloodmeal analyses indicated that tsetse flies had fed on thirteen mammalian species, with DNA confirmation based on PCR-High Resolution Melting analyses of the vertebrate genes *16S* and *Cytochrome b* and amplicon sequencing of the gene *Cytochrome Oxidase 1*. Warthogs were the preferred hosts

of tsetse flies and were associated with increased likelihood of vector infection with cattle trypanosome parasites, including *T. vivax*. Phylogenetic analyses of amplicon sequences of the *ITS-1* DNA and 18S *rRNA* genes identified endemicity of two *T. vivax* genotypes in Shimba Hills with >80.00% comprising of the virulent *Tvv4* genotype. Tsetse flies confirmed as positive for *Tvv4* included those that had blood-fed on warthogs and cattle. Using insecticide-treated fabrics (ITFs) targeted to entomologically defined trypanosome hotspots, a cluster Randomised Controlled Trial (RCT) was designed to rationalise a plan and assess ITF efficacy for the control of tsetse flies incriminated as *Tvv4* vectors and having high potential for bloodmeals on cattle and transmission of infections from warthogs to cattle. With or without a blend of waterbuck-mimicking tsetse-repellent odours on cattle, ITFs significantly reduced trypanosome risk in Shimba Hills but with greater impact on *T. congolense* than *T. vivax*. Although the intervention was initially effective, an upsurge in infection cases was observed after five months of interventions. Trypanosome infection risks were not significantly different between the intervention arm of the RCT and the control arm. *Stomoxys* were highly abundant and *T. vivax* risk was apparently high in the control arm. The present work is the most extensive in Shimba Hills in terms of spatio-temporal coverage of tsetse sample collection and reports the highest diversity of trypanosomes and animal bloodmeal hosts documented in a single epidemiological survey in the area. Spatial entomological risk of cattle trypanosome infections is described for the first time in the Shimba Hills human-wildlife-livestock interface with evidence of high risk of cattle infections from warthogs in locations close to the wildlife reserve. Unequivocal evidence is also presented for the first time of the *Tvv4* genotype endemicity in Kenya and plausible contribution to pathologies in cattle in Shimba Hills. As supplementing ITFs with tsetse-repellent did not significantly improve outcome of interventions in Shimba Hills, combined adoption of both technologies for tsetse control may be unnecessary for trypanosome vector management in the area. However, livestock

owners in smallholder communities in Shimba Hills may deploy ITFs during tsetse peak seasons while making efforts to maintain these devices for optimal performance. Livestock owners can also apply tsetse-repellent odours on cattle year-round particularly during periods of low tsetse fly abundance when ITF adoption may be unnecessary. This is important given ease-of-adoption and low-cost of the odours and previous findings in Shimba Hills of the odours being effective at reducing tsetse-cattle contacts and infection risk. *Stomoxys* possible involvement in *T. vivax* transmission in the control arm of the RCT in Shimba Hills had plausibly compromised intervention-effects on the parasite thus should be assessed in future studies for its role in trypanosome epidemiology in the area.

Dedication

To my beloved mother, Patricia

for her impact on my life in ways too deep to express in words

Acknowledgements

To my supervisors Prof. ADS Bastos, Dr MN Okal and Dr DK Masiga, thanks for the support and guidance. You helped me to successfully navigate the hurdles of doctoral training. I am happy our paths crossed and have learned so much from you within the three and half years of working together. To Dr Shewit Kalayou, I appreciate the cordiality and constructive inputs to my work.

The International Centre of Insect Physiology & Ecology *icipe* provided the platform for my PhD research under the auspices of the African Regional Postgraduate Programme in Insect Science (ARPPIS). I thank Dr Rob Skilton, Ms. Vivian Atieno, Ms. Esther Ndung'u, & Ms. Margret Ochanda at the *icipe* Capacity Building & Institutional Development (CB&ID) Unit for invaluable support. I also thank my classmates (Fairo Dzekashu, Vongai Paradza, Nadia Toukem, Kochelani Saili, Edwin Ogola, Mukundi Mukundamago & Rose Sagwe) and other colleagues too many to mention for the camaraderie.

Kevin Kidambasi Ogola provided support in laboratory analyses of samples, James Kabii assisted with logistics, Caroline Muya helped in organizing travels to the field while David Mbuvi, Peter Muasa, Richard Tumba, Philip Kolei, Oscar Esibi and Careen Kemunto provided technical support in field collection of samples.

Scholarship funding was provided by the *Deutscher Akademischer Austauschdienst* (DAAD) and research funding by the BioVision Foundation Switzerland and the German Ministry for Economic Cooperation and Development (BMZ). Many thanks to the University of Pretoria for providing funding support through the 2021 Bursary for Postgraduate Students.

Finally, I thank my lovely family, and Christian brethren in Kenya, Nigeria and Ghana for emotional support and prayers all through the time of my PhD research. To God Almighty be the glory for the gift of life and good health!

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AAT: Animal African Trypanosomiasis

ARPPIS: African Regional Postgraduate Programme in Insect Science

BGLMM: Binomial Generalised Linear Mixed Model

BMZ: The German Ministry for Economic Cooperation and Development

CB&ID: Capacity Building & Institutional Development

CI: Confidence Interval

CO1: Cytochrome Oxidase 1

CONSORT: Consolidated Standards of Reporting Randomised Trials

CV: Coefficient of Variation

Cytb: Cytochrome b oxidase

DAAD: The German Academic Exchange Service

DDT: Dichlorodiphenyltrichloroethane

DF: Degree of Freedom

DNA: Deoxy-ribo Nucleic Acid

EIR: Entomological Inoculation Rate

FAO: Food and Agriculture Organisation

FITCA: Farming in Tsetse Controlled Area

FTD: Flies per Trap per Day

GDP: Gross Domestic Product

g-HAT: gambiense Human African Trypanosomiasis

HRM: High-Resolution Melting Analysis

icipe: International Centre of Insect Physiology and Ecology

IGAD: East African Intergovernmental Authority on Development

ITF: Insecticide-treated Fabric

ITS1: Internal Transcribed-Spacer 1

KENTTEC: Kenya Tsetse and Trypanosomiasis Eradication Council

KETRI: Kenya Tsetse Research Institute

KWS: Kenya Wildlife Service

Mg/hr: Milligram per Hour

NACOSTI: National Commission for Science, Technology, and Innovation

NBGLMM: Negative Binomial Generalised Linear Mixed Model

NCBI: National Center for Biotechnology Information

NP: National Park

NR: National Reserve

BGLM: Binomial Generalised Linear Model

OIE: World Organisation for Animal Health

OR: Odd Ratio

PCR: Polymerase Chain Reaction

PCV: Packed Cell Volume

PGLM: Poisson Generalised Linear Model

RCT: Randomised Controlled Trial

r-HAT: rhodesiense Human African Trypanosomiasis

RNA: Ribonucleic Acid

SHNR: Shimba Hills National Reserve

SIT: Sterile Insect Technique

USD: United States Dollars

WB: Waterbuck

WFS: Wing Fray Score

WHO: World Health Organisation

Research Outputs

Journal articles

Ebhodaghe, F. I., Bastos, A. D. S., Shewit Kalayou, Michael N. Okal & Masiga, D. K. (2022). Trypanosome entomological risk assessment in the Shimba Hills human-wildlife-livestock interface in Kenya. Manuscript accepted for publication in *Frontiers in Veterinary Science*.

Ebhodaghe, F. I., Okal, M. N., Kalayou, S., Bastos, A. D., & Masiga, D. K. (2021). Tsetse Bloodmeal Analyses Incriminate the Common Warthog *Phacochoerus africanus* as an Important Cryptic Host of Animal Trypanosomes in Smallholder Cattle Farming Communities in Shimba Hills, Kenya. *Pathogens*, *10*(11), 1501. **https://pubmed.ncbi.nlm.nih.gov/34832656/**

Ebhodaghe, F. I., Bastos, A. D., Kidambasi, K. O., Kalayou, S., Masiga, D. K., & Okal, M. N. (2021). Molecular characterisation of *Trypanosoma vivax* in tsetse flies confirms the presence of the virulent *Tvv4* genotype in Kenya: Potential implications for the control of trypanosomiasis in Shimba Hills. *Infection, Genetics and Evolution*, 104953. **https://pubmed.ncbi.nlm.nih.gov/34091066/**

Ebhodaghe, F. I., Bastos, A. D. S., Shewit Kalayou, Masiga, D. K. & Michael N. Okal (2022). Characterising the epidemiological effect of tsetse control in trypanosome hotspots in Shimba Hills (Kenya): a cluster randomised controlled trial suing insecticide-treated fabrics with and without tsetse-repellent odours on cattle. Manuscript Prepared for submission to *PLoS Neglected Tropical Diseases*.

Oral presentations

Faith Ebhodaghe (2021). *First things first*: Characterizing the epidemiology of trypanosomiasis in Shimba Hills for effective and sustainable vector control. Meeting of the 4-Health Themes of the International Centre of Insect Physiology and Ecology *icipe* Nairobi, Kenya. 17th of February 2021.

Faith Ebhodaghe, Shewit Kalayou, Armanda Bastos, Dan Masiga & Michael Okal (2021). Characterizing the risk for tsetse-borne animal African trypanosomosis at the wildlife-livestock interface of the Shimba Hills National Reserve, East Africa: a case for targeted control. The 28th International Conference of the World Association for the Advancement of Veterinary Parasitology WAAVP, 19th to 22nd July, Dublin, Ireland.

Faith Ebhodaghe, Armanda Bastos, Michael Okal & Daniel Masiga (2021). First molecular confirmation of the pathogenic *Trypanosoma vivax* genotype *Tvv4* in wild-caught vectors of Animal African Trypanosomiasis from Kenya, East Africa. The 28th International Conference of the World Association for the Advancement of Veterinary Parasitology WAAVP, 19th to 22nd July, Dublin, Ireland.

Faith Ebhodaghe (2022). Hotspot-targeted control of tsetse flies in *push-pull* interventions in Shimba Hills (Kenya). Meeting of the 4-Health Themes of the International Centre of Insect Physiology and Ecology *icipe* Nairobi, Kenya. 17th of February 2022.

CHAPTER 1

General Introduction, Justification, & Study Aim & Objectives

1.1.0 General introduction

1.1.1 Complex epidemiology of infectious diseases in wildlife areas

Pathogens adversely affect human and animal health worldwide. They include viruses, bacteria, fungi, prions, and parasites. These infectious agents are propagated through different means including transmission by arthropod-vectors. Understanding pathogen transmission patterns are important for deciphering disease dynamics. However, pathogen transmission patterns are complex and less well understood in many disease ecologies especially sylvatic landscapes. Hence, control programs in local ecologies in wildlife areas experience difficulties when it comes to predicting infectious disease risk and strategizing interventions against pathogens.

The complexities in pathogen transmission in sylvatic environments are underpinned by a plethora of factors including anthropogenic disturbances (Hassell et al. 2017). Anthropisation affects the bioecology of disease-vectors and has been found to drive fundamental changes in infectious disease transmission risk in areas experiencing high rates of human influx (Cator et al. 2020; Wilke et al. 2021). A far more important driver of pathogen complex transmission patterns in wildlife areas is the involvement of a wide range of animal species in the epidemiology of infectious agents (Triguero-Ocaña et al. 2020). Pathogen dissemination among different animal species in wildlife areas is facilitated by the cosmopolitan blood-feeding behaviours of hematophagous vectors. Mosquitoes, ticks, tsetse flies and other arthropod-vectors that transmit human and animal pathogens in sylvatic landscapes seek nourishment from large communities of animal species (Omondi et al. 2015; Oundo et al. 2020; Channumsin et al. 2021). Hence, the vectors are able to disseminate pathogens among an extensive variety of susceptible wild fauna and, sometimes, cause spill-over of infections from wildlife to livestock along the borders of National Reserves and Game Parks (LaHue et al. 2016). The involvement of an extensive repertoire of wildlife species

in pathogen transmission cycles and adaptations of these pathogens to different animal hosts have caused many etiological agents of human and animal diseases to evolve into multiple genetic forms that have varying degrees of virulence and distinct transmission patterns. These complexities require adjusted approaches for control (Regoes et al. 2000; Azat 2021; Krücken et al. 2021).

Among human and animal infective vector-borne pathogens with complex epidemiology in wildlife areas is the extracellular flagellate protozoan *Trypanosoma* parasite commonly referred to as trypanosome (Fig. 1.1).

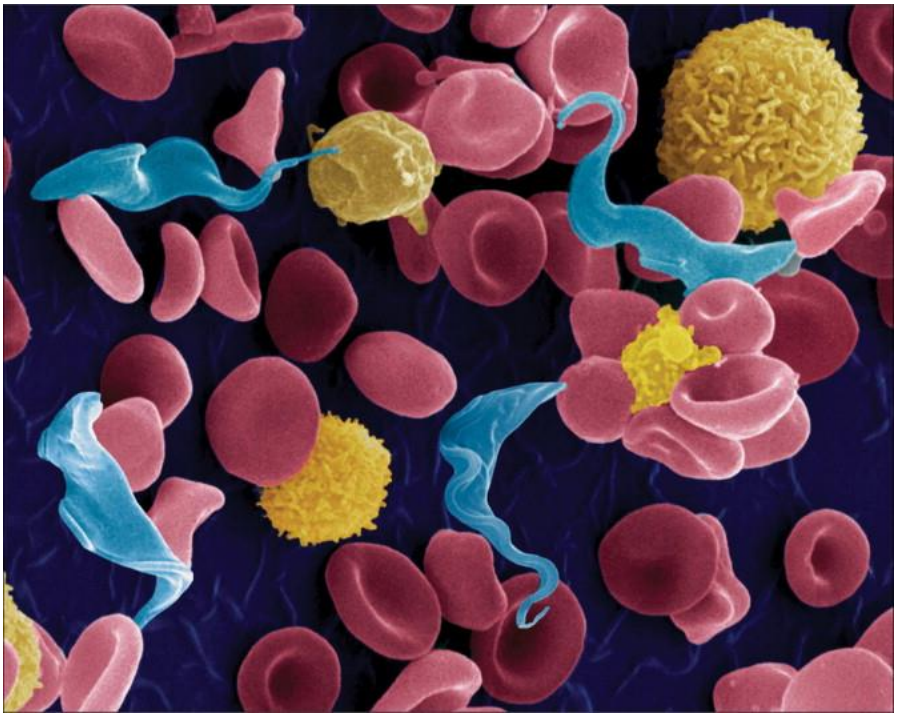


Figure 1.1: Trypanosomes in vertebrate blood. Trypanosomes are shown in blue, while red blood cells (erythrocytes) and white blood cells (leukocytes) are shown in red and yellow respectively.

Credit: M Duszenko, University of Tübingen, Germany (Source: Brun et al. 2010. *The Lancet*, 375(9709), pp. 152)

Trypanosomes have a global distribution but are primarily endemic to the tropics with Latin America and Africa being the most affected continents (Fig. 1.2; Giordani et al. 2016). The parasites, which have an extensive host range, belong to the family 'Trypanosomatidae', and possess complex transmission patterns. *Trypanosoma* species and subspecies occur in widely varying genotypes (Radwanska et al. 2018; Kasozi et al. 2021). Many trypanosome genotypes reported within the last four decades have poorly described epidemiologies though are highly virulent causing huge negative impacts on public health and driving extensive agricultural and socio-economic losses valued in billions of dollars on annual basis (Rodrigues et al. 2008, 2017; Adams et al. 2010).

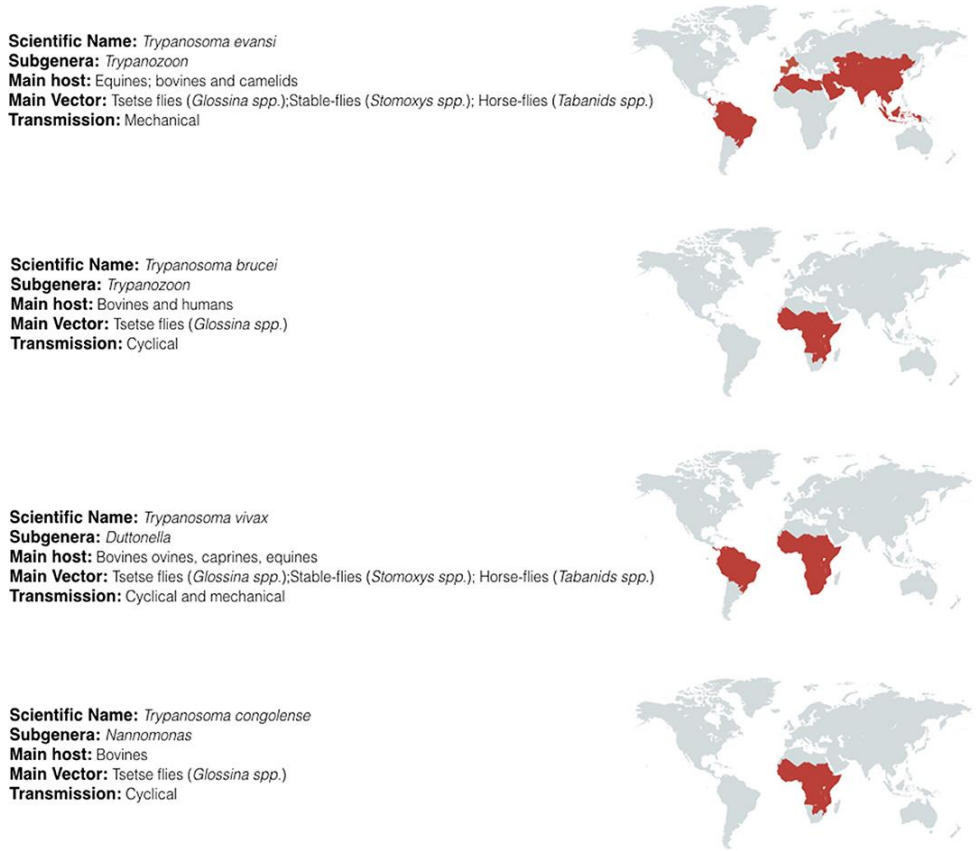


Figure 1.2: Trypanosome global distribution. The red patches on the maps have been used to indicate the continent(s) where particular trypanosomes are reportedly endemic. The texts shown on the left side of each trypanosome distribution map provides information on a trypanosome species' subgenus, vertebrate and vector hosts, and mode of transmission

Source: Radwanska et al. 2018. *Frontiers in immunology*, 9(2253), pp. 3

In Latin America, trypanosomes are biologically propagated by arthropod-vectors of different types. Triatomine bugs are responsible for disseminating *T. cruzi* the etiological agent of the Human American Trypanosomiasis otherwise called the *Chagas* disease (Ribeiro et al. 2015; Pinto et al. 2015). Biting flies such as tabanids and stable flies are also confirmed (mechanical) vectors of trypanosomes in Latin America and are incriminated in the parasite transmission in other parts of the world, including Africa (Jone & Dàvila, 2001).

In Africa, tabanids and stable flies are important vectors of animal trypanosomes north of the sub-Sahara and, to a lesser extent, within the sub-Saharan region (Baldacchino et al. 2013; Odeniran et al. 2019; Mulandane et al. 2020). In sub-Saharan Africa, tsetse flies are the main vectors of trypanosomes and the only known cyclical transmitters of the parasites that infect and cause morbidities and mortalities in humans and animals (Radwanska et al. 2018; Kasozi et al. 2021).

1.1.2 Tsetse-borne human trypanosomiasis control in sub-Saharan Africa & intractable endemicity in wildlife areas in East & Southern Africa

Tsetse-transmitted trypanosomes in humans give rise to the sleeping sickness disease also known as the chronic or *gambiense* Human African Trypanosomiasis (g-HAT) caused by *Trypanosoma brucei gambiense* in West and Central Africa and acute or *rhodesiense* HAT (*r*-HAT) caused by *T. b. rhodesiense* in Southern and East Africa (Brun et al. 2010; Franco et al. 2020) except northwest Uganda where *g*-HAT is the endemic form of the disease (Priotto et al. 2006). HAT has a long history in sub-Saharan Africa but first became a public health concern during the colonial era in the continent. The first HAT outbreaks were recorded towards the close of the nineteenth century (World Health Organisation, 2006). However, control operations in the form of extensive vegetation removal to deprive the tsetse fly vectors of conducive habitats soon caused HAT incidences to recede (Steverding 2008). Also, as part of intervention measures against HAT to curtail the transmission of the disease, the British colonial-administrators initiated programs

that aimed to eliminate animals in the wild since these provided bloodmeals for, and hence sustained, the HAT tsetse fly vectors (de Raadt 2005). There were also chemotherapeutic management programs to control HAT, but these, as it later turned out, were constrained by drug toxicities in humans that received treatments (Winkle 2005).

Tsetse flies are notorious for making a comeback to areas from which they were cleared. This occurred frequently in the first half of the twentieth century, frustrating efforts to contain HAT (Lambrecht 1964). However, renewed commitment to disease control through collaborations and partnerships between national governments in Africa and international donor agencies and organisations led to a remarkable decline in HAT incidences in many endemic foci between 1960 and 2000 (Torr et al. 2005). But recrudescence in HAT transmission and resurgence in cases soon began to be noticed. In 2009, >9000 *g*-HAT and ~200 *r*-HAT cases were officially documented but are likely to have been higher due to underreporting (Franco et al. 2020). However, thanks to coordinated control programs implemented by way of active HAT case detections and safe trypanocidal treatments supplemented with tsetse fly vector control, the annual cases of HAT infections have progressively declined in the subsequent decade thus informing the ambitious goal by the World Health Organization to eliminate the anthroponotic *g*-HAT disease transmission by 2030 (Barrett 2018; Franco et al. 2020). Whether this is achievable remains a matter of debate within the scientific and policy-making space.

The elimination of *r*-HAT, on the other hand, is still out of sight given that it is anthropo-zoonotic with wildlife intricate involvement in the disease epidemiology (Franco et al. 2020). Findings by Duffy et al. (2013) further suggest that wildlife may play an important role in driving proliferation of multiple *T. b. rhodesiense* genotypes. The authors base this on reports of high genetic diversity among *T. b. rhodesiense* isolates recovered from people infected in the HAT focus

of the Nkhotakota Wildlife Reserve in Malawi and genetic homogeneity of the parasite isolates from people in the Tororo HAT focus in Uganda, where cattle are the main reservoirs of *T. b. rhodesiense*.

1.1.3.0 Tsetse-borne animal trypanosomiasis

1.1.3.1 Constraints

Rhodesiense-HAT reported cases reduced from 190 in 2009 to 24 in 2018 (Franco et al. 2020). In spite of remarkable reductions in cases of HAT particularly *g*-HAT, Animal African Trypanosomiasis (AAT) remains highly endemic in sub-Saharan Africa (Diall et al. 2017). Importantly, the disease burden is high and unabating in areas of the continent where the animal trypanosome parasites are endemic and biologically transmitted by tsetse flies among animal hosts in local ecologies.

The World Organization for Animal Health (OIE) has AAT among its list of notifiable diseases of veterinary importance (OIE 2021). This is in recognition of its debilitating effects on health of domestic stock, the constraints on animal protein production, threat to food and economic security, and the far-reaching implications for smallholder agriculture in resource-limited settings.

In East Africa, AAT seriously impedes cattle health and production and thus agricultural and economic development. The disease effects are profound in Kenya, Ethiopia, Somalia, and the other countries (Uganda, Sudan, and South Sudan) within the East African Intergovernmental Authority on Development (IGAD) region (Shaw et al. 2014). Abro et al. (2021) in a recent economic study estimated annual government expenditures on AAT control in sub-Saharan Africa at an excess of US$ 40 million with a substantial component (~40.00%) of the budget expended in East Africa and ~25% of this (US$ 4 million) accruing to control operations in Kenya alone.

1.1.3.2 Parasites

1.1.3.2.1 *Genetic diversity*

Tsetse-borne trypanosomes are phylogenetically segregated into at least four subgenera (Fig. 1.3). They possess intraspecific genetic variations known to influence the parasite infectivity of vertebrate and vector hosts and fundamentally shape the protozoan transmission patterns. *Trypanosoma b. rhodesiense* and *T. b. brucei* are phylogenetically related *Trypanozoon* parasites of livestock (Fig. 1.3). However, *T. b. rhodesiense* possesses the Serum Resistance Associated (SRA) gene that enables it to evade immune defenses in humans and extend its host range to humans (Duffy et al. 2013). Trypanosomes transmitted by tsetse flies have the maxicircle kinetoplast DNA. However, the *Trypanozoon T. evansi* during evolution from *T. brucei* lost the maxicircle kinetoplast DNA consequently making it impossible for tsetse flies to cyclically transmit the parasite (Borst et al. 1987). Therefore, *T. evansi* vector transmission is only possible mechanically. As a result, the parasite has been able to extend its distribution to areas beyond sub-Saharan Africa where tsetse flies are exclusively endemic, to areas in Asia and Latin America where non-tsetse mechanical vectors of trypanosomes are present (Jones & Dàvila 2001).

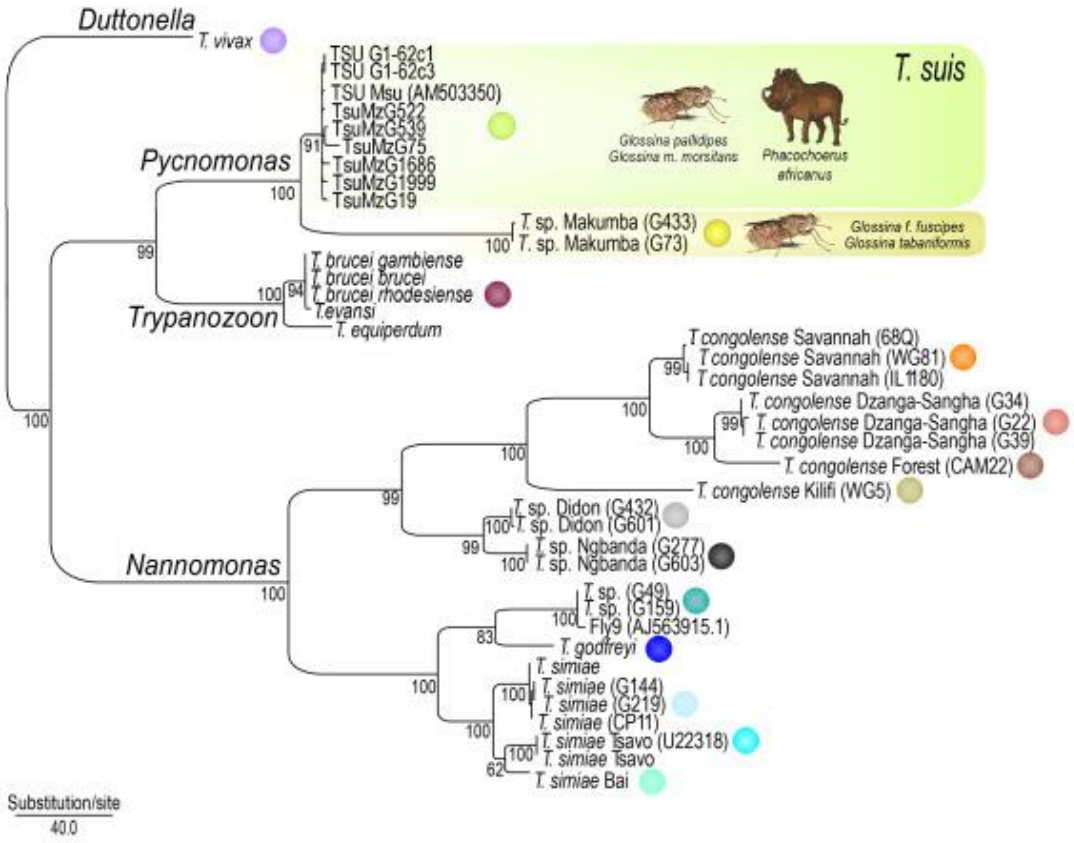


Figure 1.3: Parsimony phylogenetic tree inferred by analyses of SSU rRNA sequences and showing evolutionary relationships among tsetse-transmitted trypanosomes belonging to different subgenera.

Source: Rodrigues et al. 2020. *Infection, Genetics & Evolution*, 78(104143), pp. 6

1.1.3.2.2 *Parasite diversity*

Tsetse-transmitted trypanosome (*Nannomonas, Duttonella* and *Trypanozoon*) parasites of livestock that cause AAT in East Africa and other parts of sub-Saharan Africa are highly diverse (Adams et al. 2010). The human trypanosome *T. b. rhodesiense* responsible for *r-HAT* in East Africa also infects domestic stock in the East Africa sub-region but is relatively sparsely reported in livestock (Selby et al. 2013). In Kenya, for example, *T. b. rhodesiense* occurs only in the western part of the country (KENTTEC 2019) with infrequent reports of the parasite in livestock

(von Wissmann et al. 2011; Ng'ayo et al. 2015) and just two cases of human infections reported to the WHO from 2010 to 2020 (Franco et al. 2020).

Trypanosoma equiperdum and *T. theirleri* are examples of trypanosomes that infect livestock in sub-Saharan Africa. However, *T. equiperdum* is transmitted sexually and affects only equines. On the other hand, *T. theileri* is non-pathogenic except in immunocompromised animals, hence the parasite is regarded as being of less veterinary importance (Garcia et al. 2011, 2018). *Trypanosoma godfreyi* has some veterinary importance but is detected in tsetse flies more often than in vertebrates (McNamara et al. 1994; Auty et al. 2012). As a result, the *T. godfreyi* animal host range is far from being clearly defined and epidemiological records are limited regarding the parasite pathogenicity in livestock hosts (Adams et al. 2010). *Trypanosoma* (*Pycnomonas*) *suis* is a pathogenic trypanosome parasite of suids first discovered in 1905 (Hoare 1972) and recently rediscovered in epidemiological surveys in wildlife areas in Tanzania (Hamilton et al. 2008) and Mozambique (Rodrigues et al. 2020). However, *T. suis* remains rarely reported in epidemiological surveys and is not considered among common causes of AAT in livestock.

Trypanosoma simiae and *T. simiae* Tsavo have a host range towards suids with high mortality associated with the parasites' infections in domestic pigs (Claxton et al. 1992; Majiwa et al. 1993; Zweygarth et al. 1994; Kaare et al. 2007). Although also reported in cattle, cases are rare (Odongo et al. 2016). *Trypanosoma* (*Nannomonas*) *congolense* together with *T.* (*Duttonella*) *vivax* and *T. brucei* (not *gambiense* but *rhodesiense* and *brucei* subspecies) in the *Trypanozoon* subgenus infect cattle and are responsible for most AAT cases in sub-Saharan Africa (Bengaly et al. 2002; Selby et al. 2013; Rodrigues et al. 2017; Ebhodaghe et al. 2018). The *T. simiae* complex and *T. congolense* complex are members of the *Nannomonas* subgenus reputed to have the widest diversity of trypanosome parasites of livestock (Adams et al. 2010). To further expand the *Nannomonas* subgenus is the report of a new lineage

'*Dzanga-Sangha*' in molecular studies that screened tsetse flies for infections in wildlife areas in the Central African Republic (Votýpka et al. 2015).

1.1.3.2.3 *Biological cycle*

Trypanosoma congolense and *T. vivax* respectively in the *Nannomonas* and *Duttonella* subgenera are cyclically transmitted by tsetse flies and endemic to sub-Saharan Africa. Both parasites undergo their developmental cycles in tsetse flies (Fig. 1.4). However, *T. vivax* incubation in tsetse flies is restricted to the mouthparts (Ooi et al. 2016), whereas *T. congolense* undergoes part of its development in the midgut from where it migrates to the cibarium and finally to the mouthparts where the parasites attain the metacyclic stage of development and become infective (Peacock et al. 2012); *T. brucei* and other *Trypanozoons* attain the infective metacyclic stage in the salivary glands.

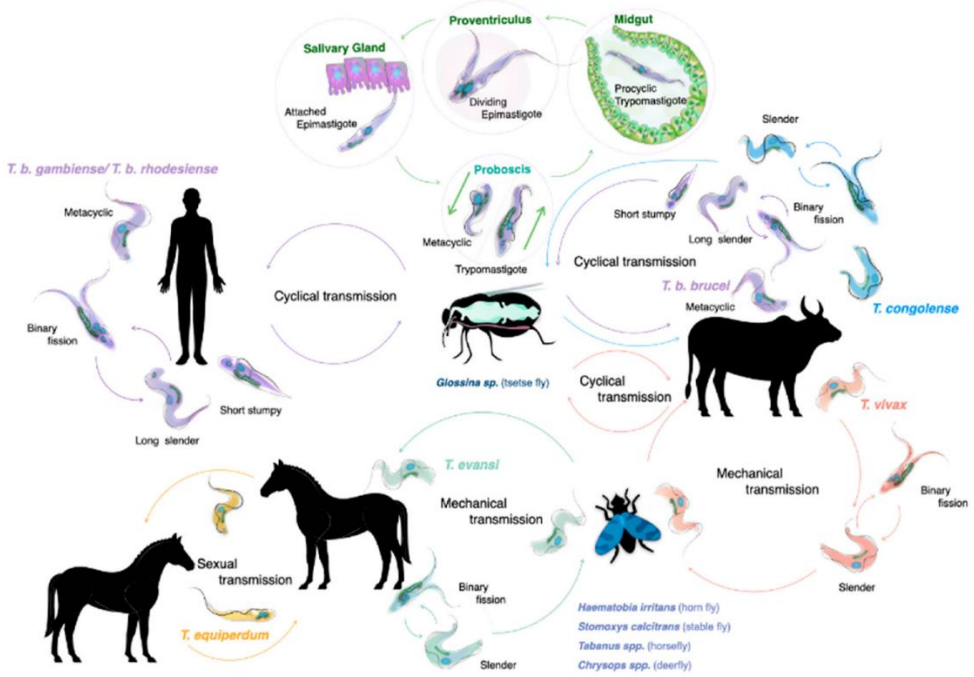


Figure 1.4: Diagrammatic illustrations of trypanosome transmission cycles. The figure shows: i.) transmission cycles for sexually transmitted and vector-borne (tsetse-transmitted and non-tsetse transmitted) trypanosomes, and ii.) developmental stages of cyclically transmitted human and animal trypanosomes.

Source: Magez et al. 2021. *Pathogens*, 10(6), pp. 4

The entire incubation period of trypanosomes in tsetse flies takes 16 days for *Duttonella* but 21 days for *Nannomonas* and 32 days for *Trypanozoon* (Bruce et al. 1910; Peacock et al. 2012). *Trypanosoma vivax* develops exclusively in the mouthparts (Vickerman 1973) and consequently is not exposed to physical structural barriers and anti-trypanosomal substances that inhibit trypanosome development in the midgut of tsetse flies (Hu & Aksoy 2006; Dyer et al. 2013). Thus, *T. vivax* has higher survival rates in tsetse flies. Consequently, it is not surprising that epidemiological surveys usually report relatively high rates of *T. vivax* in tsetse flies and high risk of cattle exposure (Ooi et al. 2016). Meanwhile, tabanids, stable flies and camel keds, among other arthropods, have mouthparts with conditions similar to that of tsetse flies. Hence, whilst *T. vivax* has not lost its maxicircle kinetoplast DNA like *T. evansi* (Borst et al. 1987), it is transmitted by mechanical vectors and therefore has a spatial distribution to areas beyond sub-Saharan Africa including Latin America and a wider spatial occurrence than *T. congolense* transmitted by tsetse flies in sub-Saharan Africa (Jones &Dàvila 2001; Radwanska et al. 2018).

1.1.3.3 Animal hosts

There is overwhelming evidence to show that different animals including wildlife species play important roles in shaping the epidemiology of tsetse-borne trypanosomes (Kaare et al. 2007; Anderson et al 2011; Auty et al. 2012). However, before they can contribute to trypanosome transmission, animal species must be present and abundant in a local ecology. Additionally, they must be selected by tsetse flies for bloodmeals and susceptible to trypanosome infections. Some animals that are abundant and yet do not meaningfully contribute to trypanosome transmission. For example, impalas are frequently sighted in the Serengeti NP but rarely contribute to the parasite infections in this area due to trypanosome-lytic antibodies in their bloods (Mulla & Rickman 1988) and scarce selection by tsetse flies for bloodmeals (Auty et al. 2016).

Waterbuck (*Kobus ellipsiprymnus*) are susceptible to trypanosomes with high rates of parasite infections reportedly observed in epidemiological studies (Anderson et al 2011). However, waterbuck emit allomonal volatiles that repel tsetse flies hence are rarely fed upon by tsetse flies and so barely contribute to the parasite transmission (Gikonyo et al. 2000; Saini et al. 2017). Caprine blood provides quality nutrients for trypanosomes (Aksoy et al. 2003). However, goats rarely contribute to trypanosome transmission because they exhibit behaviours that disallow tsetse flies to feed on them (Auty et al. 2016b). Therefore, it is important for studies that investigate animal contributions to trypanosome epidemiology to also assess host selection by tsetse flies for bloodmeals. Such studies should in addition assess the risk of tsetse fly exposure to infections. This is because some animal species, for example baboons, whilst selected by tsetse flies for bloodmeals (Bett et al. 2008; Nyawira et al. 2009), are largely refractory to trypanosome infections (Mulla & Rickman 1988) and recognised as non-important drivers of trypanosomes.

An important factor that may influence an animal species contribution to trypanosomes is the synchronisation of daily activity patterns with that of tsetse flies. For example, both warthogs (*Phacochoerus africanus*) and tsetse flies are active in the mornings and early evenings (Okiwelu 1977); epidemiological studies reveal a higher probability of trypanosome parasite detection in the peripheral blood of animals screened for infections in the morning (Maudlin et al. 2004). It is therefore not surprising that tsetse flies feed preferentially on warthogs even in environments where they are sparsely abundant (Lamprey et al. 1962). Warthogs are highly mobile and therefore are able to disseminate infections over extensive areas. However, studies have rarely shown how warthog bloodmeals are associated with trypanosome infections in tsetse flies.

Trypanosoma congolense

Trypanosoma congolense are major animal parasites endemic to sub-Saharan. They occur in different strains namely Savannah, Forest, and Kilifi having somewhat different geographical distribution patterns. The Savannah strain is cosmopolitan being commonly reported in different sub-regions across the tsetse distribution belt in Africa. On the other hand, the Forest strain and the Kilifi strain are primarily respectively endemic to West Africa and East Africa (Kaare et al. 2007; Isaac et al. 2016). However, infrequent reports are made of the Forest strain in East Africa and the Kilifi strain in West Africa (Rodrigues et al. 2020; Habeeb et al. 2021) with epidemiological studies also noting the presence of both strains in Southern Africa (Mekata et al. 2008; Gaithuma et al. 2019).

While *T. congolense* veterinary importance lies mainly with its ability to infect cattle livestock, infections are observed in wildlife hosts. In the Serengeti NP in Tanzania, the Savannah strain of *T. congolense* was reported in spotted hyaena (*Crocuta crocuta*) [GenBank Accession No.: JN673388 (ITS)] and lion [GenBank Accession No.: JN673389 (ITS)] (Auty et al. 2012). A different survey in the area (Kaare et al. 2007) detected Savannah and Kilifi *T. congolense* strains in eland (*Taurotragus oryx*), Savannah strain in buffalo (*Syncerus caffer*), giraffe (*Girraffa camelopardalis*), impala (*Aepyceros melampus*), lion, gazelle (*Gazella* species), topi (*Damaliscus lunatus*), and wildebeest (*Connochaetes* species) and Kilifi strain in reedbuck (*Redunca* species) and warthog.

The findings in the Serengeti NP show that the different strains of *T. congolense* may have variable transmission patterns with slightly different repertoire of animal hosts in their epidemiology. Aside from differences in their transmission patterns, *T. congolense* strains display different levels of virulence in infected cattle with reports of high virulence in the Savannah strain, moderate virulence in the Forest strain and low virulence or non-pathogenicity in the Kilifi strain (Bengaly et al. 2002). There are also reports of varying degrees of virulence between

parasites within the Savannah strain from different geographical locations (Masumu et al 2006) with those from wildlife areas being more virulent than those outside sylvatic ecologies (Van den Bossche et al. 2011).

Trypanosoma vivax

Epidemiological studies between 1958 and 2019 documented *T. vivax* in at least nine livestock and thirty-nine wildlife species in forty-nine countries worldwide of which twenty-seven were in Africa and twelve in Latin America (Fig. 1.5; Fetene et al. 2021). *Trypanosoma vivax* transmission by a broad diversity of fly vectors across continents and its infection of a large community of animal hosts have exposed the parasite to remarkably heterogeneous conditions (Jones & D`avila 2001). This consequently has driven *T. vivax* to evolve into a wide range of genotypes numbering over fifteen, some of which are reported in asymptomatic wild fauna hosts and found to be highly virulent in infected livestock (Rodrigues et al. 2017).

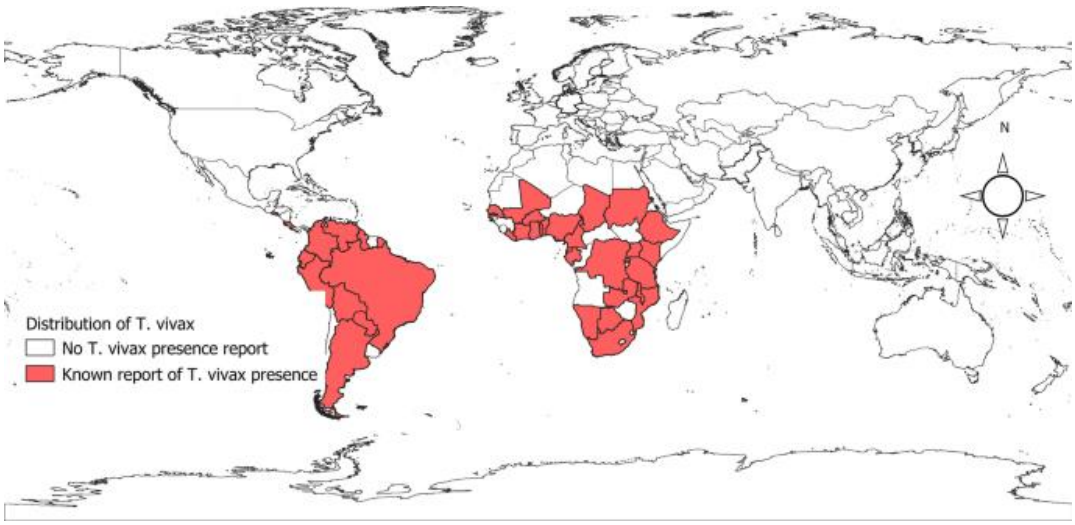


Figure 1.5: *Trypanosoma vivax* global distribution. The distributional range of *T. vivax* in West Africa and Latin America has been shown using red patches. Other areas outside the red patches on the map are considered to be *T. vivax*-free

Source: Fetene et al. 2021. *Parasites & Vectors*, 14(80), pp. 5

One example of a virulent *T. vivax* genotype harbored by asymptomatic wild animal hosts is *Tvv4* (*T. vivax* group 4, as defined by Rodrigues et al. (2017)) first reported in a nyala antelope (*Tragelaphus angasii*) in East Africa specifically Mozambique (Rodrigues et al. 2008) (Fig. 1.6). In an experimental study, *Tvv4* caused high parasitaemia, emaciation, fever, and severe anaemia in a goat, three weeks after the parasite inoculation (Rodrigues et al. 2008). Since its first characterisation in nyala, this genotype has been reported in cattle and tsetse flies in the Niassa National Reserve in Mozambique (Rodrigues et al. 2017). These findings indicate possible *Tvv4* transmission by tsetse flies between wildlife and livestock in Niassa. Recent studies in the Kafue National Park in Zambia have also reported *Tvv4* (Nakamura et al. 2021) thus suggesting broader parasite circulation in wildlife ecosystems in sub-Saharan Africa, potentially threatening livestock health and production in these areas.

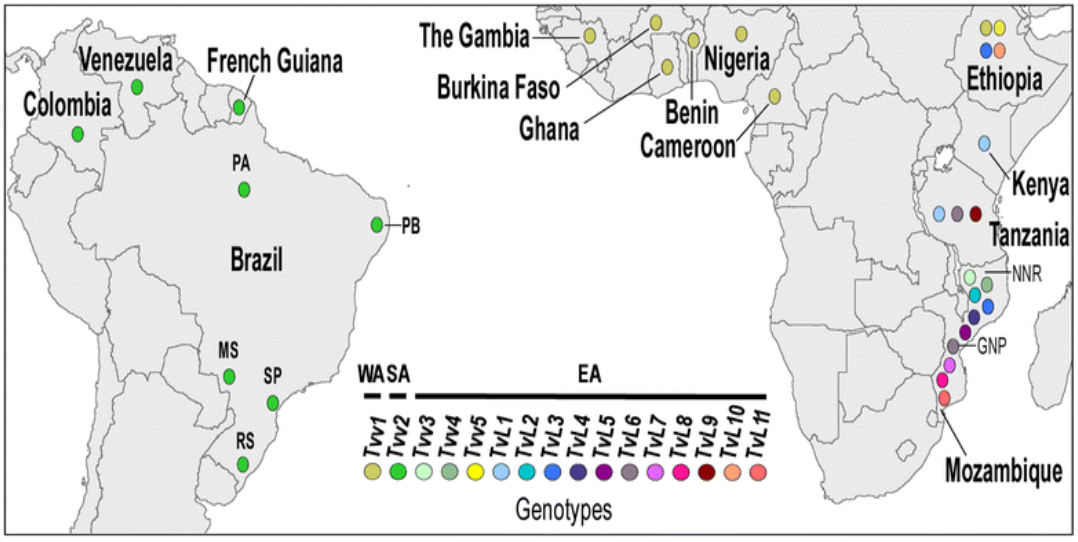


Figure 1.6: *Trypanosoma vivax* ITS rDNA genotype distribution. Different genotypes are shown using different colored circles. The green circles on the Latin American map are used to indicate that *T. vivax* isolates in the region are comprised of one genotype. Similarly, *T. vivax* isolates in West Africa belong to one genotype. Multiple colored circles in East Africa reflect a high diversity of *T. vivax* genotypes in the sub-region.

Source: Rodrigues et al. 2017. *Parasites & Vectors*, 10(337), pp. 3

Other genotypes of *T. vivax* have been reported in East Africa aside *Tvv4* (Fig. 1.6). Notably, East Africa has the highest number of documented *T. vivax* genotypes among sub-regions in Africa. To date, more than ten genotypes of *T. vivax* have been observed in epidemiological surveys carried out in the area (Rodrigues et al. 2017). The high diversity of *T. vivax* in East Africa is not unconnected to the relatively high number of surveys conducted in the area using sensitive molecular tools (Garcia et al. 2018). As several of these surveys focused on national reserves and game parks, it may be possible that the extensive repertoire of wildlife hosts of *T. vivax* in study sites in sylvatic ecologies had contributed to the proliferation of genetically diverse parasite isolates and thus informed the wide range of parasite genotypes (Jones & D`avila 2001).

Only one genotype of *T. vivax* is known in West Africa (Fig. 1.6). The low genetic diversity of *T. vivax* isolates in West Africa has been attributed to the fact that parasites seldom undergo recombination (or genetic exchange) but are mostly propagated clonally (Duffy et al. 2009). The Duffy et al (2009) study in The Gambia noted a clonal population structure for *T. vivax*. According to Silva Pereira et al (2020), clonal propagation of *T. vivax* is responsible for the conservation of genes that code for the Surface Variant Glycoprotein (SVG) that enable trypanosomes to evade host immune responses. SVG gene conservation in *T. vivax* narrows the diversity of SVG antigens in parasites and makes it possible for vertebrate hosts to develop immunity against infection over time (Silva Pereira et al 2020). This explains the phenomenon of self-cure observed in *T. vivax*-infected hosts and has probably necessitated the evolutionary adaptation of *T. vivax* parasites to a wide range of arthropod and vertebrate hosts as a strategy for survival and persistence (Silva Pereira et al 2020; Autheman et al. 2021).

1.1.3.4 Tsetse vectors

Tsetse flies are the cyclical transmitters of trypanosomes in sub-Saharan Africa and belong to the family 'Glossinidae' and genus *Glossina* (Fig. 1.7). They acquire bloodmeals for metabolic needs and, in females, for reproductive activities (Bursell et al. 1974). Among dipterous insects, female tsetse flies are unique by their larviparous reproduction (Saini et al. 1996).

Figure 1.7: Dorsal view of *Palpalis* tsetse (*Glossina fuscipes*).

Credit: Milan Kozánek. Source: IAEA (2019)

Both sexes of the vector appear in a shade of yellowish-brown to dark brown, range in body size between 6mm and 16mm and possess aristate antennae (Leak et al. 2008). They are morphologically distinguished from other dipterous insects by their possession of a 'hatchet' cell on the wings of all species of the vectors so far described. Additionally, the vectors possess forwardly projecting proboscis and a pair of aristate antennae.

Overall, 31 species and subspecies of tsetse flies have been described to date and these are all endemic to sub-Saharan Africa (Leak et al. 2008). The different species and subspecies of tsetse flies are categorised into three subgenera namely *Palpalis* (riverine ecotype), *Fusca* (forest ecotype) and *Morsitans* (savannah ecotype). The *Palpalis* and *Fusca* tsetse flies are primarily endemic to West and Central Africa (Leak et al. 2008). Patches of tsetse flies of both subgenera are also found in some

areas in East Africa (Pollock 1982). Although all species of tsetse flies have the potential to transmit trypanosomes, only a few are considered epidemiologically important under natural environmental conditions (Leak et al. 2008).

In East Africa, most tsetse flies are of the *Morsitans* subgenus. Trypanosome infections are reported in each of the seven species and subspecies of tsetse flies within the *Morsitans* subgenus (Kuzoe & Schofield 2005). However, *G. austeni* and *G. longipalpis* due to their limited distribution are of comparatively less epidemiological importance. *Glossina pallidipes* tsetse flies because of their extensive spatial distribution, high abundance, and high appetite for bloodmeals are recognized in East Africa as prolific vectors of human and especially animal trypanosomes (Bateta et al. 2020).

The epidemiological importance of tsetse flies is also largely dependent on the vector susceptibility to infections. Differential susceptibilities to trypanosomes in tsetse flies are modulated by physical and chemical defenses (Dyer et al. 2013). Furthermore, differences in susceptibility to trypanosome infections are observed between tsetse fly species and influenced by intrinsic traits including fly sex and fly age (Channumsin et al. 2018). Females are usually phenotypically larger than males and consequently have higher displacement rates, require higher energy consumption, and hence experience higher metabolic activity levels (Vale et al. 2014). High displacements in female tsetse flies increase the vector likelihood to encounter and blood-feed on animals. This increases exposure to trypanosome infections. That adult females (four months) would normally outlive males (three weeks) (https://www.britannica.com/animal/tsetse-fly. Assessed 3rd March 2022) has been identified as an additional reason for higher infection rates in the former (Woolhouse et al. 1993; Peacock et al. 2012; Channumsin et al. 2018). Females also may not feed up to the full capacity of their abdomen so as to create space for larva development. As a result, female tsetse flies usually take in small amounts of blood which they digest quickly due to their high metabolic needs (Hargrove et

al. 2011). This causes them to feed frequently at short intervals, thus increasing vector exposure to infections.

Meanwhile, laboratory experiments show that younger tsetse flies are more susceptible to infections than older flies (Kaaya & Darjt 1988). A likely explanation is that young flies have under-developed immunities. This includes physical immunity provided by the peritrophic membrane. The peritrophic membrane hardens over time with age, thus makes it increasingly difficult for trypanosomes to penetrate the mid-gut into the bloodstream of older tsetse flies (Dyer et al. 2013). Also, young flies experience starvation and this increases the propensity to feed hence exposing the vectors to infections (Hargrove et al. 2011).

1.1.3.5 Anthropisation effects on transmission dynamics

Increase in deforestation results from escalation in human activities and leads to reduction in humidity conditions required for tsetse fly reproduction, development, and survival. Hence, increasing fragmentation of natural vegetations has aggravated stress in tsetse flies and caused the vectors to disappear from erstwhile infested areas (Reid et al. 2000). Where human-induced stress was moderate and tsetse infestation remained, field studies report a break-up of the vector population into sub-populations (Ducheyne et al. 2009; Mweempwa et al. 2015). Splitting populations of tsetse flies into units creates trypanosome transmission hotspots which can become candidates for targeted control in interventions to reduce trypanosome transmission risk (Auty et al. 2016b).

Deforestation resulting from anthropogenic pressures also increases temperature levels. According to Mweempwa et al. (2015), vegetation fragmentation of tsetse fly habitats increased the temperature conditions in Chisulo in eastern Zambia and was associated with high trypanosome infection rates in tsetse flies. To further corroborate the linkage between temperature and tsetse fly trypanosome

infections, Sy (2011) discovered that trypanosomes matured faster at higher temperatures. Furthermore, *G. morsitans centralis* and *G. brevipalpis* had higher infection rates if they emerged from pupae incubated at 29°C instead of 25°C (Ndegwa et al. 1992). In another experiment, *T. rhodesiense* infection rates were higher in *G. morsitans* from pupae incubated at 40°C than in the vectors from un-incubated pupae (Burtt 1946). The same investigator observed higher levels of success in trypanosome transmissions between animals for experimentally infected tsetse flies which emerged from incubated pupae when compared with tsetse flies from un-incubated pupae. These laboratory findings have also been confirmed in field studies, for example, in the Mouhoun river in Burkina Faso where infection rates in riverine tsetse flies maintained a significant relationship with temperature (Bouyer et al. 2013).

Other ways by which anthropogenic disturbances of the environment may influence trypanosome transmission are by driving changes in the physiology and biological traits of tsetse flies and altering animal species composition.

Higher temperatures in anthropised sites in Chisulo (Eastern Zambia) (Mweempwa et al. 2015) may have accelerated digestion rates in tsetse flies, hence increased starvation, and the vector's quest for bloodmeals from cattle, including infected animal hosts. Importantly, starved tsetse flies have lower amounts of anti-trypanosomal chemicals such as glutamic acid-proline produced in the midgut and thus are more susceptible to infections (Akoda et al. 2009). An alternative or complementary hypothesis could be that stress conditions in anthropised areas, as have been reported for several arthropod-vectors (Guo et al. 2019), selects for phenotypically larger tsetse individuals that are believed to have higher levels of competence than smaller counterparts.

However, as Mweempwa et al. (2015) did not screen tsetse flies for animal bloodmeals they could not determine the proportion of cattle-fed flies which is an important parameter for appraising trypanosome entomological risk (Leak et al.

1990). Furthermore, no assessment was done to measure tsetse phenotypic sizes to determine the plausible anthropisation-stress effect and the consequence for trypanosome risk. However, Mweempwa et al. (2015) were able to provide evidence to anthropisation-induced stress selecting for older female tsetse flies (~40 days in markedly anthropised Chisulo and 26 days in less disturbed Lusandwa). According to the investigators, the presence of older female flies in highly disturbed areas contributed to the high parasitological rates of infections in cattle in those areas where tsetse populations were sparse. This, however, contradicts speculations in the Serengeti NP in Tanzania (Lord et al. 2018) and the Akagera National Reserve in Rwanda (Gashururu et al. 2021) of higher domestic animal risk near wildlife protectorates where tsetse flies occur in high abundance.

According to Anderson et al. (2011), increasing human migration of livestock into the Luangwa Valley in Zambia is causing gradual shifts in trypanosome transmission from wildlife towards cattle. It is also possible though insufficiently demonstrated that this will drive changes in the species diversity of trypanosomes in tsetse infested areas within and outside the Luangwa Valley.

A further consequence of anthropogenically-driven changes on trypanosome epidemiology in sylvatic environments is a likely increase in *HAT* risk in human populations. In East and Southern Africa where the *r-HAT* is endemic, introduction of cattle to wildlife areas exposes the livestock to the *T. b. rhodesiense* etiological agent (Auty et al. 2016b). Cattle in this case may serve as amplifier hosts of the parasite. This has potential to increase *r-HAT* risk in humans in sylvatic environments where tsetse flies feed on humans and cattle, in addition to wildlife. But there are possibilities that human-cattle interactions will reduce human infection risk in locations endemic to savannah tsetse flies since these vectors after repelled by odours from humans are attracted to cattle by kairomones released by the livestock (Vale 1979). Tsetse fly attraction to and infection of cattle, however, still portend undesirable consequences for the wellbeing of rural dwellers in local

communities following tsetse-borne trypanosome adverse effects on livestock health and accompanying economic losses and constraints on animal and agricultural production (Ohaga et al. 2007).

1.1.3.6 Control

1.1.3.6.1 *Tools & Techniques*

Chemotherapy

Trypanosomes are managed using chemoprophylactic and chemotherapeutic drugs that respectively prevent the parasite infection and kill the parasite in animal hosts. However, trypanosome drug use is greatly hampered by widespread circulation of fake and substandard trypanocides in many sub-Saharan African countries (Tekle et al. 2018). The problem of trypanoresistance which perhaps constitutes the greatest threat to effective drug-use for trypanosome control is also on the increase (Assefa & Shibeshi 2018). This has continued to worsen due to failure of livestock owners in areas of high tsetse challenge to apply sanative pairs of available drugs but rather engage the monotonous use of drugs having the same mode of action. Efforts to develop new drugs are at low ebb with no safe and effective new drugs developed for animal trypanosome control since the 1960s (Richards et al. 2021). Meanwhile, trypanosomes exhibit differential responses to drugs. However, in many locations endemic to trypanosomes, treatments are carried out without consideration of the type of trypanosomes responsible for infections (Ngumbi & Silayo 2017; Richards et al. 2021). Over the last four decades, novel trypanosomes have been reported for which knowledge remains limited regarding their responses to drugs (Adams et al. 2010). As a result, targeting treatment of these parasites is difficult. In wildlife areas, chemotherapy as an option of trypanosome control is not sustainable because livestock are continuously exposed to reinfections from the parasite wildlife reservoirs, to which it is practically difficult to extend treatment.

Trypanotolerance

Production of trypanotolerant livestock breeds has been practised as an alternative strategy for trypanosome control in sub-Saharan Africa. Cattle breeds with the innate ability of trypanotolerance harbour trypanosomes without succumbing to parasite infections even in the absence of trypanocides (Naessens 2006). However, trypanotolerant cattle such as N'Dama, Muturu, Keteku and West African Short Horn (WASH) provide less traction power compared to trypanosusceptible zebu cattle and may lose their trypanotolerance trait when subjected to intense stressful conditions or introduced into wildlife areas where the risk of trypanosome infections is high (Agyemang 2005).

Hotspot avoidance

Migration from and avoidance of high-risk areas were adopted as strategies to manage trypanosomes during the first widespread outbreaks of the parasite infections in human and animal populations towards the close of the 19th century in sub-Saharan Africa (Kuzoe & Scofield 2004). Fortunately, this helped to reduce contacts between tsetse flies and livestock and minimised cattle infection cases. Since then, livestock owners have preferred to settle outside and have avoided wildlife areas where trypanosome infection risk is high (Muriuki et al. 2005). However, this has excluded swathes of agriculturally productive land spaces from exploitation for cattle grazing as well as food crop production (Ilemobade 2009). Furthermore, avoidance of areas at high risk of animal trypanosomes has not been sustainable because pastoralists during prolonged drought often travel long distances in search for grazing resources and, sometimes, transverse tsetse-infested areas where cattle are exposed to trypanosome infections with accompanying high mortality rates in livestock populations (Majekodunmi et al. 2013; Meyer et al. 2016). Meanwhile and as the human population continues to grow in sub-Saharan Africa and other parts of the world, the need to produce more food and increase livestock production has necessitated the expansion of

agricultural activities to areas around wildlife where tsetse-borne trypanosome infection risk is high (Wittemyer et al. 2008).

Tsetse population reduction

Trypanosome transmission are reduced and maintained below epidemiologically important thresholds by reducing the vector abundance in locations where contacts between tsetse flies and cattle cannot be ruled out and livestock production must continue. The abundance of tsetse flies is reduced using a variety of tools and techniques. Initially, abundance of tsetse flies was reduced through vegetation clearance and game destruction (Scott et al. 1966; Hocking et al. 1963). Vegetation clearance was aimed at depriving tsetse flies of favourable breeding and resting sites while game destruction was carried out to reduce sources of nourishment for the vectors and eliminate animal sources of trypanosomes. However, the benefits of vegetation clearance were temporary and non-sustainable because cleared vegetation soon grew back. Destruction of game needed the elimination of large animal populations over extensive areas before the desired results could be achieved. It also had adverse repercussions on biodiversity.

Despite initially driving declines in the incidence of trypanosomes, vegetation clearance and game destruction, due to their adverse effects on the environment, were eventually discouraged and banned as forms of tsetse fly vector control (Kuzoe & Schofield 2004). Dichloro-Diphenyl-Trichloroethane (DDT), discovered in the 1930s, was applied extensively in large-scale interventions in aerial-wide tsetse control programmes until the 1970s (Winkle 2005; de Raadt 2005). However, release of DDT into the environment was also banned due to the chemical lethal and sublethal effects on non-target organisms. But before it was banned, DDT applications drove remarkable reductions in the abundance of tsetse flies and, together with drug treatments, brought down trypanosome transmission not only

in livestock but also in human populations (Steverding 2008). However, trypanosome incidence began to peak once DDT usage was stopped.

The Sterile Insect Technique (SIT) for insect pest and vector control has been extensively applied in interventions against tsetse flies. SIT programmes release sterilized male tsetse flies to mate with females in the wild (Vreysen et al. 2000). Female tsetse flies mate once in a lifetime, but males are able to mate repeatedly. Females that mate with sterilized males give birth to non-viable offspring. This makes a strong case for SIT adoption for control of tsetse flies. However, SIT requires a lot of expertise, is expensive and less effective where tsetse flies occur in high abundance (Torr et al. 2005). In addition,, male tsetse flies, unlike many arthropod-vectors of diseases, are epidemiologically important and involved in trypanosome transmission (Bouyer et al. 2019). Hence, there are ethical concerns that mass releases of sterilized male tsetse flies in SIT interventions could actually increase instead of reduce infection risk.

Tsetse flies especially members of the Savannah ecotype are attracted to kairomones released from cattle urine. For the Riverine ecotype of tsetse flies, olfactory cue may play a lesser role eliciting attractions (Gibson & Torr 1999). However, control programs in a bid to enhancing tsetse collection have exploited this understanding of tsetse fly ecology to manage the vectors have combined odour and visual attractants in large-scale interventions (Meyer et al. 2016). Control of tsetse flies in donor-funded large-scale interventions between the 1970s up to 2000 deployed stationary bait technologies over extensive land areas (Torr et al. 2005). These technologies assumed the form of insecticide-treated or (un)treated traps constructed in different designs (Fig. 1.8). The biconical trap, for example, was developed in 1973 for the management of riverine tsetse flies in West Africa (Challier & Laveissiere 1973), but this was also found to effectively control savannah tsetse flies in East Africa (especially when treated with acetone

and/or cow urine) and are presently widely used across sub-Saharan Africa in entomological surveillance of tsetse flies (Leak et al. 2008).

Figure 1.8: a) Insecticide-treated fabrics and b) biconical traps for tsetse control

Source: Mbewe et al. 2018. *Parasites & Vectors*, 11(268), pp. 4

Bait technologies also came in the form of targets comprising of pieces of blue fabrics (used to attract tsetse flies) held together with insecticide-treated black fabrics (used to elicit tsetse fly landing for exposure to lethal chemicals) (Fig. 1.8a; Kuzoe & Schofield 2004). Another form of bait called the live-bait (insecticide-treated cattle) has also been developed for control of tsetse flies (Torr et al. 2005). Tsetse flies are naturally attracted to cattle but acquire lethal chemical doses by feeding on cattle treated with insecticides. Presently, the live-bait technique is recognised as a cost-effective technique for tsetse fly control (Thomson et al. 1987; Shaw et al. 2013). However, there are a number of constraints associated with its

use. One of the major constraints is that tsetse flies in sylvatic environments are exposed to a wide range of animal species aside from cattle for bloodmeals. The vectors in many wildlife locations will barely feed on cattle including those treated with insecticides (Torr et al. 2005). Hence, tsetse flies have a low probability of feeding on cattle and remain highly abundant being nourished by wild animal species.

In several interventions, traps and targets were combined with insecticide spray with the goal of eradicating tsetse flies hence completely interrupting trypanosome transmission (Meyer et al. 2016). A major motivation for large-scale vector control between the 1980s and 2000 was that insecticides (e.g., deltamethrin) safer than DDT had been discovered (Torr et al. 2005). Meanwhile, chemical resistance was building up increasingly among trypanosomes but not tsetse flies (Kuzoe & Schofield 2004); there is still no report of tsetse fly resistance to chemicals to date. Additionally, no effective vaccines were available (neither are they available today) and vector control was and remains the only means of preventing trypanosome infections.

1.1.3.6.2 *Experiences in selected programmes in East Africa*

Large-scale tsetse interventions to reduce trypanosome burden on livestock were implemented between the early 1980s up to 2000s in different parts of sub-Saharan Africa (Brightwell et al. 2001; Meyer et al. 2016). In East Africa, local communities adopted environmentally friendly strategies to control tsetse flies in donor-funded programmes (Barrett & Okali 1998), a classical example of which is the EU-funded FITCA programme (1997 to 2004) carried out in four countries in the sub-region (Kenya, Uganda, Ethiopia, and Tanzania) (FAO 2002).

In Uganda, for example, FITCA, in partnership with local communities, controlled tsetse flies between 1999 to 2004 within an area of 2,000 km2 in Busoga out of the 50,000 km^2 targeted for tsetse control in the country (FAO 2002; Meyer et al. 2016).

FITCA interventions achieved ~90.00% reduction of the vector abundance in Busoga, but farmers eventually relaxed interventions thus tsetse flies reinvaded controlled areas (Meyer et al. 2016). According to Oloo & Bauer in FAO (2002), FITCA activities in Kenya extended to a 6,500 km^2 area. In Kenya, FITCA provided expert advice while farmers owned the intervention. Pyrethroid-impregnated nets in the FITCA programme were used to protect livestock from tsetse infectious bites and reduced trypanosome infection rates from 64.00% to 2.00% in zero-grazing cattle. However, Oloo & Bauer in their report did not indicate the duration of the intervention.

Community-led tsetse control operations have a long history in Kenya and were implemented long before the FITCA programme (Barrett & Okali 1998). Following failure of an aerial-wide intervention in 1981 to eliminate tsetse flies in Ruma in Western Kenya (Turner 1986), local communities in 1989 in collaboration with the Kenya Tsetse and Trypanosomiasis Research Institute (KETRI) led a tsetse control operation over an area of ~100 km^2 in the Ruma National Park (Brightwell et al. 2001). Tsetse abundance of >470 flies per trap per day (FTD) in 1989 before the intervention was reduced by 99.00% in 1990. In one intervention implemented by the Kenya Wildlife Service (KWS) in Ruma, control operations were implemented for only a brief period (Brightwell et al. 2001). According to Brightwell et al. (2001), the KWS deployed too many insecticide-treated fabrics which became difficult to maintain. Brightwell et al. (2001) argued that fewer numbers of one odour-baited target per km^2 where tsetse infestations are high could have done the job of keeping tsetse numbers low and would have increased the lifespan of interventions in the area. Further, field and simulation data from interventions in Zimbabwe showed that a density of four traps or insecticide-treated fabrics per km^2 is suitable to effectively control tsetse flies specifically the savannah species (Vale et al. 1988; Hargrove 2003).

Local community engagements in tsetse control in Ruma were borne out of the huge adverse effects of trypanosomes in the area. These were also occasioned by the apparent impacts of interventions. Excited about the success of tsetse control carried out by the International Centre of Insect Physiology and Ecology *icipe*, farmers in Nyaboro in Ruma expressed a desire for *icipe*'s continuation of operations in the area (Barrett & Okali 1998). However, the Brightwell et al. (2001) study noted that tsetse control near the Ruma National Park kept low abundance of the fly in Nyaboro at a time interventions had ceased in the latter. This clearly demonstrates that interventions in wilderness areas and along their boundaries, as was the case in Ruma, can have spill-over effects in reducing tsetse fly abundance in adjoining areas. This was reported for *G. pallidipes* and *G. morsitans morsitans* in the Zambezi Valley of Zimbabwe where baited targets in less than 12 months reduced tsetse fly abundance up to 10 km outside of the intervention sites (Vale et al. 1988).

In the Ziwani Ranch on the Kenyan Coast, about 50% of livestock, not long after they were introduced into the area, were lost to AAT (Brightwell et al. 2001). With technical support from *icipe* and KETRI, the ranch owner led an intervention in the area focusing on areas where tsetse infestations were highest. In 1994, tsetse flies were significantly reduced (by 99.00%) within 12 months of intervention by using traps (stationary baits) in combination with insecticide-treated livestock (live-baits) and bush clearing (Brightwell et al. 2001). AAT incidence was also reduced. In 1998, 30% of the total number of insecticide-treated traps that the ranch owner set to use in 1994 were still being retained and these were sufficient to maintain low abundance of tsetse flies at below 1 FTD compared to >500 FTD before interventions were initiated on the ranch (Brightwell et al. 2001).

Tsetse control in Ziwani provides a classic example of how strategic deployment of tools for the vector management can fast-track both effective and efficient trypanosome vector control. Additionally, it demonstrates how tsetse control can

be taken seriously when farmers are threatened by the risk of losing their livestock to AAT and how interventions can be sustained when livestock owners derive private benefits from carrying out interventions and have access to cheap and easy-to-use tools.

1.2 Justification

Millions of rural dwellers in Africa depend on smallholder agriculture for livelihood and food security. In East Africa alone, ~60.00% of the >350 million inhabitants, many of whom are rural farmers, have employment in the livestock agricultural sector (FAO 2019). Furthermore, livestock production in the East African region accounts for >USD 1 billion of foreign earnings annually. In Ethiopia and Somalia, the livestock industry respectively contributes 47% and 85% to national Gross Domestic Product (GDP) (FAO 2019). However, livestock contribution to national GDP in Kenya is merely 14% which is less than half the figure in Ethiopia and even one-fifth that of Somalia. Different reasons ranging from scarcity of quality feeds to drought and pests and diseases are identified as factors responsible for the comparatively low livestock contribution to national GDP in Kenya (Onono et al. 2013).

AAT is endemic in countries within the East African Intergovernmental Authority on Development (IGAD) region. In these countries, economic losses due to the disease infections are most severe in local communities close to National Reserves and Game Parks. Economic losses associated with AAT across tsetse-infested areas in sub-Saharan Africa exceeds USD 3 billion annually and within the IGAD region, it averages at USD 175 per annum per km square (Shaw et al. 2014). The losses arising from AAT in the IGAD region are highest in areas in southwest Ethiopia and in western, central, and coastal regions of Kenya where the greatest socio-economic benefits are also expected following successful control of the disease.

Cattle production is a major economic enterprise in Kenya. The total cattle population size in the country is ~18 million (KNBS 2009). However, ~46% of these cattle are at risk of animal trypanosome infections (Abro et al. 2021). Animal trypanosomes transmitted by different types of arthropod-vectors are widespread in Kenya from the arid north to the semi-arid central region down to the coastal south (Kidambasi et al. 2019; Ngari et al. 2020). However, the parasites biologically transmitted by tsetse flies are present in only the south and parts of the central region.

Kwale County in the southeast coast of Kenya has enormous potential for cattle production, but it is also a major hotspot of tsetse flies in East Africa (Shaw et al. 2014; Saini et al. 2017). Kwale has extensive savannah grasslands for cattle grazing fields but holds less than 2.00% of the overall number of cattle at the national level (GoK 2017). Livestock owners in Kwale have consistently identified tsetse-transmitted trypanosomes as a major hinderance to sustainable livestock production in the area (Machila et al. 2003; Muriithi et al. 2021). Some epidemiological surveys in Kwale county report trypanosome infections in almost half the number of animals in cattle herds in the Shimba Hills area (Saini et al. 2017).

Farmers in Shimba Hills expressed a willingness to pay for and adopt a novel tool for tsetse fly control (Muriithi et al. 2021) after a field trial in Shimba Hills reduced trypanosome rates in cattle by >80.00% using a novel tool (Saini et al. 2017). The tool developed by *icipe* scientists is a collar worn around the neck of cattle and has a dispenser that gradually releases a blend of synthetic tsetse fly repellent odours, mimicking allomonal volatiles emitted by waterbuck. Application of the tsetse repellent odour collars (hereafter referred to as the collar) to 50% of cattle at risk of trypanosomes in East Africa will, according to projections, result in potential economic benefits worth USD 386 million per annum in the sub-region with

almost half of these benefits accruing to Kenya where total annual expenditures on trypanosome control is in excess of USD 11 million (Abro et al. 2021).

The *icipe* tsetse-repellent collars are cost-effective, environmentally safe, and easy to adopt in rural contexts. Unlike stationary baits for trypanosome vector control, the collars protect cattle from tsetse fly infectious bites under different livestock management systems. Modelling studies indicate that integration of the collars with stationary baits could deliver better intervention-effects (Bett et al. 2013). However, this remains understudied in field conditions. The intervention by Saini et al. (2017) in Shimba Hills attempted a combination of both technologies for trypanosome vector control. However, random deployment of stationary baits blurred a clear understanding of the intervention-effects.

A community-led intervention to reduce trypanosome transmission is underway in Shimba Hills. Across sub-Saharan Africa, community involvement in trypanosome management in tsetse interventions has helped to reduce cost and prolong the lifespan of control operations (Barrett & Okali 1998; Meyer et al. 2016). However, trypanosome epidemiological hotspots for targeted tsetse control in Shimba Hills are not well known. Meanwhile, Shimba Hills has experienced increased anthropogenic pressures over time (Schmidt 1992), but it remains poorly understood how these pressures are affecting trypanosome transmission patterns in farming communities in the area. Addressing these knowledge-gaps would require providing data that describe locations where cattle are highly exposed to trypanosomes from tsetse flies. Profiling of wildlife reservoirs of trypanosomes will provide insights into the parasite transmission patterns among animals in the Shimba Hills National Reserve. More importantly, it will allow us to identify key animal reservoirs of the parasites and areas where they are present and co-exist with cattle. This will, eventually, contribute to a clearer understanding of trypanosome spatial risk and identification of the parasite hotspot locations in Shimba Hills. As not all trypanosomes are pathogenic to

cattle, the intervention in Shimba Hills will generate better impact if designed to target tsetse flies that feed on cattle and expose the livestock to pathogenic and virulent genotypes of trypanosomes that give rise to clinical conditions in livestock. But trypanosome diversity is largely understudied in Shimba Hills. Hence, tsetse flies that disseminate pathogenic species and virulent genotypes of the parasite are yet to be properly identified.

1.3 Overarching aim of the thesis

To rationalise a plan for community-led control of tsetse flies and trypanosomiasis in Shimba Hills (Kenya) based on reliable epidemiological data using environmentally safe and low-cost technologies deployed to trypanosome hotspots and targeted at tsetse flies that expose cattle to pathogenic and virulent strains and genotypes of the parasite.

1.4 Specific objectives of the thesis

1. To investigate cattle trypanosome infection risk and assess anthropisation effects on tsetse fly bioecology and trypanosome epidemiology,
2. To profile bloodmeal hosts of tsetse flies, unravel trypanosome transmission patterns among animal species and identify wildlife sources of cattle trypanosome parasites,
3. To characterise trypanosome (*Trypanosoma vivax*) genotype diversity in tsetse flies, and
4. To strategize application and evaluate the epidemiological effect of stationary bait-technologies (insecticide-treated fabrics) for tsetse control with and without tsetse-repellent odours.

References

Abro Z, Kassie M, Muriithi B, Okal M, Masiga D, Wanda G, Gisèle O, Samuel A, Nguertoum E, Nina RA, Mansinsa P. The potential economic benefits of controlling trypanosomiasis using waterbuck repellent blend in sub-Saharan Africa. PloS One. 2021 Jul 20;16(7):e0254558.

Adams ER, Hamilton PB, Gibson WC. African trypanosomes: celebrating diversity. Trends in Parasitology. 2010 Jul 1;26(7):324-8.

Agyemang K. Trypanotolerant livestock in the context of trypanosomiasis intervention strategies. Food & Agriculture Organisation.; 2005.

Akoda K, Van den Bossche P, Marcotty T, Kubi C, Coosemans M, De Deken R, Van den Abbeele J. Nutritional stress affects the tsetse fly's immune gene expression. Medical and Veterinary Entomology. 2009 Sep;23(3):195-201.

Aksoy S. Control of tsetse flies and trypanosomes using molecular genetics. Veterinary parasitology. 2003 Jul 25;115(2):125-45 *In:* Wongserepipatana M. *Prevalence and associations of Trypanosoma spp. and Sodalis glossinidius with intrinsic factors of tsetse flies* (Doctoral dissertation, University of Glasgow).

Anderson NE, Mubanga J, Fevre EM, Picozzi K, Eisler MC, Thomas R, Welburn SC. Characterisation of the wildlife reservoir community for human and animal trypanosomiasis in the Luangwa Valley, Zambia. PLoS Neglected Tropical Diseases. 2011 Jun 21;5(6):e1211.

Assefa S, Shibeshi W. Drug resistance in African animal trypanosomes: A review. African Journal of Microbiology Research. 2018 May 7;12(17):380-6.

Autheman D, Crosnier C, Clare S, Goulding DA, Brandt C, Harcourt K, Tolley C, Galaway F, Khushu M, Ong H, Romero-Ramirez A. An invariant Trypanosoma vivax vaccine antigen induces protective immunity. Nature. 2021 Jul;595(7865):96-100.

Auty H, Anderson NE, Picozzi K, Lembo T, Mubanga J, Hoare R, Fyumagwa RD, Mable B, Hamill L, Cleaveland S, Welburn SC. Trypanosome diversity in wildlife species from the Serengeti and Luangwa Valley ecosystems. PLoS Negl Trop Dis. 2012 6(10): e1828.

Auty H, Cleaveland S, Malele I, Masoy J, Lembo T, Bessell P, Torr S, Picozzi K, Welburn SC. Quantifying heterogeneity in host-vector contact: tsetse (Glossina swynnertoni and *G. pallidipes*) host choice in Serengeti National Park, Tanzania. PloS One. 2016a Oct 5;11(10):e0161291.

Auty H, Morrison LJ, Torr SJ, Lord J. Transmission dynamics of Rhodesian sleeping sickness at the interface of wildlife and livestock areas. Trends in Parasitology. 2016b Aug 1;32(8):608-21.

Azat C. Not just a pathogen: The importance of recognizing genetic variability to mitigate a wildlife pandemic. Molecular Ecology Resources. 2021;21

Baldacchino F, Muenworn V, Desquesnes M, Desoli F, Charoenviriyaphap T, Duvallet G. Transmission of pathogens by *Stomoxys* flies (Diptera, Muscidae): a review. Parasite. 2013;20.

Barrett K, Okali C. Community participation in the management of Tsetse: a comparative assessment of impact and sustainability. Final Technical Report. 1998.

Barrett MP. The elimination of human African trypanosomiasis is in sight: Report from the third WHO stakeholders meeting on elimination of gambiense human African trypanosomiasis. PLoS Neglected Tropical Diseases. 2018 Dec 6;12(12):e0006925.

Bateta R, Saarman NP, Okeyo WA, Dion K, Johnson T, Mireji PO, Okoth S, Malele I, Murilla G, Aksoy S, Caccone A. Phylogeography and population structure of

the tsetse fly *Glossina pallidipes* in Kenya and the Serengeti ecosystem. PLoS Neglected Tropical Diseases. 2020 Feb 24;14(2):e0007855.

Bengaly Z, Sidibé I, Ganaba R, Desquesnes M, Boly H, Sawadogo L. Comparative pathogenicity of three genetically distinct types of Trypanosoma congolense in cattle: clinical observations and haematological changes. Veterinary Parasitology. 2002 Aug 30;108(1):1-9.

Bett B, Hargrove J, Randolph TF, Saini R, Hassanali A, Ndung'u JM, McDermott JJ. Modelling the impact of tsetse-repellent on the transmission of cattle trypanosomosis by *Glossina pallidipes* at Nguruman, Kenya. 2013. Proceedings of the 10th International Symposium on Veterinary Epidemiology and Economics.

Bett B, Irungu P, Nyamwaro SO, Murilla G, Kitala P, Gathuma J, Randolph TF, McDermott J. Estimation of tsetse challenge and its relationship with trypanosomosis incidence in cattle kept under pastoral production systems in Kenya. Veterinary Parasitology. 2008 Aug 17;155(3-4):287-98.

Borst P, Fase-Fowler F, Gibson WC. Kinetoplast DNA of Trypanosoma evansi. Molecular and Biochemical Parasitology. 1987 Feb 1;23(1):31-8.

Bouyer J, Carter NH, Batavia C, Nelson MP. The ethics of eliminating harmful species: The case of the tsetse fly. Bioscience. 2019 Feb 1;69(2):125-35.

Bouyer J, Koné N, Bengaly Z. Dynamics of tsetse natural infection rates in the Mouhoun river, Burkina Faso, in relation with environmental factors. Frontiers in Cellular and Infection Microbiology. 2013 Aug 29;3:47.

Brightwell B, Dransfield B, Maudlin I, Stevenson P, Shaw A. Reality vs. rhetoric–a survey and evaluation of tsetse control in East Africa. Agriculture and Human Values. 2001 Jun;18(2):219-33.

Bruce D, Hamerton AE, Bateman HR, Mackie FP. The development of trypanosomes in tsetse flies. Proceedings of The Royal Society of London. Series B, Containing Papers of a Biological Character. 1910 May 10;82(556):368-88.

Brun R, Blum J, Chappuis F, Burri C. Human african trypanosomiasis. The Lancet. 2010 Jan 9;375(9709):148-59.

Bursell E, Billing KC, Hargrove JW, Mccabe CT, Slack E. Metabolism of the bloodmeal in tsetse flies.(A review.). Acta Tropica. 1974;31(4):297-320.

Burtt E. Incubation of tsetse pupae: Increased transmission-rate of Trypanosoma rhodesiense in Glossina morsitans. Annals of Tropical Medicine & Parasitology. 1946 Apr 1;40(1):18-28.

Cator LJ, Johnson LR, Mordecai EA, El Moustaid F, Smallwood TR, LaDeau SL, Johansson MA, Hudson PJ, Boots M, Thomas MB, Power AG. The role of vector trait variation in vector-borne disease dynamics. Frontiers in Ecology and Evolution. 2020 Jul 10;8:189.

Challier A, Laveissiere C. A new trap for catching Glossina: description and field trials. Cahiers ORSTOM, Serie Entomologie Medicale et Parasitologie. 1973;11(4):251-62.

Channumsin M, Ciosi M, Masiga D, Auty H, Turner CM, Kilbride E, Mable BK. Blood meal analysis of tsetse flies (*Glossina pallidipes*: Glossinidae) reveals higher host fidelity on wild compared with domestic hosts. Wellcome Open Research. 2021;6.

Channumsin M, Ciosi M, Masiga D, Turner CM, Mable BK. Sodalis glossinidius presence in wild tsetse is only associated with presence of trypanosomes in complex interactions with other tsetse-specific factors. BMC Microbiology. 2018 Nov;18(1):69-82.

Claxton JR, Faye JA, Rawlings P. Trypanosome infections in warthogs (Phacochoerus aethiopicus) in the Gambia. Veterinary Parasitology. 1992 Mar 1;41(3-4):179-87.

de Raadt P: The history of sleeping sickness. 2005, World Health Org,. In: Steverding D. The history of African trypanosomiasis. Parasites & Vectors. 2008 Dec;1(1):1-8.

Diall O, Cecchi G, Wanda G, Argilés-Herrero R, Vreysen MJ, Cattoli G, Viljoen GJ, Mattioli R, Bouyer J. Developing a progressive control pathway for African animal trypanosomosis. Trends in Parasitology. 2017 Jul 1;33(7):499-509.

Ducheyne E, Mweempwa C, De Pus C, Vernieuwe H, De Deken R, Hendrickx G, Van den Bossche P. The impact of habitat fragmentation on tsetse abundance on the plateau of eastern Zambia. Preventive Veterinary Medicine. 2009 Sep 1;91(1):11-8.

Duffy CW, Morrison LJ, Black A, Pinchbeck GL, Christley RM, Schoenefeld A, Tait A, Turner CM, MacLeod A. *Trypanosoma vivax* displays a clonal population structure. International Journal for Parasitology. 2009 Nov 1; 39 (13): 1475-83.

Duffy CW, MacLean L, Sweeney L, Cooper A, Turner CM, Tait A, Sternberg J, Morrison LJ, MacLeod A. Population genetics of Trypanosoma brucei rhodesiense: clonality and diversity within and between foci. PLoS Neglected Tropical Diseases. 2013 Nov 14;7(11):e2526.

Dyer NA, Rose C, Ejeh NO, Acosta-Serrano A. Flying tryps: survival and maturation of trypanosomes in tsetse flies. Trends in Parasitology. 2013 Apr 1;29(4):188-96.

Ebhodaghe F, Ohiolei JA, Isaac C. A systematic review and meta-analysis of small ruminant and porcine trypanosomiasis prevalence in sub-Saharan Africa (1986 to 2018). Acta Tropica. 2018 Dec 1;188:118-31.

FAO. East Africa Animal Feed Action Plan. Rome: Food and Agriculture Organization of the United Nations (FAO) and Intergovernmental Authority on Development (IGAD). 2019:50.

FAO. tsetse and trypanosomiasis information quarterly. 2002. Part 3. Pp 79-80. https://www.fao.org/3/CA0968EN/ca0968en.pdf (Accessed on 13th December 2021).

Fetene E, Leta S, Regassa F, Büscher P. Global distribution, host range and prevalence of *Trypanosoma vivax*: a systematic review and meta-analysis. Parasites & Vectors. 2021 Dec;14(1):1-20.

Franco JR, Cecchi G, Priotto G, Paone M, Diarra A, Grout L, Simarro PP, Zhao W, Argaw D. Monitoring the elimination of human African trypanosomiasis at continental and country level: Update to 2018. PLoS Neglected Tropical Diseases. 2020 May 21;14(5):e0008261.

Gaithuma AK, Yamagishi J, Martinelli A, Hayashida K, Kawai N, Marsela M, Sugimoto C. A single test approach for accurate and sensitive detection and taxonomic characterisation of trypanosomes by comprehensive analysis of internal transcribed spacer 1 amplicons. PLoS Neglected Tropical Diseases. 2019 Feb 25;13(2):e0006842.

Garcia HA, Rodrigues AC, Martinkovic F, Minervino AH, Campaner M, Nunes VL, Paiva F, Hamilton PB, Teixeira MM. Multilocus phylogeographical analysis of Trypanosoma (Megatrypanum) genotypes from sympatric cattle and water buffalo populations supports evolutionary host constraint and close phylogenetic relationships with genotypes found in other ruminants. International Journal for Parasitology. 2011 Nov 1;41(13-14):1385-96.

Garcia HA, Rodrigues CM, Rodrigues AC, Pereira DL, Pereira CL, Camargo EP, Hamilton PB, Teixeira MM. Remarkable richness of trypanosomes in tsetse flies (Glossina morsitans morsitans and *Glossina pallidipes*) from the Gorongosa

National Park and Niassa National Reserve of Mozambique revealed by fluorescent fragment length barcoding (FFLB). Infection, Genetics and Evolution. 2018 Sep 1;63:370-9.

Gashururu RS, Githigia SM, Gasana MN, Habimana R, Maingi N, Cecchi G, Paone M, Zhao W, Masiga DK, Gashumba J. An update on the distribution of Glossina (tsetse flies) at the wildlife-human-livestock interface of Akagera National Park, Rwanda. Parasites & Vectors. 2021 Dec;14(1):1-3.

Gibson G, Torr SJ. The responses of haematophaous Diptera to physical and olfactory host stimuli. Medical and Veterinary Entomology. 1999;13:2-3.

Gikonyo NK, Hassanali A, Njagi PG, Saini RK. Behaviour of Glossina morsitans morsitans Westwood (Diptera: Glossinidae) on waterbuck Kobus defassa Ruppel and feeding membranes smeared with waterbuck sebum indicates the presence of allomones. Acta Tropica. 2000 Dec 1;77(3):295-303.

Giordani F, Morrison LJ, Rowan TG, De Koning HP, Barrett MP. The animal trypanosomiases and their chemotherapy: a review. Parasitology. 2016 Dec;143(14):1862-89.

GoK. Government of Kenya. 2017. https://www.opendata.go.ke/datasets/7acafcec6b2d41e990c200dbb045768b/explore?showTable=true (Accessed 20th December 2021).

Guo F, Bonebrake TC, Gibson L. Land-use change alters host and vector communities and may elevate disease risk. Ecohealth. 2019 Dec;16(4):647-58.

Habeeb IF, Chechet GD, Kwaga JK. Molecular identification and prevalence of trypanosomes in cattle distributed within the Jebba axis of the River Niger, Kwara state, Nigeria. Parasites & vectors. 2021 Dec;14(1):1-2.

Hamilton PB, Adams ER, Malele II, Gibson WC. A novel, high-throughput technique for species identification reveals a new species of tsetse-transmitted

trypanosome related to the *Trypanosoma brucei* subgenus, Trypanozoon. Infection, Genetics and Evolution. 2008 Jan 1;8(1):26-33.

Hargrove JW, Ouifki R, Ameh JE. A general model for mortality in adult tsetse (Glossina spp.). Medical and Veterinary Entomology. 2011 Dec;25(4):385-94.

Hargrove JW. Optimized simulation of the control of tsetse flies *Glossina pallidipes* and G. m. morsitans (Diptera: Glossinidae) using odour-baited targets in Zimbabwe. Bulletin of Entomological Research. 2003 Jan;93(1):19-29.

Hassell JM, Begon M, Ward MJ, Fèvre EM. Urbanization and disease emergence: dynamics at the wildlife–livestock–human interface. Trends in Ecology & Evolution. 2017 Jan 1;32(1):55-67.

Hoare CA. *The Trypanosomes of Mammals*, Blackwell Science Publishers In: Adams ER, Hamilton PB, Gibson WC. African trypanosomes: celebrating diversity. Trends in Parasitology. 2010 Jul 1;26(7):324-8.

Hu C, Aksoy S. Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans*. Molecular Microbiology. 2006 Jun;60(5):1194-204.

IAEA. International Research Project Explores Novel Strategies to Improving the Sterile Insect Technique to Eradicate Tsetse Flies Through Enhancing Males Refractoriness to Trypanosome Infection. 2019. https://www.iaea.org/newscenter/news/international-research-project-explores-novel-strategies-to-improving-the-sterile-insect-technique-to-eradicate-tsetse-flies-through-enhancing-males-refractoriness-to-trypanosome-infection (Accessed 20th December 2021).

Ilemobade AA. Tsetse and trypanosomosis in Africa: the challenges, the opportunities. Onderstepoort Journal of Veterinary Research. 2009;76(1):35-40.

Isaac C, Ciosi M, Hamilton A, Scullion KM, Dede P, Igbinosa IB, Nmorsi OP, Masiga D, Turner CM. Molecular identification of different trypanosome species and subspecies in tsetse flies of northern Nigeria. Parasites & Vectors. 2016 Dec;9(1):1-7.

Jones TW, Dávila AM. *Trypanosoma vivax*–out of Africa. Trends in Parasitology. 2001 Feb 1;17(2):99-101.

Kaare MT, Picozzi K, Mlengeya T, Fèvre EM, Mellau LS, Mtambo MM, Cleaveland S, Welburn SC. Sleeping sickness—a re-emerging disease in the Serengeti?. Travel Medicine and Infectious Disease. 2007 Mar 1;5(2):117-24.

Kaaya GP, Darjt N. The humoral defense system in tsetse: differences in response due to age, sex and antigen types. Developmental & Comparative Immunology. 1988 Mar 1;12(2):255-68.

Kasozi KI, Zirintunda G, Ssempijja F, Buyinza B, Alzahrani KJ, Matama K, Nakimbugwe HN, Alkazmi L, Onanyang D, Bogere P, Ochieng JJ. Epidemiology of Trypanosomiasis in Wildlife—Implications for Humans at the Wildlife Interface in Africa. Frontiers in Veterinary Science. 2021 Jun 14;8:565.

KENTTEC. Kenya Tsetse & Trypanosomiasis Eradication Council. 2019. https://www.kenttec.go.ke/tsetse-and-trypanosomiasis-problem-in-kenya/ (Accessed 10th December 2021).

Kidambasi KO, Masiga DK, Villinger J, Carrington M, Bargul JL. Detection of blood pathogens in camels and their associated ectoparasitic camel biting keds, Hippobosca camelina: the potential application of keds in xenodiagnosis of camel haemopathogens. AAS Open Research. 2019;2.

KNBS. Kenya National Bureau of Statistics. 2009. https://www.knbs.or.ke/livestock-population/ (Accessed 20th December 2021).

Krücken J, Czirják GÁ, Ramünke S, Serocki M, Heinrich SK, Melzheimer J, Costa MC, Hofer H, Aschenborn OH, Barker NA, Capodanno S. Genetic diversity of vector-borne pathogens in spotted and brown hyenas from Namibia and Tanzania relates to ecological conditions rather than host taxonomy. Parasites & Vectors. 2021 Dec;14(1):1-20.

Kuzoe FA, Schofield C. Strategic review of traps and targets for tsetse and African trypanosomiasis control. 2005.

LaHue NP, Baños JV, Acevedo P, Gortázar C, Martínez-López B. Spatially explicit modeling of animal tuberculosis at the wildlife-livestock interface in Ciudad Real province, Spain. Preventive Veterinary Medicine. 2016 Jun 1;128:101-11.

Lambrecht FL. Aspects of evolution and ecology of tsetse flies and trypanosomiasis in prehistoric African environment. The Journal of African History. 1964 Mar;5(1):1-24.

Lamprey HF, Glasgow JP, Lee-Jones F, Weitz B. A simultaneous census of the potential and actual food sources of the tsetse fly *Glossina swynnertoni* Austen. The Journal of Animal Ecology. 1962 Feb 1:151-6.

Leak SG, Collardelle C, Coulibaly L, Dumont P, Feron A, Hecker P, d'Ieteren GD, Jeannin P, Minengu M, Minja S, Mulatu W. Relationships between tsetse challenge and trypanosome prevalence in trypanotolerant and susceptible cattle. International Journal of Tropical Insect Science. 1990 Jun;11(3):293-9.

Leak SG, Ejigu D, Vreysen MJ. Collection of entomological baseline data for tsetse area-wide integrated pest management programmes. Food and Agriculture Organization of the United Nations (FAO); 2008.

Lord, J.S., Torr, S.J., Auty, H.K., Brock, P.M., Byamungu, M., Hargrove, J.W., Morrison, L.J., Mramba, F., Vale, G.A. and Stanton, M.C., 2018. Geostatistical models using remotely-sensed data predict savanna tsetse decline across the

interface between protected and unprotected areas in Serengeti, Tanzania. Journal of Applied Ecology, *55*(4), pp.1997-2007.

Machila N, Wanyangu SW, McDermott J, Welburn SC, Maudlin I, Eisler MC. Cattle owners' perceptions of African bovine trypanosomiasis and its control in Busia and Kwale Districts of Kenya. Acta Tropica. 2003 Apr 1;86(1):25-34.

Magez S, Pinto Torres JE, Oh S, Radwanska M. Salivarian Trypanosomes Have Adopted Intricate Host-Pathogen Interaction Mechanisms That Ensure Survival in Plain Sight of the Adaptive Immune System. Pathogens. 2021 Jun;10(6):679.

Majekodunmi AO, Fajinmi A, Dongkum C, Picozzi K, Thrusfield MV, Welburn SC. A longitudinal survey of African animal trypanosomiasis in domestic cattle on the Jos Plateau, Nigeria: prevalence, distribution and risk factors. Parasites & Vectors. 2013 Dec;6(1):1-0.

Majiwa PA, Maina M, Waitumbi JN, Mihok S, Zweygarth E. Trypanosoma (Nannomonas) congolense: molecular characterisation of a new genotype from Tsavo, Kenya. Parasitology. 1993 Feb;106(2):151-62.

Masumu J, Marcotty T, Geysen D, Geerts S, Vercruysse J, Dorny P, Van den Bossche P. Comparison of the virulence of Trypanosoma congolense strains isolated from cattle in a trypanosomiasis endemic area of eastern Zambia. International Journal for Parasitology. 2006 Apr 1;36(4):497-501.

Maudlin I, Holmes PH, Miles MA. The Trypanosomiases. Wallingford, UK. 2004. *In:* Saini RK, Orindi BO, Mbahin N, Andoke JA, Muasa PN, Mbuvi DM, Muya CM, Pickett JA, Borgemeister CW. Protecting cows in small holder farms in East Africa from tsetse flies by mimicking the odor profile of a non-host bovid. PLoS Neglected Tropical Diseases. 2017 Oct 17;11(10):e0005977.

McNamara JJ, Mohammed G, Gibson WC. Trypanosoma (Nannomonas) godfreyi sp. nov. from tsetse flies in The Gambia: biological and biochemical characterisation. Parasitology. 1994 Nov;109(4):497-509.

Mekata H, Konnai S, Simuunza M, Chembensofu M, Kano R, Witola WH, Tembo ME, Chitambo H, Inoue N, Onuma M, Ohashi K. Prevalence and source of trypanosome infections in field-captured vector flies (*Glossina pallidipes*) in southeastern Zambia. Journal of Veterinary Medical Science. 2008;70(9):923-8.

Meyer A, Holt HR, Selby R, Guitian J. Past and ongoing tsetse and animal trypanosomiasis control operations in five African countries: a systematic review. PLoS Neglected Tropical Diseases. 2016 Dec 27;10(12):e0005247.

Mulandane FC, Snyman LP, Brito DR, Bouyer J, Fafetine J, Van Den Abbeele J, Oosthuizen M, Delespaux V, Neves L. Evaluation of the relative roles of the Tabanidae and Glossinidae in the transmission of trypanosomosis in drug resistance hotspots in Mozambique. Parasites & Vectors. 2020 Dec;13:1-6.

Mulla AF, Rickman LR. How do African game animals control trypanosome infections?. Parasitology Today. 1988 Dec 1;4(12):352-4.

Muriithi BW, Gathogo NG, Diiro GM, Kidoido MM, Okal MN, Masiga DK. Farmer perceptions and willingness to pay for novel livestock pest control technologies: A case of tsetse repellent collar in Kwale County in Kenya. PLoS Neglected Tropical Diseases. 2021 Aug 17;15(8):e0009663.

Muriuki GW, Njoka TJ, Reid RS, Nyariki DM. Tsetse control and land-use change in Lambwe valley, south-western Kenya. Agriculture, Ecosystems & Environment. 2005 Mar 30;106(1):99-107.

Mweempwa C, Marcotty T, De Pus C, Penzhorn BL, Dicko AH, Bouyer J, De Deken R. Impact of habitat fragmentation on tsetse populations and trypanosomosis risk in Eastern Zambia. Parasites & Vectors. 2015 Dec;8(1):1-0.

Naessens J. Bovine trypanotolerance: a natural ability to prevent severe anaemia and haemophagocytic syndrome?. International Journal for Parasitology. 2006 May 1;36(5):521-8.

Nakamura Y, Hayashida K, Delesalle V, Qiu Y, Omori R, Simuunza M, Sugimoto C, Namangala B, Yamagishi J. Genetic Diversity of African Trypanosomes in Tsetse Flies and Cattle From the Kafue Ecosystem. Frontiers in Veterinary Science. 2021 Jan 27;8:5.

Ndegwa PN, Irungu LW, Moloo SK. Effect of puparia incubation temperature: increased infection rates of Trypanosoma congolense in Glossina morsitans centralis, G. fuscipes fuscipes and *G. brevipalpis*. Medical and Veterinary Entomology. 1992 Apr;6(2):127-30.

Ngari NN, Gamba DO, Olet PA, Zhao W, Paone M, Cecchi G. Developing a national atlas to support the progressive control of tsetse-transmitted animal trypanosomosis in Kenya. Parasites & Vectors. 2020 Dec;13:1-2.

Ng'ayo MO, Njiru ZK, Kenya EU, Muluvi GM, Osir EO, Masiga DK. Detection of trypanosomes in small ruminants and pigs in western Kenya: important reservoirs in the epidemiology of sleeping sickness?. Kinetoplastid Biology and Disease. 2005 Dec;4(1):1-7.

Ngumbi AF, Silayo RS. A cross-sectional study on the use and misuse of trypanocides in selected pastoral and agropastoral areas of eastern and northeastern Tanzania. Parasites & Vectors. 2017 Dec;10(1):1-9.

Nyawira NC. *Analysis of tsetse fly (Diptera: Glossinidae) blood meals using mitochondrial cytochrome genes for vertebrate host identification* (master's degree, Egerton University).

Odeniran PO, Macleod ET, Ademola IO, Welburn SC. Molecular identification of bloodmeal sources and trypanosomes in *Glossina* spp., *Tabanus* spp. and *Stomoxys*

spp. trapped on cattle farm settlements in southwest Nigeria. Medical and Veterinary Entomology. 2019 Jun;33(2):269-81.

Odongo S, Delespaux V, Ngotho M, Bekkele SM, Magez S. Comparative evaluation of the nested ITS PCR against the 18S PCR-RFLP in a survey of bovine trypanosomiasis in Kwale County, Kenya. Journal of Veterinary Diagnostic Investigation. 2016 Sep;28(5):589-94.

Ohaga SO, Kokwaro ED, Ndiege IO, Hassanali A, Saini RK. Livestock farmers' perception and epidemiology of bovine trypanosomosis in Kwale District, Kenya. Preventive Veterinary Medicine. 2007 Jun 15;80(1):24-33.

OIE. World Organisation for Animal Health. 2021. https://www.oie.int/en/what-we-do/animal-health-and-welfare/animal-diseases/?_alphabet=T (Accessed 10th December 2021).

Okiwelu SN. Host preference and trypanosome infection rates of Glossina morsitans morsitans Westwood in the Republic of Zambia. Annals of Tropical Medicine & Parasitology. 1977 Mar 1;71(1):101-7. *In:* Munang'andu HM, Siamudaala V, Munyeme M, Nalubamba KS. A review of ecological factors associated with the epidemiology of wildlife trypanosomiasis in the Luangwa and Zambezi valley ecosystems of Zambia. Interdisciplinary Perspectives on Infectious Diseases. 2012 May 27;2012.

Omondi D, Masiga DK, Ajamma YU, Fielding BC, Njoroge L, Villinger J. Unraveling host-vector-arbovirus interactions by two-gene high resolution melting mosquito bloodmeal analysis in a Kenyan wildlife-livestock interface. PloS One. 2015 Jul 31;10(7):e0134375.

Onono JO, Wieland B, Rushton J. Constraints to cattle production in a semiarid pastoral system in Kenya. Tropical Animal Health and Production. 2013 Aug;45(6):1415-22.

Ooi CP, Schuster S, Cren-Travaillé C, Bertiaux E, Cosson A, Goyard S, Perrot S, Rotureau B. The cyclical development of *Trypanosoma vivax* in the tsetse fly involves an asymmetric division. Frontiers in Cellular and Infection Microbiology. 2016 Sep 28;6:115.

Oundo JW, Villinger J, Jeneby M, Ong'amo G, Otiende MY, Makhulu EE, Musa AA, Ouso DO, Wambua L. Pathogens, endosymbionts, and blood-meal sources of host-seeking ticks in the fast-changing Maasai Mara wildlife ecosystem. PloS One. 2020 Aug 31;15(8):e0228366.

Peacock L, Cook S, Ferris V, Bailey M, Gibson W. The life cycle of Trypanosoma (Nannomonas) congolense in the tsetse fly. Parasites & Vectors. 2012 Dec;5(1):1-3.

Peacock L, Ferris V, Bailey M, Gibson W. The influence of sex and fly species on the development of trypanosomes in tsetse flies. PLoS Neglected Tropical Diseases. 2012 Feb 14;6(2):e1515.

Pinto CM, Ocaña-Mayorga S, Tapia EE, Lobos SE, Zurita AP, Aguirre-Villacís F, MacDonald A, Villacís AG, Lima L, Teixeira MM, Grijalva MJ. Bats, trypanosomes, and triatomines in Ecuador: new insights into the diversity, transmission, and origins of Trypanosoma cruzi and Chagas disease. PloS One. 2015 Oct 14;10(10):e0139999.

Pollock JN, editor. Training manual for tsetse control personnel. Food and Agriculture Organization of the United Nations; 1982.

Priotto G, Fogg C, Balasegaram M, Erphas O, Louga A, Checchi F, Ghabri S, Piola P. Three drug combinations for late-stage Trypanosoma brucei gambiense sleeping sickness: a randomized clinical trial in Uganda. PLoS Clinical Trials. 2006 Dec 8;1(8):e39.

Radwanska M, Vereecke N, Deleeuw V, Pinto J, Magez S. Salivarian trypanosomosis: a review of parasites involved, their global distribution and their

interaction with the innate and adaptive mammalian host immune system. Frontiers in Immunology. 2018 Oct 2;9:2253.

Regoes RR, Nowak MA, Bonhoeffer S. Evolution of virulence in a heterogeneous host population. Evolution. 2000 Feb;54(1):64-71.

Reid RS, Kruska RL, Deichmann U, Thornton PK, Leak SG. Human population growth and the extinction of the tsetse fly. Agriculture, Ecosystems & Environment. 2000 Feb 1;77(3):227-36.

Ribeiro Jr G, Gurgel-Gonçalves R, Reis RB, Santos CG, Amorim A, Andrade SG, Reis MG. Frequent house invasion of Trypanosoma cruzi-infected triatomines in a suburban area of Brazil. PLoS Neglected Tropical Diseases. 2015 Apr 24;9(4):e0003678.

Richards S, Morrison LJ, Torr SJ, Barrett MP, Manangwa O, Mramba F, Auty H. Pharma to farmer: field challenges of optimizing trypanocide use in African animal trypanosomiasis. Trends in Parasitology. 2021 May 4.

Rodrigues AC, Neves L, Garcia HA, Viola LB, Marcili A, Da Silva FM, Sigauque I, Batista JS, Paiva F, Teixeira MM. Phylogenetic analysis of *Trypanosoma vivax* supports the separation of South American/West African from East African isolates and a new *T. vivax*-like genotype infecting a nyala antelope from Mozambique. Parasitology. 2008 Sep;135(11):1317-28.

Rodrigues CM, Garcia HA, Rodrigues AC, Costa-Martins AG, Pereira CL, Pereira DL, Bengaly Z, Neves L, Camargo EP, Hamilton PB, Teixeira MM. New insights from Gorongosa National Park and Niassa National Reserve of Mozambique increasing the genetic diversity of *Trypanosoma vivax* and *Trypanosoma vivax*-like in tsetse flies, wild ungulates and livestock from East Africa. Parasites & Vectors. 2017 Dec;10(1):1-6.

Rodrigues CM, Garcia HA, Rodrigues AC, Pereira DL, Pereira CL, Viola LB, Neves L, Camargo EP, Gibson W, Teixeira MM. Expanding our knowledge on African trypanosomes of the subgenus Pycnomonas: a novel Trypanosoma suis-like in tsetse flies, livestock and wild ruminants sympatric with Trypanosoma suis in Mozambique. Infection, Genetics and Evolution. 2020 Mar 1;78:104143.

Saini RK, Hassanali A, Andoke J, Ahuya P, Ouma WP. Identification of major components of larviposition pheromone from larvae of tsetse fliesGlossina morsitans morsitans Westwood and Glossina morsitans centralis Machado. Journal of Chemical Ecology. 1996 Jul 1;22(7):1211-20.

Saini RK, Orindi BO, Mbahin N, Andoke JA, Muasa PN, Mbuvi DM, Muya CM, Pickett JA, Borgemeister CW. Protecting cows in small holder farms in East Africa from tsetse flies by mimicking the odor profile of a non-host bovid. PLoS Neglected Tropical Diseases. 2017 Oct 17;11(10):e0005977.

Silva Pereira S, de Almeida Castilho Neto KJ, Duffy CW, Richards P, Noyes H, Ogugo M, Rogério André M, Bengaly Z, Kemp S, Teixeira MM, Machado RZ. Variant antigen diversity in Trypanosoma vivax is not driven by recombination. Nature communications. 2020 Feb 12;11(1):1-3.

Schmidt R. Degradation of a forest in the Shimba Hills National Reserve, Kenya, as initiated by man and maintained by wildlife. InTropical forests in transition 1992 (pp. 85-104). Birkhäuser Basel.

Selby R, Bardosh K, Picozzi K, Waiswa C, Welburn SC. Cattle movements and trypanosomes: restocking efforts and the spread of Trypanosoma brucei rhodesiense sleeping sickness in post-conflict Uganda. Parasites & Vectors. 2013 Dec;6(1):1-2.

Shaw AP, Cecchi G, Wint GR, Mattioli RC, Robinson TP. Mapping the economic benefits to livestock keepers from intervening against bovine trypanosomosis in Eastern Africa. Preventive Veterinary Medicine. 2014 Feb 1;113(2):197-210.

Shaw AP, Torr SJ, Waiswa C, Cecchi G, Wint GR, Mattioli RC, Robinson TP. Estimating the costs of tsetse control options: an example for Uganda. Preventive Veterinary Medicine. 2013 Jul 1;110(3-4):290-303.

Steverding D. The history of African trypanosomiasis. Parasites & Vectors. 2008 Dec;1(1):1-8.

Sy I. L'effet de la température ambiante à la sensibilité de Glossina morsitans morsitans aux infections à Trypanosoma congolense. Master. Antwerp: MSSAT, ITM; 2011 *In*: Mweempwa C, Marcotty T, De Pus C, Penzhorn BL, Dicko AH, Bouyer J, De Deken R. Impact of habitat fragmentation on tsetse populations and trypanosomosis risk in Eastern Zambia. Parasites & Vectors. 2015 Dec;8(1):1-0.

Tekle T, Terefe G, Cherenet T, Ashenafi H, Akoda KG, Teko-Agbo A, Van Den Abbeele J, Gari G, Clausen PH, Hoppenheit A, Mattioli RC. Aberrant use and poor quality of trypanocides: a risk for drug resistance in south western Ethiopia. BMC Veterinary Research. 2018 Dec;14(1):1-8.

Thomson MC. The effect on tsetse flies (Glossina spp.) of deltamethin applied to cattle either as a spray or incorporated into ear-tags. International Journal of Pest Management. 1987 Jan 1;33(4):329-35.

Torr SJ, Hargrove JW, Vale GA. Towards a rational policy for dealing with tsetse. Trends in Parasitology. 2005 Nov 1;21(11):537-41.

Triguero-Ocaña R, Martínez-López B, Vicente J, Barasona JA, Martínez-Guijosa J, Acevedo P. Dynamic network of interactions in the wildlife-livestock interface in mediterranean Spain: an epidemiological point of view. Pathogens. 2020 Feb;9(2):120.

Turner DA. Tsetse and trypanosomiasis in the Lambwe Valley, Kenya. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1986 Jan 1;80(4):592-5.

Vale GA, Hargrove JW, Solano P, Courtin F, Rayaisse JB, Lehane MJ, Esterhuizen J, Tirados I, Torr SJ. Explaining the host-finding behavior of blood-sucking insects: computerized simulation of the effects of habitat geometry on tsetse fly movement. PLoS Neglected Tropical Diseases. 2014 Jun 12;8(6):e2901.

Vale GA, Lovemore DF, Flint S, Cockbill GF. Odour-baited targets to control tsetse flies, Glossina spp.(Diptera: Glossinidae), in Zimbabwe. Bulletin of Entomological Research. 1988 Mar;78(1):31-49.

Vale GA. Field responses of tsetse flies (Diptera: Glossinidae) to odours of men, lactic acid and carbon dioxide. Bulletin of Entomological Research. 1979 Sep;69(3):459-67.

Van den Bossche P, Chitanga S, Masumu J, Marcotty T, Delespaux V. Virulence in Trypanosoma congolense Savannah subgroup. A comparison between strains and transmission cycles. Parasite Immunology. 2011 Aug;33(8):456-60.

Vickerman K. The mode of attachment of *Trypanosoma vivax* in the proboscis of the tsetse fly *Glossina fuscipes*: an ultrastructural study of the epimastigote stage of the trypanosome. The Journal of Protozoology. 1973 Aug;20(3):394-404.

Von Wissmann B, Machila N, Picozzi K, Fèvre EM, deC. Bronsvoort BM, Handel IG, Welburn SC. Factors associated with acquisition of human infective and animal infective trypanosome infections in domestic livestock in western Kenya. PLoS Neglected Tropical Diseases. 2011 Jan 18;5(1):e941.

Votýpka J, Rádrová J, Skalický T, Jirků M, Jirsová D, Mihalca AD, D'Amico G, Petrželková KJ, Modrý D, Lukeš J. A tsetse and tabanid fly survey of African great apes habitats reveals the presence of a novel trypanosome lineage but the absence of Trypanosoma brucei. International Journal for Parasitology. 2015 Oct 1;45(12):741-8.

Vreysen MJ, Saleh KM, Ali MY, Abdulla AM, Zhu ZR, Juma KG, Dyck VA, Msangi AR, Mkonyi PA, Feldmann HU. *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. Journal of Economic Entomology. 2000 Feb 1;93(1):123-35.

Wilke AB, Benelli G, Beier JC. Anthropogenic changes and associated impacts on vector-borne diseases. Trends in Parasitology. 2021 Oct 19.

Winkle S: Geißeln der Menschheit. Kulturgeschichte der Seuchen. 2005, Düsseldorf: Artemis & Winkler In: Steverding D. The history of African trypanosomiasis. Parasites & Vectors. 2008 Dec;1(1):1-8.

Wittemyer G, Elsen P, Bean WT, Burton AC, Brashares JS. Accelerated human population growth at protected area edges. Science. 2008 Jul 4;321(5885):123-6.

Woolhouse ME, Hargrove JW, McNamara JJ. Epidemiology of trypanosome infections of the tsetse fly *Glossina pallidipes* in the Zambezi Valley. Parasitology. 1993 Jun;106(5):479-85.

World Health Organization. African trypanosomiasis or sleeping sickness. 2006. http://www.who.int/mediacentre/factsheets/fs259/en/ (Accessed 10th December 2021).

Zweygarth E, Mihok S, Majiwa PA, Kaminsky R. A new Nannomonastype trypanosome: Isolation, in vitro cultivation and partial characterisation. Malaysian Society of Parasitology and Tropical Medicine. Kuala Lumpur. 1994 Aug 24.

CHAPTER 2

Trypanosome entomological risk assessment in the Shimba Hills human-wildlife-livestock interface, Kenya

---

This chapter has been accepted for publication in the Journal ***Frontiers in Veterinary Science*** as:

Ebhodaghe, F. I., Bastos, A. D. S., Shewit Kalayou, Michael N. Okal & Masiga, D. K. (2022). Trypanosome entomological risk assessment in the Shimba Hills human-wildlife-livestock interface, Kenya.

2.1 Abstract

The Shimba Hills National Reserve in southeast Kenya is a major focus of tsetse-borne trypanosomes in East Africa. However, epidemiological hotspots in the area where livestock experience high risk of trypanosome infections have not been systematically investigated hence are not clearly known. This study investigated spatial risk and entomological drivers of cattle trypanosome infection in smallholder communities at the Shimba Hills human-wildlife-livestock interface. The objective was to identify trypanosome hotspots for targeted interventions. Tsetse flies (n = 10,996) collected in entomological surveys (November 2018 to September 2019) were morphologically identified and sorted according to species and sex. The fly samples (n = 696) were later characterised for chronological age and phenotypic size respectively based on the wing fray and wing length methods and screened for trypanosome infection and cattle bloodmeal using molecular tools. In addition, cattle blood samples (n = 1,417) were screened for infection using the buffy coat technique. Entomological risk of cattle trypanosome infection was expressed as the product of tsetse abundance and molecular rates of vector-infection and confirmed cattle bloodmeals in tsetse flies. Results revealed that *Glossina pallidipes* has a wider distribution and greater abundance than two other tsetse fly species (*G. brevipalpis* and *G. austeni*) endemic in Shimba Hills. *Glossina pallidipes* also had a greater likelihood of infectious bites on cattle than the other tsetse flies. Cattle exposures to bites from trypanosome-positive tsetse flies were similar between smallholder communities in Shimba Hills and across landscapes with disparate levels of anthropogenic disturbance. There was, however, a significant association between cattle infection risk and proximity to the wildlife reserve and between tsetse abundance and reserve proximity. Tsetse age and phenotypic sizes were similar between the fly populations irrespective of location or proximity to the reserve and unassociated with the vector infection risk and likelihood of cattle bloodmeals. Trypanosomes in tsetse flies included parasites

that infect wild-suids but which are rarely reported in cattle. Findings indicate weak relationships between trypanosome entomological risk and parasitological rates in cattle, screened for infections in contemporaneous surveys in Shimba Hills. This weak relationship was plausibly due to the limited temporal scale of data collection. Tsetse control programmes tailored to target *G. pallidipes* in sites close to wildlife protectorates may assist in effectively managing trypanosomes in Shimba Hills. Finally, studies that screen wildlife for trypanosomes or infected tsetse flies for bloodmeals could provide further insights into trypanosome epidemiology in the area.

2.2 Introduction

African trypanosomiasis is a neglected tropical disease of humans and animals caused by tsetse-borne trypanosomes in sub-Saharan Africa. The disease is also known as sleeping sickness in humans and *nagana* in livestock. Humans and animals are exposed to African trypanosomiasis when bitten by tsetse flies that are positive for matured trypanosome parasites in the metacyclic stage of development (Auty et al. 2012). Epidemiological risk of trypanosome infections from tsetse flies is spatially heterogenous in many African trypanosomiasis endemic foci and largely determined by the extent of tsetse-trypanosome interactions and the frequency of tsetse-host contacts (Auty et al. 2016). However, field studies that investigate tsetse-trypanosome interactions are limited. Moreover, there are yet fewer studies that explore tsetse-host interactions, particularly in wildlife areas such as Shimba Hills (Kenya) where Channumsin et al. (2021) observed human and wildlife but not cattle bloodmeals in tsetse flies.

Shimba Hills is a major tsetse-borne trypanosome hotspot in East Africa and one of the areas in the sub-region where trypanosomes constrain agricultural production and rural livelihoods (Shaw et al. 2014; Odongo et al. 2016; Saini et al 2017; Muriithi et al. 2021). Epidemiological surveys in Shimba Hills report trypanosome infection rates of ~50.00% in cattle populations (Saini et al. 2017). However, rates of trypanosome infections in cattle are widely heterogeneous in Shimba Hills (Mbahin et al. 2013) therefore implying that cattle in the area are exposed to variable spatial risk of trypanosome infections from tsetse flies.

Understanding of trypanosome spatial risk patterns in the Shimba Hills wildlife area will assist with identifying epidemiological hotspots where cattle are exposed to high risk of infections. However, trypanosome epidemiology and transmission risk patterns are largely understudied in Shimba Hills. Although different investigations in the area have assessed trypanosome diversity and rates in tsetse

flies (Wamwiri et al. 2013; Channumsin et al. 2018, 2021; Kimenyi et al. 2021), none of these extended the entire stretch of the human-wildlife-livestock interface or evaluated infection rates in tsetse flies in relation to vector abundance and cattle bloodmeals to determine entomological inoculation rates of trypanosome infections.

Cattle trypanosome infection rates and tsetse entomological inoculation rates maintained significantly positive relationships in Zaire, Gabon, Côte d'Ivoire, and Ethiopia (Leak et al. 1990). In Eastern Zambia, Mweempwa et al. (2015) observed that tsetse entomological inoculation rates were influenced by the vector demographics. The investigators found that anthropogenic pressures affected age structure of tsetse flies across landscapes experiencing varying levels of vegetation fragmentation. Older female individuals dominated populations of tsetse flies in markedly anthropised locations. According to Mweempwa et al. (2015), high entomological inoculation rates, hence high incidence of cattle infections in markedly anthropised study sites where tsetse flies were sparse, were due to the high proportion of older tsetse flies in those areas.

Indeed, studies show that intrinsic traits such as age and also sex are important determinants of arthropod-vector competence in pathogen transmission (Channumsin et al. 2018; Cator et al. 2020). However, conflicting information exists in field studies regarding the effect of sex on trypanosome risk in tsetse flies. For example, Channumsin et al. (2018) discovered higher trypanosome infection rates in male tsetse flies in Sampu in southern Kenya while Isaac et al. (2016) found higher rates in female tsetse flies in Yankari in northern Nigeria. According to Isaac et al. (2016), average longer lifespan probably increased risk of infection in female tsetse flies in Yankari. However, data describing the relationship between age and infection risk in tsetse flies under natural conditions are limited. It is also widely accepted that phenotypic body sizes of arthropod-vectors influence pathogen transmission (Nasci et al. 1986; Alto et al. 2008), however, investigations

of the epidemiological importance of this intrinsic trait in tsetse flies are limited, particularly in the context of variable anthropogenic pressures which are reported to alter environmental resources of tsetse flies and thus drive changes in the vector phenotypic sizes (Chilongo et al. 2021).

This study characterised tsetse entomological inoculation rates in the Shimba Hills human-wildlife-livestock interface with the aim of identifying trypanosome hotspots for targeted vector control. To better understand factors influencing trypanosome dynamics, I investigated the effect of differential anthropogenic pressures across landscapes in Shimba Hills on tsetse flies and the implications for cattle trypanosome parasite risk in the area. This work reports the most extensive survey on trypanosome infections in tsetse flies in Shimba Hills. It is also the first to evaluate trypanosome entomological inoculation rates for tsetse fly species in Shimba Hills, as well as systematically assess the relationship between trypanosome entomological risk and cattle parasitological infection rates.

2.3.0 Materials and methods

2.3.1 Study Area

Shimba Hills where the present study was conducted is a wildlife area located in Kwale County, southeast Kenya (latitude: −4.174°S and longitude: 39.4602 °E) (Fig. 2.1). The area is unique for its high elephant (*Loxodonta africana*) density and extensive faunal diversity including rare and endangered species such as the sable antelope *Hippotragus niger* (Knickerbocker & Waithaka 2005; Kenya Wildlife Service KWS 2021, http://www.kws.go.ke/content/shimba-hills-national-reserve, assessed on 16th December 2021).

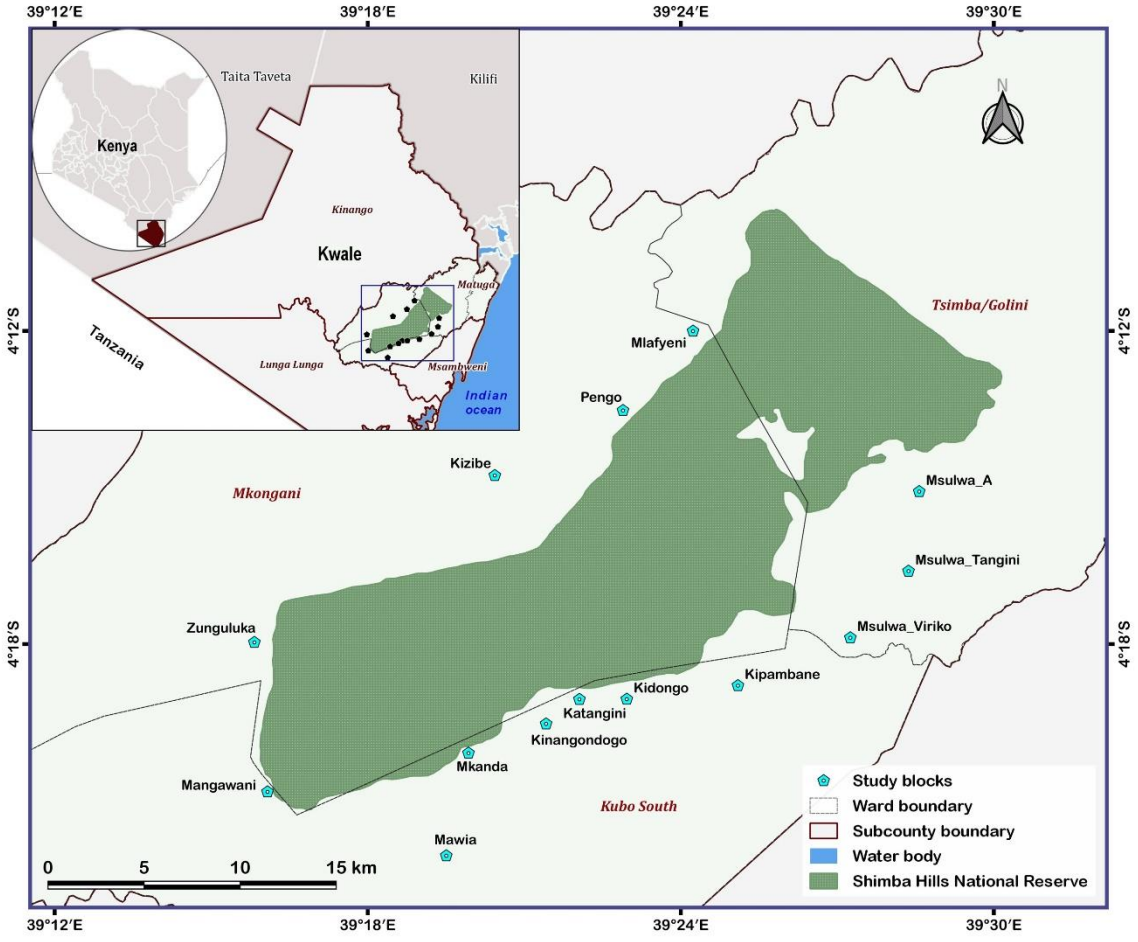


Figure 2. 1: Map showing the 14 study blocks situated within 5 km of the Shimba Hills National Reserve, Kwale county, Kenya

Average annual rainfall and temperature in Shimba Hills is 1150 mm and ~24 °C, respectively. Rainfall in the area is bimodal with long rains from March to May (occasionally extending to July), and short rains from October to December. Vegetation in Shimba Hills ranges from savannah woodlands to shrubby forests and open grasslands interspersed with trees, shrubs, and thickets. The human population in communities surrounding the Shimba Hills National Reserve is ~300,000 people, many of whom are farmers engaging in food crop and livestock production (Kenya Water Towers 2020, https://watertowers.go.ke/wp-content/uploads/2020/11/SHIMBA-Hills-Status-Report.pdf, assessed on 16th December 2021). Livestock management in local communities in Shimba Hills is extensive but without cattle migration outside the area since grazing fields remain green almost throughout the year.

2.3.2 Collection and identification of tsetse flies

Tsetse flies were surveyed in the Shimba Hills human-wildlife-livestock interface using odour-baited biconical traps (Challier & Laveissierie 1973). Biconical traps were baited using cow urine and acetone at respective release rates of 1000 mg/hr and 500 mg/hr. Collections of tsetse flies were carried out over a 231 km^2 area. The entire area was partitioned into 14 blocks (Fig. 2.1) and each block was further partitioned into grid-cells of 1 km^2. A biconical trap was deployed within each grid-cell totaling 231 and records were taken of tsetse presence or absence in traps. Spatial distribution of tsetse flies in km square were inferred based on the number of traps that caught at least one tsetse fly throughout the sampling period. Each trap was used to represent one km square. Tsetse flies were collected bimonthly from November 2018 to September 2019 and in different vegetation landscapes and locations within 5 km from the wildlife reserve. However, initial collections (November 2018 to April 2019) were limited to three blocks (Mlafyeni, Pengo, and Kizibe; Fig. 2.1) but thereafter extended to an additional 11 blocks. During a sampling exercise, collections of tsetse flies were carried out over a four-day

period and the vector abundance expressed as the number of flies per trap per day. Tsetse flies harvested from traps were morphologically identified, sorted according to sex and species using taxonomic keys (Pollock 1982) and preserved in 95% ethanol. Tsetse fly samples for analyses were randomly selected from the total collections in traps. The number of fly samples selected per trap depended on the total collections made. On average, 6 fly individuals were selected per trap. This summed up to 696 tsetse flies caught in 113 biconical traps spread across the entire study period and 14 blocks. Right wings were later carefully detached from each of the 696 tsetse flies. Each wing was assessed for serrations on the trailing edge and the extent of serrations scored on a scale of 1 to 6 to assess age of tsetse flies based on the wing fray scoring technique developed by Jackson (1946). The number on the scale increased with age of tsetse flies. Linear measurements were also taken on each wing as proxy for tsetse fly phenotypic size following the procedure adopted by Hargrove et al. (2019).

2.3.3 Molecular identification of cattle bloodmeals in tsetse flies

Each of the 696 tsetse flies assessed for age and phenotypic sizes were screened for cattle bloodmeals using Genomic DNA extraction kits (Bioloine, London, UK) according to the manufacturer's instructions for animal tissues. Individual tsetse flies were sterilized in alcohol, air-dried, and crushed using a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK, USA). Two vertebrate mitochondrial genes were then amplified in separate Polymerase Chain Reactions (PCRs): i) the 16S ribosomal RNA gene amplified with Vert 16S For: 5′-GAGAAGACCCTRTGGARCTT-3′ and Vert 16S Rev: 5′-CGCTGTTATCCCTAGGGTA-3′ primers targeting an ~200 bp region (Omondi et al. 2015), and ii) the cytochrome *b* gene amplified with the Cyt b For: 5′-CCCCTCAGAATGATATTTGTCCTCA-3′ and Cyt b Rev: 5′-CATCCAACATCTCAGCATGATGAAA-3′ primers targeting an ~383 bp region (Boakye et al. 1999). Each PCR-reaction contained 0.5 µM of each Forward and Reverse primer (Macrogen, Europe, Amsterdam, The Netherlands), 1 µL template

DNA and 2 µL of pre-formulated 5X HOT FIREPol® EvaGreen® HRM Mix, (Solis BioDyne, Tartu, Estonia) in a 10 µL reaction-volume. DNA amplifications were carried out for 16S ribosomal RNA and cytochrome *b* respectively in a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) and QuantStudio 3 Real-Time PCR System thermal cycler (MicroAmp®; Applied Biosystems, Inc., Foster city, CA, USA). Thermal cycling conditions for DNA amplifications were: initial denaturation for 15 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 20 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. High-Resolution Melting analysis of amplicons followed immediately with gradual melting from 75 °C to 95 °C. A non-template (negative) control was included in each PCR-HRM run. Cattle DNA in tsetse flies were identified by comparing melting profiles for alignment with HRM profiles of cattle DNA positive controls. Melting profiles were analysed in the software Rotor-Gene Q v2.1 and QuantStudioTM Design & Analysis v1.5.1 depending on the machine used for PCR-HRM analysis. Amplification and amplicon sequencing of the CO1 gene (Ivanova et al. 2007) was carried out to confirm positive cases of cattle bloodmeals in tsetse flies. A ~750 bp region of the CO1 gene. was targeted for amplification with 0.5 µM of each Forward and Reverse primer (Macrogen, Europe) (VF1d For: TCTCAACCAACCACAARGAYATYGG; VR1d Rev: TAGACTTCTGGGTGGCCRAARAAYCA) (Ivanova et al. 2007) in a 15 µL reaction-volume containing, 2 µL template DNA, 3 µL of 5X HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia). Cycling conditions for the amplification were: initial denaturation for 15 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 57 °C for 30 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 7 min. Success of DNA amplification was ascertained by electrophoresis of PCR-products for 30 min in a 1.5% agarose-gel stained with 5 µg/mL ethidium bromide at 120 V. Unincorporated dNTPs and PCR primers were removed from amplicons using

Exo-SAP (USB Corporation, Cleveland, OH, USA). Purified amplicons were then submitted for unidirectional Sanger sequencing at Macrogen in Europe.

2.3.4 Molecular detection and characterisation of trypanosomes in tsetse flies

The same DNA extracts prepared from tsetse fly homogenates using the Genomic DNA extraction kits (Bioloine, London, UK) for cattle bloodmeal analysis were screened for trypanosome DNA. A segment of the Internal Transcribed Spacer (ITS) region of the trypanosome genome was amplified using 0.5 μM of each of Forward and Reverse ITS-1 primers (CF: CCGGAAGTTCACCGATATTG, BR: TTGCTGCGTTCTTCAACGAA) (Njiru et al. 2005) in a 10 μL reaction-volume containing 1 μL DNA template, and 5 μL DreamTaq Master Mix (2X) (Thermo Scientific, UK). Cycling conditions for trypanosome ITS-1 DNA amplification were: initial denaturation for 1 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 20 s, and extension at 72 °C, and a final extension at 72 °C for 7 min. Amplicons were sized against a molecular weight marker (Gene-Ruler 100 bp DNA ladder, Thermo Scientific, Lithuania) on a 1.5% agarose-gel stained with ethidium bromide (5 μg/mL). The following unique band sizes were used to characterize trypanosomes: *T. vivax* ~250 bp, *T. godfreyi* ~300 bp, *T. simiae* Tsavo ~370 bp, *T. simiae* ~400 bp, Trypanozoon (*T. brucei* sp.) ~480 bp, *T. congolense* Kilifi ~620 bp, and *T. congolense* Savannah/Forest ~700 bp (Njiru et al. 2005). Further analyses to confirm trypanosome identity were carried out based on amplicon sequencing. Cleaning of amplicon to remove unincorporated dNTPs and PCR primers was performed using Exo-SAP (USB Corporation, Cleveland OH) and purified products were submitted for unidirectional Sanger sequencing at Macrogen in Europe.

2.3.5 Trypanosome parasitological surveys in cattle

Cattle in Shimba Hills were screened for trypanosomes at two different seasons during entomological surveys. The first screening was carried out in June 2019 and the second from September to October 2019. Cattle recruitment was by the

single-stage household-cluster sampling technique. For the parasitological survey, cattle were assembled in central crush-pens in each of the 14 blocks where tsetse flies were collected. Cattle were pricked on their ear veins using sterilized lancets and blood samples were collected into capillary tubes for trypanosome examination in the buffy coat (Murray et al. 1977). Most blood sample collections in Shimba Hills were done in the morning but sometimes extended into the afternoons. Cattle were also assessed for anemia based on the Packed Cell Volume (PCV) using a microhaematocrit reader (Hawksley Ltd., UK). Body weight was estimated based on heart girth measurements using calibrated bands (Saini et al. 2017).

2.3.6 Data analyses

Cattle and trypanosome DNA sequence chromatograms were inspected for quality, edited in the BioEdit software v7.2.5 (Hall 1999), and submitted to BLAST analysis for comparison to nucleotide sequences in the NCBI GeneBank-*nr* database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Cattle and trypanosome DNA sequences were identified based on a homology cut-off of at least 80.00%. Trypanosome DNA sequence alignments were implemented online in Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustao/) and the unaligned regions trimmed off prior to further analyses in *MEGA-X* software (Kumar et al. 2018). A Maximum-Likelihood phylogenetic tree to show trypanosome diversity was estimated based on 1000 bootstrap replications using default parametres in *MEGA-X*. The Smart Model Selection in PhyLM (Lefort et al. 2017) selected the Hasegawa-Kishino-Yano HKY model of sequence evolution (Hasegawa et al. 1985) as the best-fit model used in tree construction. The tree was rooted using a sequence of the Kinetoplastid *Bodo caudatus* (GenBank accession number: AY028450).

Statistical analyses were conducted in the *R* statistical environment (Team, 2013). Negative Binomial Generalized Linear Mixed Models (NB-GLMMs) (Brooks et al.

2017) with '*trap_ID*' as random-effect were used to assess significant differences in the abundance of tsetse flies with 'sex', 'species', 'landscape vegetation' and 'distance from wildlife reserve' as predictor variables. Entomological risk (Entomological Inoculation Rate EIR) of cattle trypanosome infections was expressed as the product of tsetse abundance and rates of infection and confirmed cattle bloodmeals in sampled tsetse flies. EIRs were multiplied by 365 to derive estimates of annual [*a*]-EIRs. Mean *a*-EIRs were used to reflect the average number of trypanosome-positive tsetse flies expected to feed on cattle per year in Shimba Hills.

Mean *a*-EIRs, Wing Fray Scores (WFS) and phenotypic sizes of tsetse flies were significantly different from normal distribution (P <0.05) using the Shapiro-Wilk's test. Consequently, a-EIRs, WFS and phenotypic size variations between tsetse flies were assessed using the non-parametric Mann-Whitney *U* test to examine for significant difference between fly sex and the Kruskal-Wallis test to examine for significant difference between fly species, 'landscape vegetation' and 'distance from wildlife reserve'. Probabilities of tsetse infection and cattle bloodmeals were each assessed in Binomial-GLMMs using '*trap_ID*' as random-effect and 'fly_sex', 'fly_species', 'fly_WFS' and 'fly_phenotypic_size' as predictor variables.

Differences between cattle sex, trypanosome species and blocks in the proportion of cattle infection (infected vs uninfected) were assessed for significance using Binomial-Generalized Linear Models (B-GLMs) (Dunn et al. 2018). Cattle PCVs and girth measurements were significantly different from normal distribution (P <0.05) using the Shapiro-Wilk's test. Therefore, comparisons of mean PCVs and mean girth measurements between infected and uninfected cattle were done using the Mann-Whitney *U* test. The Spearman Correlation Coefficients (*rho*) were calculated to assess the relationship between mean a-EIRs and cattle trypanosome infection rates across blocks. An *alpha*-level of 0.95 was selected in all analyses. Pairwise comparisons for significant difference in Kruskal-Wallis tests having

P<0.05 were done using Dunn's post-hoc tests (Dinno 2017). Furthermore, Tukey's post-hoc tests were carried out in the *'multcomp'* R package (Hothorn et al. 2016) for GLMMs and GLMs having two or more predictor variables and *P* <0.05.

2.3.7 Ethical consent

The study received ethical clearance from the Kenyan National Commission for Science, Technology, and Innovation (License No.: NACOSTI/P/20/7344). Field collection of tsetse flies were carried out in collaboration with the Kenya Wildlife Service (KWS), the Kenya Tsetse and Trypanosomiasis Eradication Council (KENTTEC) and local communities in Shimba Hills. Verbal consent was sought and obtained from cattle owners prior to collection of blood samples from animals. Technical field staff made every effort to minimize pain and discomfort to animals during blood sample collection. Positive cases of trypanosome infections in animals were treated using diminazene diaceturate (Veriben® manufactured in France by Ceva Sante Animale) and without payments from owners.

2.4.0 Results

I collected a total of 10,996 tsetse flies in the entomological survey in Shimba Hills. This comprised of 22.45% males (n=2,469) and 77.55% females (n=8,527). Morphological identification confirmed that *G. pallidipes* was the most abundant species (95.11%, n=10,458), followed by *G. brevipalpis* (3.58%, n=394) and *G. austeni* (1.31%, n=144). Almost all tsetse flies (96.71%, 10,634/10,996) collected in the entomological survey at the human-wildlife-livestock interface were trapped in sites within 1000m from the Shimba Hills National Reserve (NR). *Glossina austeni* were trapped only within 1000m of the Shimba Hill NR. For the other species, tsetse fly abundance decreased with distance from the reserve, irrespective of sex. Male and female tsetse flies were respectively collected in an estimated 44% (102 km^2) and 62% (143 km^2) of the entire 231km^2 area surveyed while *G. pallidipes* were collected in 61% (140 km^2), *G. austeni* in 15% (35 km^2) and *G. brevipalpis* in 25% (58 km^2) of the same area.

2.4.1 Abundance of tsetse flies across landscapes

Female tsetse flies (1.50 FTD. 95% CI: 1.17 to 1.83) were significantly more abundant than males (0.43 FTD. 95% CI: 0.33 to 0.54) (NB-GLMM: P <0.0001), and *G. pallidipes* (1.84 FTD. 95% CI: 1.42 to 2.26) was significantly more abundant than *G. brevipalpis* (0.07 FTD. 95% CI: 0.06 to 0.08) and *G. austeni* (0.03 FTD. 95% CI: 0.02 to 0.03) (NB-GLMM: P <0.0001) (Fig. 2.2).

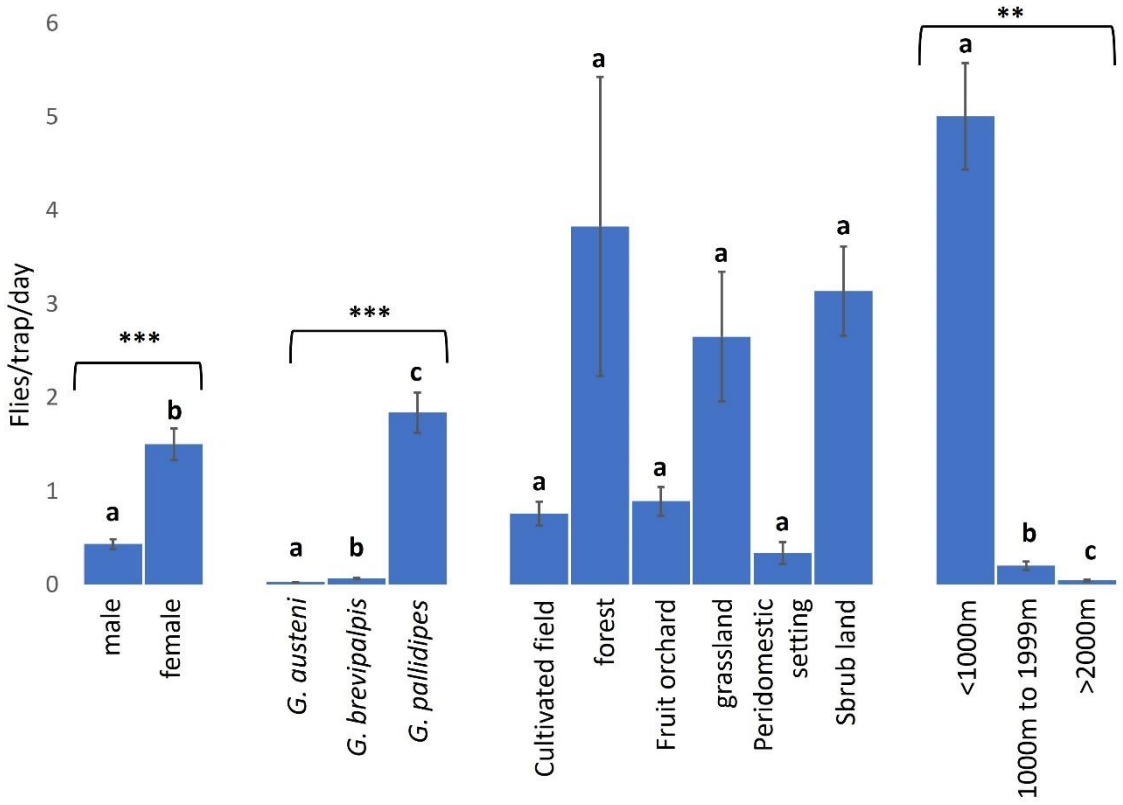


Figure 2. 2: Abundance of tsetse flies according to fly sex and species, and collection sites (landscape type and proximity to Shimba Hills National Reserve). '*' and '**' correspond to *P* values <0.0001 and <0.01 respectively. Error bars indicate the standard error of the mean (SEM).**

Forested areas (3.83 FTD. 95% CI: 0.66 – 4.49) had the highest abundance of tsetse flies among vegetation landscapes, but this was not significantly different from the vector abundance in the other locations (NB-GLMM: P >0.05), including cultivated fields (0.76 FTD. 95% CI: 0.51 – 1.27) and peridomestic settings (0.34 FTD. 95% CI: 0.10 – 0.44) where tsetse fly abundance was least (Fig. 2.2). Tsetse abundance was significantly higher within 1000m (5.00 FTD. 95% CI: 3.89 – 8.89) of the reserve than in other areas 1000-1999m (0.20 FTD. 95% CI: 0.11 – 0.32) and >2000m (0.04 FTD. 95% CI: 0.03 – 0.07) from the reserve (NB-GLMM: P <0.01).

2.4.2 Epidemiological importance of tsetse flies

Out of 696 tsetse flies screened in molecular analyses, 11.35% (95% CI: 8.99 – 13.71) and 8.62% (95% CI: 6.53 – 10.71) were respectively positive for cattle bloodmeals (Fig. 2.3) and trypanosome infections (Fig. 2.4, Table 2.1). Among the trypanosomes species identified in tsetse flies, *T. vivax* (2.44%. 95% CI: 1.29 – 3.59) was the most prevalent (Table 2.1). Furthermore, tsetse flies were positive for the double infections *T. congolense* Savannah and *T. brucei sl*. (0.29%. 95% CI: -0.11 – 0.69), *T. congolense* Kilifi and *T. congolense* Savannah (0.14%. 95% CI: -0.14 – 0.43) and *T. brucei sl*. and *T. vivax* (0.14%. 95% CI: -0.14 – 0.43) and the triple infections *T. simiae*, *T. simiae* Tsavo and *T. godfreyi* (0.14%. 95% CI: -0.14 – 0.43) and *T. simiae*, *T. simiae* Tsavo and *T. vivax* (0.14%. 95% CI: -0.14 – 0.43).

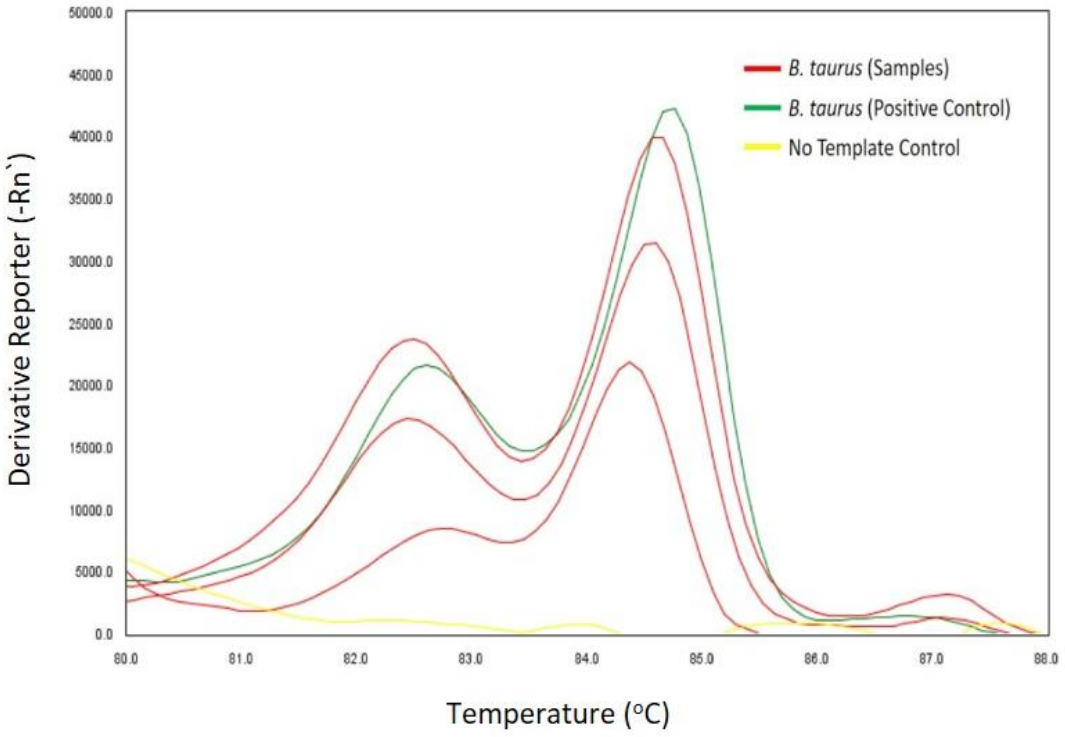


Figure 2. 3: HRM profiles showing melting curves of cattle DNA

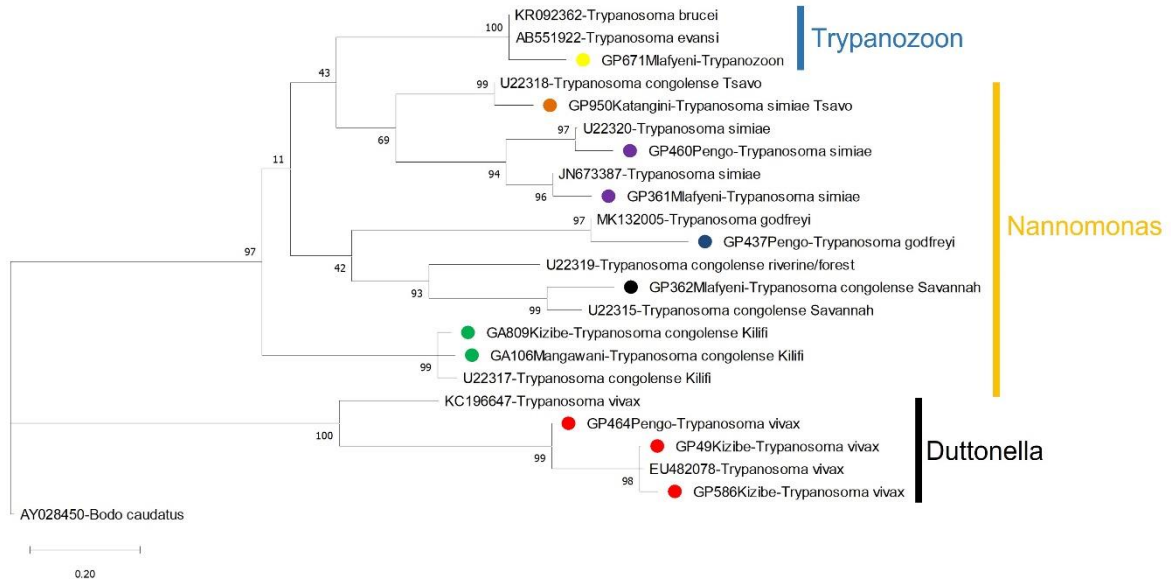


Figure 2. 4: A Maximum-Likelihood phylogenetic tree showing the species of trypanosomes detected in tsetse flies in Shimba Hills in Kenya (November 2018 to September 2019). Sequences from the study are bulleted using different colours to indicate trypanosome parasites. Other sequences shown on the tree were obtained from GenBank. Vertical bars are used to depict subgenera of trypanosomes. Nodal support values based on 1000 bootstrap replicates are indicated next to each node. The branch length represents substitution per site.

Table 2. 1: Identification of trypanosome nucleic acid sequences recovered from tsetse fly samples in Shimba Hills (2018 to 2019)

Sample ID	Block	Latitude	longitude	Fly species	Sex	Sequence length (bp)	Closest match on GenBank (host, location)	species	Sequence identity (%)
GP437	**Pengo**	-4.25076	39.36938	*G. pallidipes*	Female	116	MK131956 (Tsetse fly, Zambia)	*T. godfreyi*	91.67
GP362	**Mlafyeni**	-4.2508491	39.36904	*G. pallidipes*	Female	313	U22315 (Rat, Kenya)	*T. congolense* Savannah	94.49
GA106	**Mangawani**	-4.35776	39.25443	*G. austeni*	Female	536	MK756200 (Tsetse fly, Nigeria)	*T. congolense* Kilifi	82.51
GA693	**Kizibe**	-4.2894689	39.27341	*G. austeni*	Female	459	MK756200 (Tsetse fly, Nigeria)	*T. congolense* Kilifi	82.37
GA809	**Kizibe**	-4.2894689	39.27341	*G. austeni*	Female	527	MK756200 (Tsetse fly, Nigeria)	*T. congolense* Kilifi	89.85
GP671	**Mlafyeni**	-4.2000908	39.40392	*G. pallidipes*	Male	326	KR092362 (Colobus, Cote d'Ivoire)	*T. brucei sl*	97.45
GP950	**Katangini**	-4.3202	39.36612	*G. pallidipes*	Female	340	U22318 (Tsetse fly, Kenya)	*T. simiae* Tsavo	92.46
GP361	**Mlafyeni**	-4.1745264	39.39222	*G. pallidipes*	Female	345	JN673387 (Warthog, Tanzania)	*T. simiae*	93.08
GP460	**Pengo**	-4.21669	39.3731	*G. pallidipes*	Male	362	U22320 (?, Kenya)	*T. simiae*	91.69
GP464	Pengo	-4.24723	39.36326	*G. pallidipes*	Male	221	KX584844 (Tsetse fly, Mozambique)	*T. vivax*	100
GP49	Kizibe	-4.27402	39.30951	*G. pallidipes*	Female	209	KX584844 (Tsetse fly, Mozambique)	*T. vivax*	99.42
GP586	Kizibe	-4.2715603	39.33925	*G. pallidipes*	Male	208	KX584844 (Tsetse fly, Mozambique)	*T. vivax*	100

Overall, 0.86% (95% CI: 0.17 – 1.55) of screened tsetse flies were positive for both trypanosome infections and cattle bloodmeals. The overall rate of confirmed cattle bloodmeals was higher in trypanosome-positive female flies (mean *a*EIR: 14.19. 95% CI: -7.72 – 36.09) than male flies (mean *a*EIR: 9.17. 95% CI: -9.78 – 28.12) (Mann-Whitney *U* test: $P = 0.0331$) and in *G. pallidipes* (mean *a*EIR: 29.26. 95% CI: -27.10 – 85.62) than *G. austeni* (mean *a*EIR: 0.27. 95% CI: -0.19 – 0.73) and *G. brevipalpis* (mean *a*EIR: 0.05. 95% CI: -0.01 – 0.11) (Fig. 2.5 Kruskal-Wallis test: $H = 11.92$, $d.f = 2$, $P < 0.01$).

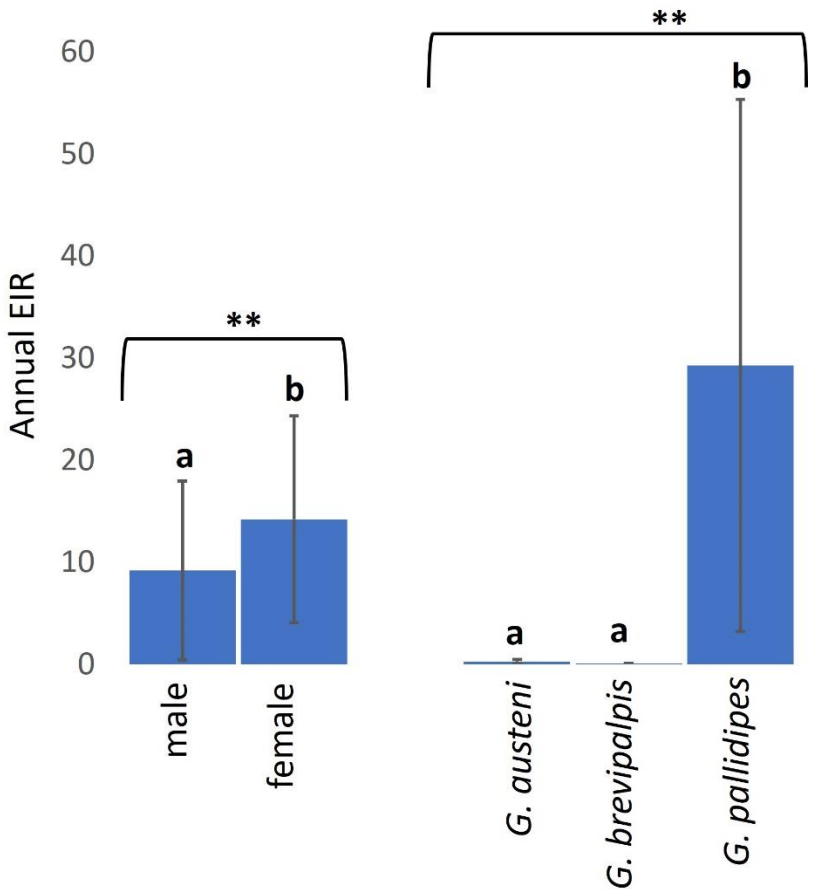


Figure 2. 5: Annual EIRs of tsetse flies according to fly sex and species. Error bars indicate the standard error of the mean (SEM).

2.4.3 Spatial entomological risk of cattle trypanosome infections

Mean *a*EIR in Shimba Hills was 14.42 (95% CI: -1.65 – 30.49). Entomological risk of cattle trypanosome infections though relatively high in Kinangodongo (mean *a*EIR: 140.89. 95%CI: -1649.29 – 1931.07) was not significantly different between study-blocks (Fig. 2.6, Kruskal-Wallis test: H = 14.52, *d.f* = 13, P = 0.3385).

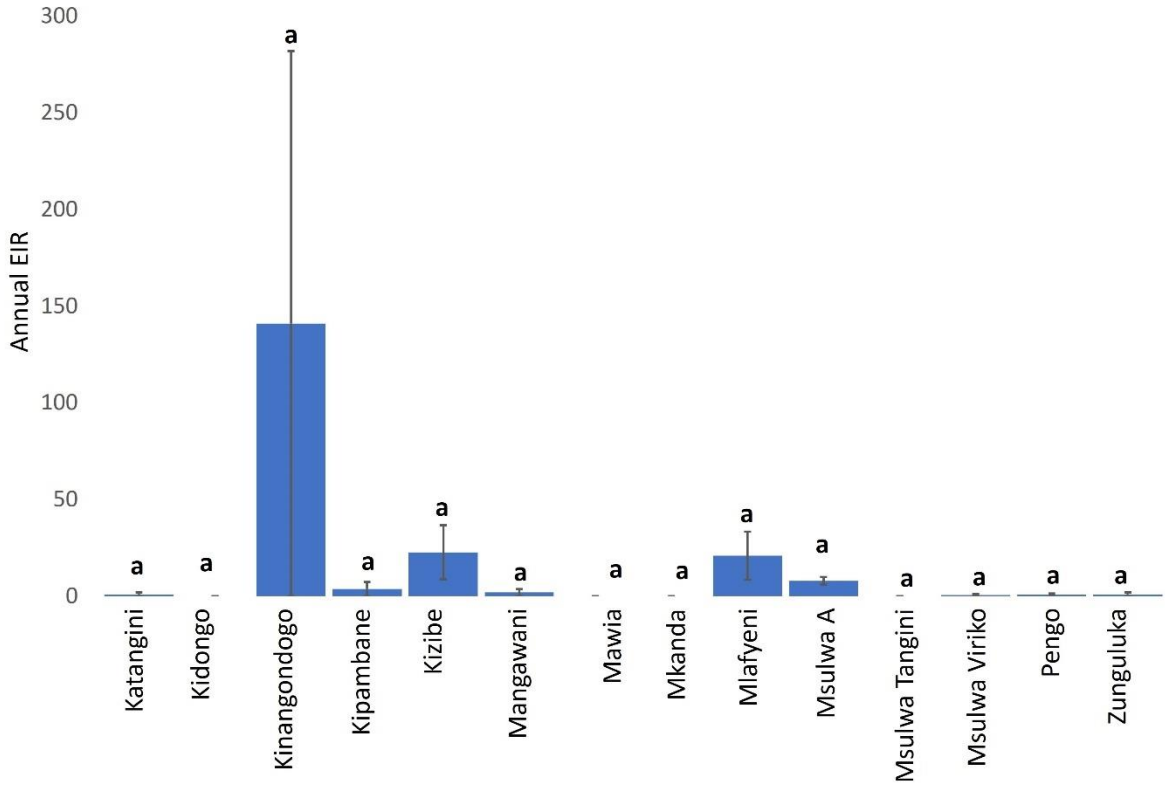


Figure 2. 6: Annual EIR of tsetse flies according to study-block. Error bars indicate the standard error of the mean (SEM).

Trypanosome-infected tsetse flies fed on cattle more frequently in shrub-lands (mean *a*EIR: 75.31. 95% CI: -83.13 – 233.74) than other landscapes and sparsely in cultivated fields (mean *a*EIR: 0.62. 95% CI: -0.10 – 1.33) and peridomestic settings (mean *a*EIR: 0.51. 95% CI: -0.66 – 1.68) (Fig. 2.7, Kruskal-Wallis test: H = 6.01, *d.f* = 5, P = 0.3057). Finally, infected tsetse flies were more likely to feed on cattle in locations within

1000m (mean *a*EIR: 28.75. 95% CI: -11.79 – 69.28) of the wildlife reserve in Shimba Hills than 1000-1999m (mean *a*EIR: 2.14. 95% CI: -2.16 – 6.45) and >2000m (mean *a*EIR: 0.30. 95% CI: -0.37 – 0.97) from the reserve (Fig. 2.6, Kruskal-Wallis test: H = 10.30, *d.f* = 2, P <0.01).

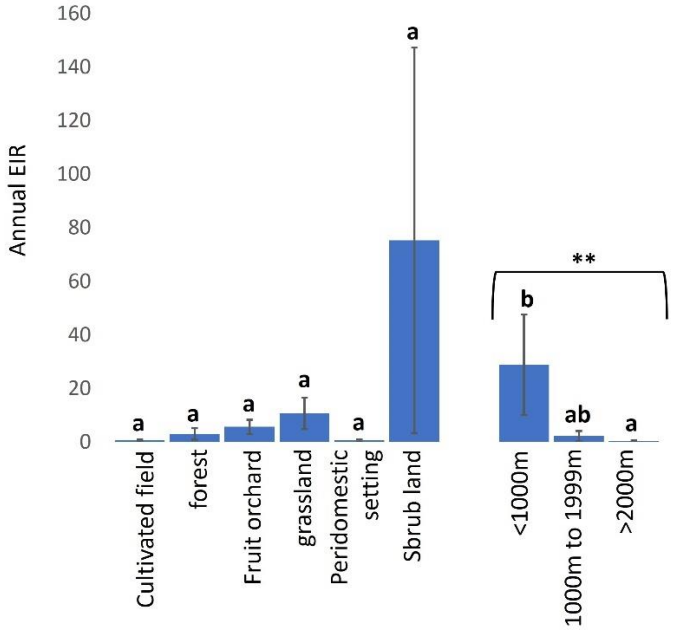


Figure 2. 7: Annual EIR of tsetse flies according to vegetation landscape and proximity to wildlife reserve in Shimba Hills. Error bars indicate the standard error of the mean (SEM).

2.4.4 Age structure of tsetse flies across landscapes

Wing fray scores (WFS) used to assess age of tsetse flies were not significantly higher in female (WFS: 2.94. 95% CI: 2.80 – 3.09) than male (WFS: 2.82. 95% CI: 2.59 – 3.04) tsetse flies (Mann-Whitney U test: P = 0.09502) but were significantly higher in *G. pallidipes* (WFS: 3.03. 95% CI: 2.89 – 3.18) than *G. brevipalpis* (WFS: 2.52. 95% CI: 2.25 – 2.78) and *G. austeni* (WFS: 2.38. 95% CI: 1.82 – 2.93) (Fig. 2.8, Kruskal-Wallis test: H =

17.63, *d.f* = 2, P < 0.01). Tsetse flies were, on average, youngest in grasslands (WFS: 2.68 (95% CI: 2.45 – 2.92) and oldest in peridomestic settings (WFS: 4.00. 95% CI: 2.88 – 5.12) than in other landscapes (Fig. 2.8, Kruskal-Wallis test: H = 9.08, *d.f* = 5, P = 0.1059). The age of tsetse flies was similar between locations <1000m (WFS: 2.89 (95% CI: 2.76 – 3.02), 1000-1999m (WFS: 3.03. 95% CI: 2.58 – 3.48) and >2000m (WFS: 2.86. 95% CI: 2.04 – 3.68) from the reserve (Fig. 2.8, Kruskal-Wallis test: H = 0.36, *d.f* =2, P = 0.8338).

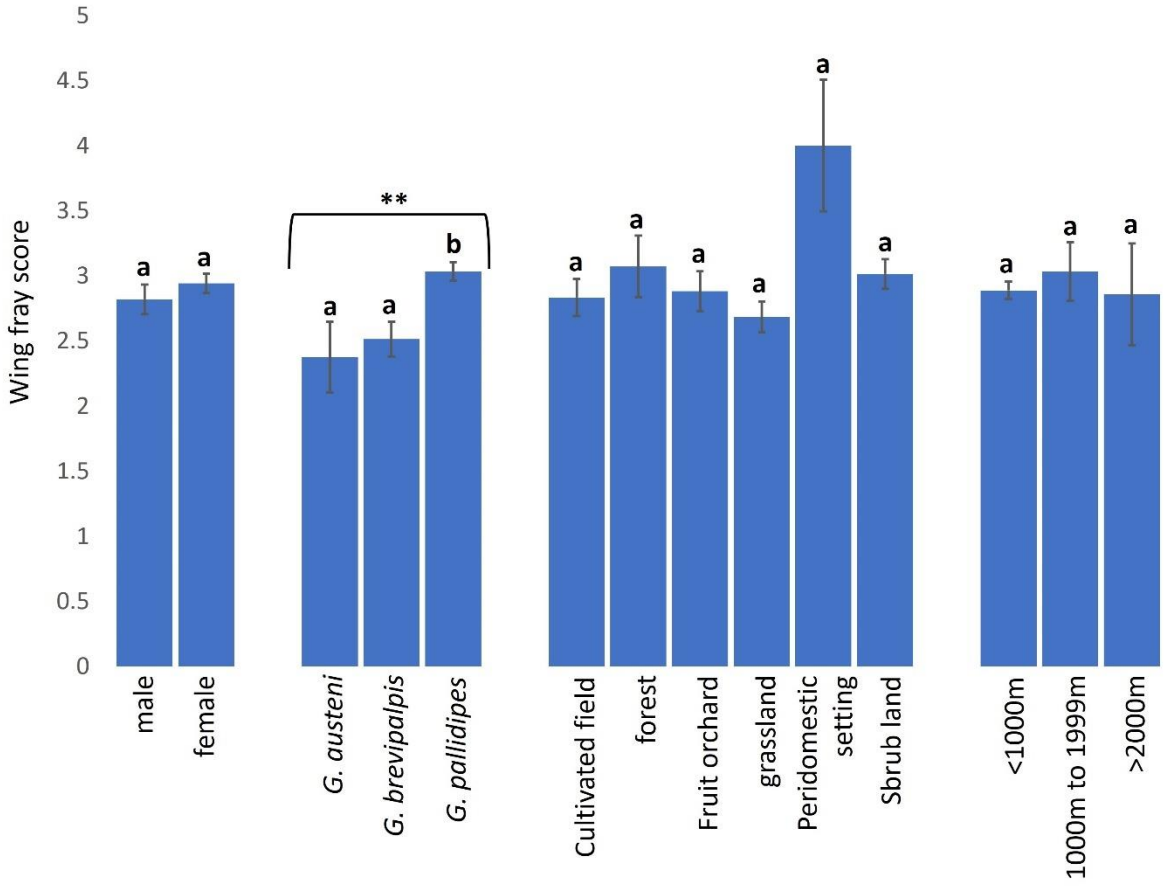


Figure 2. 8: Wing fray scores of tsetse flies according to fly sex and species and collection site. Error bars indicate the standard error of the mean (SEM).

2.4.5 Phenotypic sizes of tsetse flies across landscapes

Tsetse phenotypic sizes were significantly higher in female (8.41mm. 95% CI: 8.31 – 8.51) than male (7.67mm. 95% CI: 7.57 – 7.77) (Mann-Whitney *U* test: P <0.0001) and in *G. brevipalpis* (10.15mm. 95% CI: 10.05 – 10.24) than *G. pallidipes* (7.78mm. 95% CI: 7.75 – 7.81) and *G. austeni* (6.68mm. 95% CI: 6.58 – 6.77) (Fig.2. 8, Kruskal-Wallis test: H = 288.43, *d.f* = 2, *P* < 0.0001). Phenotypic sizes of tsetse flies were similar between landscapes, ranging from 8.02mm (95% CI: 7.82 – 8.23) in peri-domestic settings to 8.29mm (95% CI: 7.93 – 8.65) in forests (Fig. 2.9, Kruskal-Wallis test: H = 2.68, *d.f* = 5, P = 0.7487). Tsetse phenotypic sizes were also similar between the vector populations in areas <1000m (8.18mm. 95% CI: 8.09 – 8.26), 1000-1999m (7.99mm. 95% CI: 7.80 – 8.18) and >2000m (8.11mm. 95% CI: 7.64 – 8.57) from the reserve (Fig. 2.9, Kruskal-Wallis test: H = 0.03, *d.f* =2, *P* = 0.9842).

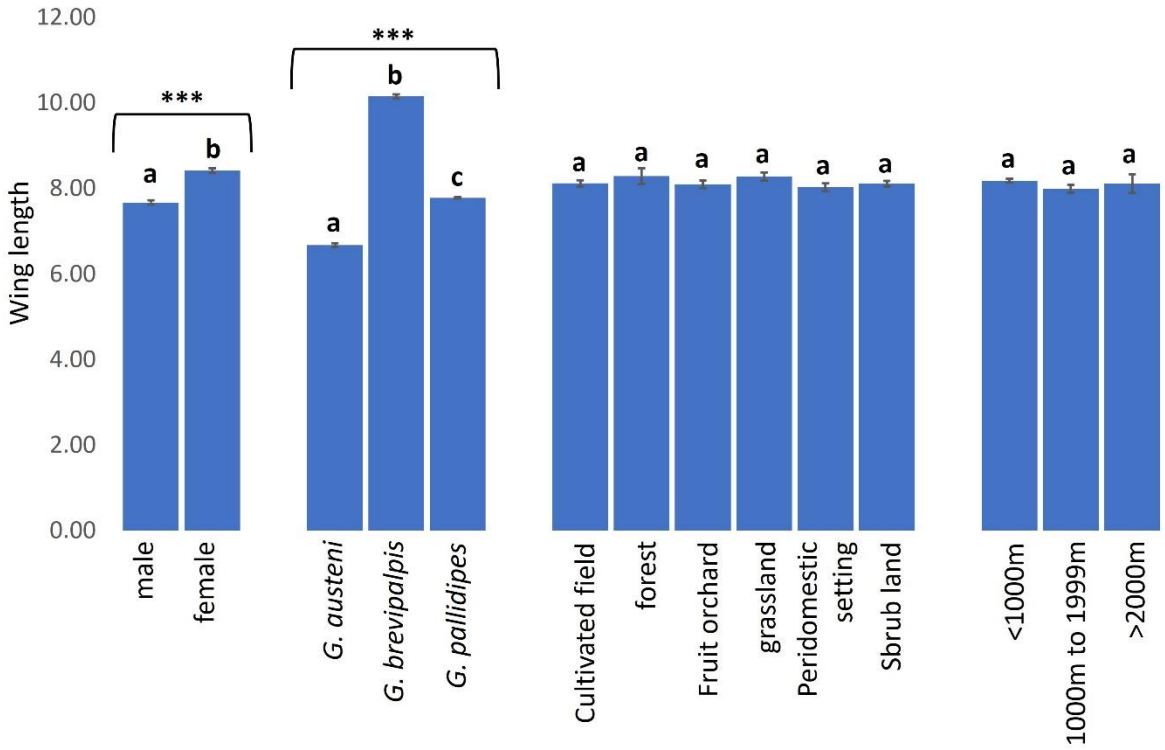


Figure 2. 9: Wing length of tsetse flies according to fly sex and species and collection site. Error bars indicate the standard error of the mean (SEM).

2.4.6 Effects of vector intrinsic traits on trypanosome infections and cattle bloodmeals in tsetse flies

Trypanosome rate was higher in female (0.09. 95% CI: 0.07 – 0.16) than male (0.07. 95% CI: 0.04 – 0.11) tsetse flies and in *G. austeni* (0.20. 95% CI: 0.07 – 0.27) than *G. pallidipes* (0.09. 95% CI: 0.06 – 0.15) and *G. brevipalpis* (0.05. 95% CI: 0.01 – 0.06). Tsetse fly species (NB-GLMM: P <0.05) but not sex (Binomial-GLMM: P >0.05) had an effect on the likelihood of the vector infection. Tsetse age (B-GLMM: P >0.05) and phenotypic size (after controlling for fly species effect) (B-GLMM: P >0.05) were also not significantly associated with the probability of trypanosome infection. Furthermore, female tsetse flies (0.13. 95% CI: 0.10 – 0.24) had a higher rate of cattle bloodmeals than male tsetse flies (0.08. 95% CI: 0.04 – 0.12). Cattle feeding rates were

higher in *G. austeni* (0.18. 95% CI: 0.05 – 0.23) than *G. pallidipes* (0.11. 95% CI: 0.08 – 0.19) and *G. brevipalpis* (0.11. 95% CI: 0.05 – 0.16). However, fly sex (NB-GLMM: P <0.05) but not fly species (B-GLMM: P >0.05) was significantly associated with probability of detecting a cattle bloodmeal in tsetse flies. Neither tsetse age (B-GLMM: P >0.05) nor phenotypic size (B-GLMM: P >0.05) was associated with probability of cattle bloodmeals in tsetse flies.

2.4.7 Cattle trypanosome infections and association with trypanosome entomological inoculation rates

A total of 185 (13.06%. 95% CI: 11.30 – 14.81) out of 1,417 cattle screened for trypanosomes were positive for infection in Shimba Hills (Fig. 2.10). Male cattle (16.86%. 95% CI: 13.87 – 19.85) had a significantly higher proportion of infection than female cattle (10.22%. 95% CI: 8.13 – 12.31) (BGLM: P = 0.0003). Cattle were infected with *T. congolense* (6.92%. 95% CI: 5.59 – 8.24) and *T. vivax* (6.21%. 95% CI: 4.95 – 7.47) (BGLM: P = 0.4483). The proportion of infection was highest in Mkanda (43.24%. 95% CI: 31.69 – 54.80) and significantly different between study-blocks (BGLM: P <0.05). Average Packed Cell Volume was significantly lower in infected (22.71. 95% CI: 21.95 – 23.47) than uninfected (27.26. 95% CI: 27.00 – 27.52) cattle (Mann-Whitney U test: P < 0.0001). However, average girth measurements were similar between infected (173.02 cm. 95% CI: 165.55 – 180.48) and uninfected (167.03 cm. 95% CI: 163.86 – 170.19) cattle (Mann-Whitney U test: P > 0.05). Trypanosome entomological risk across study-blocks lacked association with cattle trypanosome infection rates whether overall (Fig. 2.11. Spearman's Correlation Coefficient (*rho*) = 0.13. P = 0.6657) or during the long rains in May-June (*rho* = -0.02. P = 0.9505) or dry season in August-October (*rho* =-0.25. P = 0.3817).

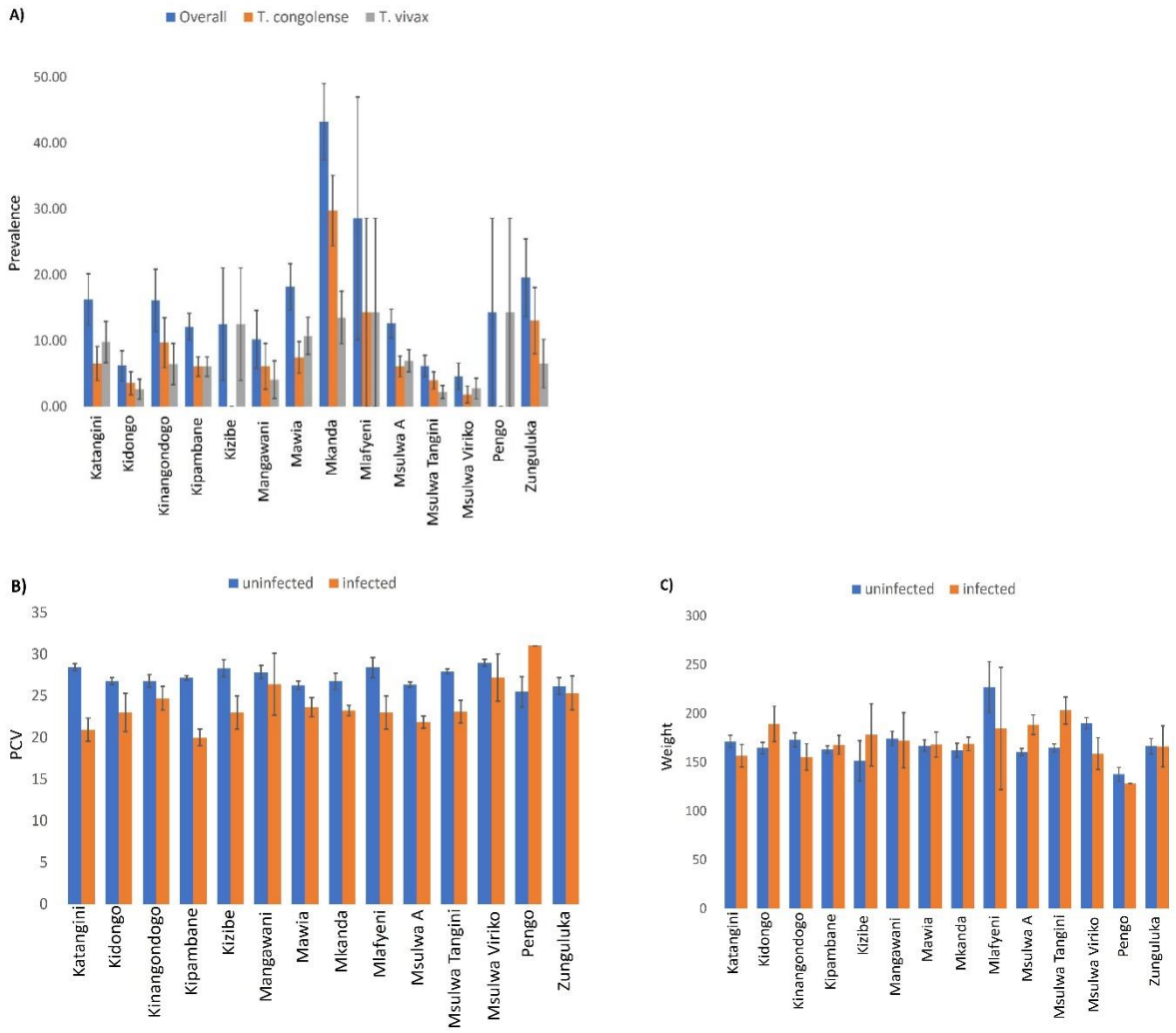


Figure 2. 10: Bar Charts showing: a) Trypanosome infection rates, b) packed cell volume (PCV) and c) girth measurements in cattle populations in Shimba Hills. Error bars indicate the standard error of the mean (SEM).

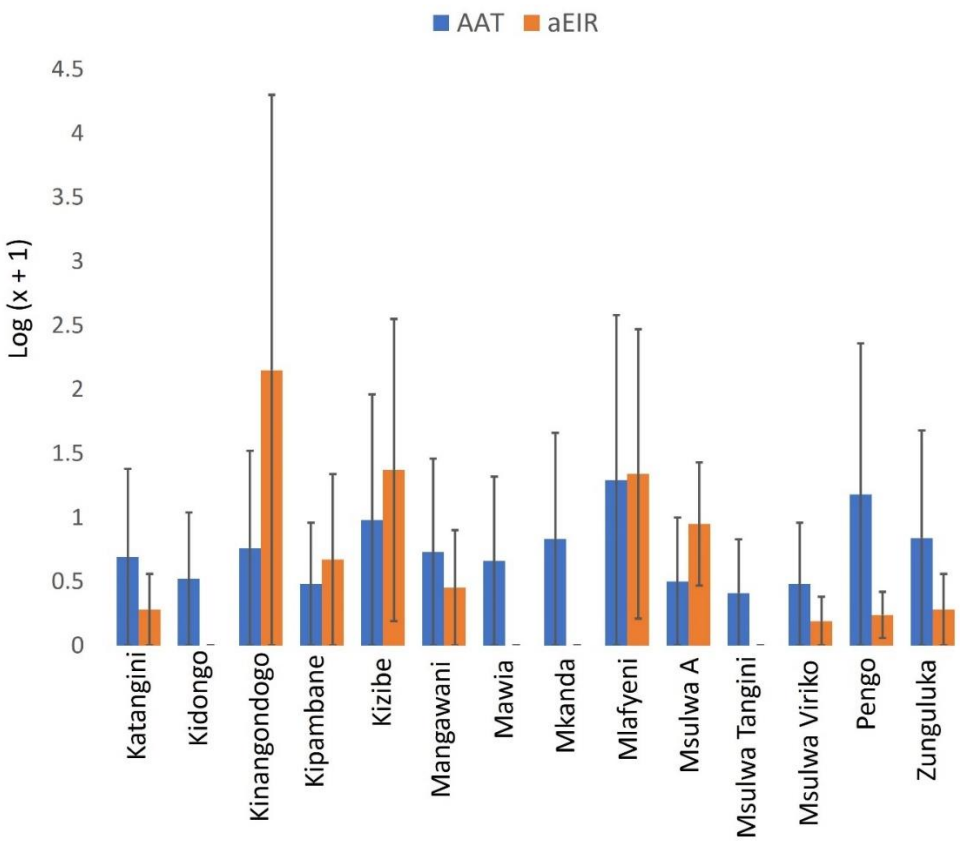


Figure 2. 11: Annual EIR of tsetse flies and trypanosome parasitological rates in cattle. Error bars are used to indicate the standard error of the mean (SEM).

2.5.0 Discussion

The present study provides insights into trypanosome spatial risk in Shimba Hills and reassessed the species diversity and abundance of tsetse flies at the human-wildlife-livestock interface. For the first time, the study provides empirical data to show evidence of ongoing interactions between tsetse flies, trypanosomes, and cattle in Shimba Hills and identified grasslands close to the wildlife reserve as hotspots for cattle infections. The study incriminated female tsetse flies and *G. pallidipes* as being responsible for most cattle exposures to trypanosome infections in Shimba Hills and thus corroborates previous reports of female and *G. pallidipes* tsetse flies as epidemiologically important vectors of trypanosomes (Ouma et al. 2005; Vale et al. 2014). Epidemiological importance of female tsetse flies and *G. pallidipes* in Shimba Hills is further supported by data from the present work showing extensive distribution and high abundance of these vectors in the area as well as the relatively high average lifespan of *G. pallidipes* and the high likelihood of females to feed on cattle.

The average annual entomological inoculation rate obtained for tsetse flies in Shimba Hills indicates that cattle in the area are exposed to bites from one trypanosome-positive tsetse fly every 26 days, an almost two-fold higher rate than the 50 days reported in the Ghibe Valley in Ethiopia (Lemecha et al. 2006). However, this frequency of tsetse-cattle contacts in Shimba Hills is clearly an under-estimation considering that tsetse flies in certain locations were characterised to have annual entomological inoculation rates of >14.42 suggesting increased frequent encounters between tsetse flies and cattle in these sites. Kinangodongo, one of the study-blocks in Shimba Hills where I assessed tsetse flies for the degree of trypanosome risk posed to cattle, is located close to the Shimba Hills National Reserve precisely within one thousand metres of the wildlife reserve. It was therefore not surprising that

Kinangodongo is among study-blocks recorded to have the highest average annual entomological inoculation rate of 140.89 implying cattle exposure to attack from one infected tsetse fly at least every three days.

Glossina pallidipes, G. austeni and *G. brevipalpis* collections in traps show further evidence of the endemicity of these fly species in Shimba Hills (Mbahin et al. 2013; Channumsin et al. 2018). Tsetse high infestations close to the wildlife reserve were unequivocally influenced by the vector high abundance within the National Reserve and contributed to the high entomological inoculation rates of cattle trypanosome infections uncovered in sites near the reserve. Studies in the Serengeti National Park in Tanzania (Lord et al. 2018) and the Akagera National Reserve in Rwanda (Gashururu et al. 2021) among other wildlife areas in East Africa (Malele et al. 2011; Salekwa et al. 2014) reported high abundance of tsetse flies within wildlife protected areas. However, as with Serengeti and Akagera, the number of tsetse flies in Shimba Hills were observed to progressively decline from wildlife protectorates following decline in vegetation cover providing resting sites as well as animal species providing bloodmeals for the vectors.

To show the effect of vegetation cover and the absence thereof on tsetse flies in Shimba Hills were the findings of high abundance of tsetse flies in forests and shrub lands and sparse abundance of the vectors in cultivated fields and peridomestic settings. However, tsetse flies in different taxa respond non-uniformly to disturbances inflicted on the environment by human activities (Reid et al. 2000; Cecchi et al. 2008; Ngari et al. 2020). *Glossina austeni* tsetse flies, for example, are highly sensitive to environmental disturbances. Therefore, they are reported in only pristine habitats (Ngari et al. 2020). The high sensitivity of *G. austeni* to habitat degradation very likely accounts for the fly species low abundance in Shimba Hills and limited distribution to only areas close to the wildlife reserve where

anthropogenic activities are extremely sparse or absent. The low abundance of *G. austeni* in Shimba Hills underpinned the low *a*-EIR for the fly species in the present work, even though *G. austeni* had the highest rates of infection and cattle blood-feeding among tsetse flies.

Contrary to expectation, populations of tsetse flies across disparately anthropised landscapes had similar wing fray scores and wing lengths. This perhaps is because the surveyed anthropised sites where stress conditions are expected to select for older and phenotypically larger tsetse flies were not sufficiently distant from the wildlife reserve. A study in eastern Zambia that reported significant difference in the average age of *G. morsitans morsitans* populations collected tsetse flies along a transect of over 20 km stretching from anthropogenically undisturbed Lusandwa to markedly anthropised Chisulo (Mweempwa et al. 2015). A different study in north-eastern Zambia observed significant variations in phenotypic sizes of tsetse flies collected along a transect line of 15 km in Rufunsa, 45 km in Mpika, and 46 km in Lundazi extending from human residential areas to wildlife protected areas (Chilongo et al. 2021). Tsetse flies in Shimba Hills were collected over an area of 5 km from human settlements to the edge of the wildlife reserve. Tsetse flies are probably migrating and mixing freely within this short distance where samplings were done and might explain the homogeneity in age and phenotypic sizes of the vectors observed in Shimba Hills.

My study successfully characterised a wide diversity of trypanosomes in tsetse flies and is the first single epidemiological study to report up to seven species and subspecies of the parasite in Shimba Hills. Extensive spatio-temporal range of tsetse collection in Shimba Hills and the application of sensitive molecular diagnostic tools for infection detection and characterisation allowed a capture of a broad animal trypanosome diversity at the wildlife-livestock interface. The broad diversity of trypanosomes in Shimba Hills portends a complex

epidemiology for the *nagana* cattle disease caused by tsetse-borne trypanosomes in the area. *Trypanosoma congolense* has been described as a major trypanosome parasite of cattle in Shimba Hills (Mbahin et al. 2013; Odongo et al. 2016). In this study, I was able to characterise two (Savannah and Kilifi) strains of *T. congolense*; the Forest strain of *T. congolense* is primarily endemic to *Palpalis* tsetse infested riverine ecologies in West and Central Africa (Isaac et al. 2016), hence the parasite was not detected in Shimba Hills where *Savannah* tsetse are the main trypanosome vectors; moreover, *T. congolesne* Forest is largely absent in East Africa except for sporadic detections made in tsetse flies (Garcia et al. 2018). Additionally, I made an observation of differential clustering with strong bootstrap support for isolates of *T. simiae* (GP361 and GP460) and *T. vivax* (GP464 and GP49/586) on the phylogenetic tree. This is indicative of a likely existence of multiple genotypes for each of the trypanosomes in Shimba Hills. Multiple genotypes for *T. simiae* and *T. vivax* could further complicate *nagana* epidemiology in Shimba Hills thus should be investigated.

Trypanosomes detected in tsetse flies were more diverse close to the wildlife reserve. Among parasites detected in tsetse flies collected close to the reserve were *T. simiae Tsavo* and *T. godfreyi* reported commonly in wildlife and sparsely in livestock. Tsetse flies plausibly had acquired these parasites from wildlife bloodmeals in Shimba Hills. A previous work reported wildlife bloodmeals in tsetse flies in Shimba Hills (Channumsin et al. 2021). However, samplings were done in Buffalo Ridge within the reserve and Zunguluka along the wildlife interface thus it was not possible to have a clear assessment of animal bloodmeal sources of tsetse flies across the wildlife interface or reliably decipher wildlife sources of trypanosomes in the vectors.

The cattle trypanosome infection rate of 13.06% in Shimba Hills and detection of only two *Trypanosoma* species is likely an under-estimation considering that I utilised the buffy coat diagnostic technique unlike a previous epidemiological survey in the area that used sensitive molecular tools to screen cattle bloodmeals for trypanosomes with report of an infection rate of 32.70% and five *Trypanosoma* species (Odongo et al.

2016). After using the PCR-High-Resolution Melting technique was used to screen samples cattle in the Ruma wildlife-livestock interface (Kenya), Kalayou et al. (2021) recorded an infection rate of 27.90% with detection of four *Trypanosoma* species, as against 11.00% when the investigators applied the buffy coat diagnostic technique. Application of sensitive tools in subsequent studies in Shimba Hills will not only allow for accurate and reliable assessment of trypanosome infection rates and species diversity in cattle but could assist with unravelling parasite intraspecific diversity. However, a parallel study is assessing *T. vivax* diversity in the tsetse fly samples analysed in this study with an objective to provide insights into trypanosome genotype diversity and associated clinical conditions in cattle in the area.

The finding of significantly lower anaemia scores in trypanosome-infected than uninfected cattle show clearly that the parasites are a burden on livestock health in Shimba Hills. However, it was not possible to show a clear relationship between trypanosome parasitological rates of cattle infections and tsetse entomological inoculation rates. Similar studies revealed that spatio-temporal scale of data collection could affect apparent relationships between cattle parasitological rates and trypanosome entomological rates (Fall et al. 1999; Bett et al. 2008). In the Fall et al. (1999) study in Senegal, data were collected over a four-year period and a significant association between cattle parasitological rates and trypanosome entomological rates was observed only after aggregating monthly datasets collected over the entire study period and lagging entomological data by three months. Bett et al. (2008) obtained statistically significant relationship for both variables in Nkuruman in southwest Kenya after pooling monthly datasets collected over a period of seventeen months. In Shimba Hills, logistical challenges constrained parasitological and entomological data collection on a monthly basis and over a longer period; entomological data were collected bimonthly for ten months and parasitological data collected once in the long

rain season and once in the dry season. Otherwise, it may have been possible to detect significant relationships between cattle parasitological rates and trypanosome entomological rates in Shimba Hills.

2.5.1 Conclusion

Cattle in Shimba Hills are exposed to a high risk of trypanosome infections from female tsetse flies and *G. pallidipes* in grazing fields close to the wildlife reserve. The present study provides no evidence that landscape anthropisation has an influence on trypanosome risk in the area but show that tsetse flies exist at high infestation levels close to the wildlife reserve unequivocally on account of favourable living conditions and with the likelihood that the vectors are feeding on wild fauna species in these locations and thus potentially exposing cattle to infections from wildlife reservoirs of trypanosomes. I recommend tsetse control programmes in the Shimba Hills wildlife-livestock interface to target operations to trypanosome hotspots close to the National Reserve. Meanwhile, findings from the present study highlight the need for further investigations to screen wildlife for trypanosomes or tsetse flies for animal bloodmeal sources in cattle farming communities in Shimba Hills. This could improve understanding of trypanosome epidemiology in the area. Further studies spanning several years will be important to better understand the relationship between cattle parasitological rates and trypanosome entomological rates in Shimba Hills.

References

1. Alto BW, Reiskind MH, Lounibos LP. Size alters susceptibility of vectors to dengue virus infection and dissemination. The American Journal of Tropical Medicine and Hygiene. 2008 Nov;79(5):688.

2. Alto BW, Reiskind MH, Lounibos LP. Size alters susceptibility of vectors to dengue virus infection and dissemination. The American Journal of Tropical Medicine and Hygiene. 2008 Nov;79(5):688.

3. Auty H, Morrison LJ, Torr SJ, Lord J. Transmission dynamics of Rhodesian sleeping sickness at the interface of wildlife and livestock areas. Trends in Parasitology. 2016 Aug 1;32(8):608-21.

4. Auty HK, Picozzi K, Malele I, Torr SJ, Cleaveland S, Welburn S. Using molecular data for epidemiological inference: assessing the prevalence of Trypanosoma brucei rhodesiense in tsetse in Serengeti, Tanzania. PLoS Neglected Tropical Diseases. 2012 Jan 31;6(1):e1501.

5. Bett B, Irungu P, Nyamwaro SO, Murilla G, Kitala P, Gathuma J, Randolph TF, McDermott J. Estimation of tsetse challenge and its relationship with trypanosomosis incidence in cattle kept under pastoral production systems in Kenya. Veterinary Parasitology. 2008 Aug 17;155(3-4):287-98.

6. Boakye DA, Tang J, Truc P, Merriweather A, Unnasch TR. Identification of bloodmeals in haematophagous Diptera by cytochrome B heteroduplex analysis. Medical and Veterinary Entomology. 1999 Sep;13(3):282-7.

7. Brooks ME, Kristensen K, Van Benthem KJ, Magnusson A, Berg CW, Nielsen A, Skaug HJ, Machler M, Bolker BM. glmmTMB balances speed and flexibility

among packages for zero-inflated generalized linear mixed modeling. The R Journal. 2017;9(2):378-400.

8. Cator LJ, Johnson LR, Mordecai EA, El Moustaid F, Smallwood TR, LaDeau SL, Johansson MA, Hudson PJ, Boots M, Thomas MB, Power AG. The role of vector trait variation in vector-borne disease dynamics. Frontiers in Ecology and Evolution. 2020 Jul 10;8:189.

9. Cecchi G, Mattioli RC, Slingenbergh J, De La Rocque S. Land cover and tsetse fly distributions in sub-Saharan Africa. Medical and Veterinary Entomology. 2008 Dec;22(4):364-73.

10. Challier A, Laveissiere C. A new trap for catching Glossina: description and field trials. Cahiers ORSTOM, Serie Entomologie Medicale et Parasitologie. 1973;11(4):251-62.

11. Channumsin M, Ciosi M, Masiga D, Auty H, Turner CM, Kilbride E, Mable BK. Blood meal analysis of tsetse flies (*Glossina pallidipes*: Glossinidae) reveals higher host fidelity on wild compared with domestic hosts. Wellcome Open Research. 2021;6.

12. Channumsin M, Ciosi M, Masiga D, Turner CM, Mable BK. Sodalis glossinidius presence in wild tsetse is only associated with presence of trypanosomes in complex interactions with other tsetse-specific factors. BMC Microbiology. 2018 Nov;18(1):69-82.

13. Chilongo K, Manyangadze T, Mukaratirwa S. Effects of human settlements and spatial distribution of wing vein length, wing fray categories and hunger stages in Glossina morsitans morsitans (Diptera: Glossinidae) and *Glossina*

pallidipes (Diptera: Glossinidae) in areas devoid of cattle in North-Eastern Zambia. Journal of Medical Entomology. 2021 Mar;58(2):891-9.

14. Dinno A. dunn. test: Dunn's test of multiple comparisons using rank sums. R Package Version. 2017;1(5).

15. Dunn PK, Smyth GK. Models for Proportions: Binomial GLMs. InGeneralized Linear Models With Examples in R 2018 (pp. 333-369). Springer, New York, NY.

16. Fall A, Diack A, Diaite A, Seye M, d'Ieteren GD. Tsetse challenge, trypanosome and helminth infection in relation to productivity of village Ndama cattle in Senegal. Veterinary Parasitology. 1999 Mar 1;81(3):235-47.

17. Garcia HA, Rodrigues CM, Rodrigues AC, Pereira DL, Pereira CL, Camargo EP, Hamilton PB, Teixeira MM. Remarkable richness of trypanosomes in tsetse flies (Glossina morsitans morsitans and *Glossina pallidipes*) from the Gorongosa National Park and Niassa National Reserve of Mozambique revealed by fluorescent fragment length barcoding (FFLB). Infection, Genetics and Evolution. 2018 Sep 1;63:370-9

18. Gashururu RS, Githigia SM, Gasana MN, Habimana R, Maingi N, Cecchi G, Paone M, Zhao W, Masiga DK, Gashumba J. An update on the distribution of Glossina (tsetse flies) at the wildlife-human-livestock interface of Akagera National Park, Rwanda. Parasites & Vectors. 2021 Dec;14(1):1-3.

19. Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. InNucleic Acids Symp. Ser. 1999 (Vol. 41, pp. 95-98).

20. Hargrove J, English S, Torr SJ, Lord J, Haines LR, Van Schalkwyk C, Patterson J, Vale G. Wing length and host location in tsetse (Glossina spp.): implications for control using stationary baits. Parasites & Vectors. 2019 Dec;12(1):1-3.

21. Hasegawa M, Kishino H, Yano T.A.. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol., 22 (2) (1985 Oct 1), pp. 160-174

22. Hothorn T, Bretz F, Westfall P, Heiberger RM, Schuetzenmeister A, Scheibe S, Hothorn MT. Package 'multcomp'. Simultaneous inference in general parametric models. Project for Statistical Computing, Vienna, Austria. 2016 Jul 14.

23. Isaac C, Ciosi M, Hamilton A, Scullion KM, Dede P, Igbinosa IB, Nmorsi OP, Masiga D, Turner CM. Molecular identification of different trypanosome species and subspecies in tsetse flies of northern Nigeria. Parasites & Vectors. 2016 Dec;9(1):1-7.

24. Ivanova NV, Zemlak TS, Hanner RH, Hebert PD. Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes. 2007 Jul;7(4):544-8.

25. Jackson CH. An artificially isolated generation of tsetse flies (Diptera). Bulletin of Entomological Research. 1946 Sep;37(2):291-9.

26. Kalayou S, Okal MN, Odhiambo PO, Mathenge K, Gamba DO, Kariuki E, McOdimba F, Masiga D. Prevalence of trypanosome species in cattle near Ruma National Park, Lambwe valley, Kenya: An update from the historical focus for African trypanosomosis. Frontiers in Veterinary Science. 2021 Nov 2:1273.

27. Kimenyi NN, Kimenyi KM, Amugune NO, Getahun MN. Genetic connectivity of trypanosomes between tsetse-infested and tsetse-free areas of Kenya. Parasitology. 2021:1-3.

28. Knickerbocker TJ, Waithaka J. People and elephants in the Shimba Hills, Kenya. CONSERVATION BIOLOGY SERIES-CAMBRIDGE-. 2005 Aug 25;9:224.

29. S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol., 35 (6) (2018 Jun 1), pp. 1547-1549

30. Leak SG, Collardelle C, Coulibaly L, Dumont P, Feron A, Hecker P, d'Ieteren GD, Jeannin P, Minengu M, Minja S, Mulatu W. Relationships between tsetse challenge and trypanosome prevalence in trypanotolerant and susceptible cattle. International Journal of Tropical Insect Science. 1990 Jun;11(3):293-9.

31. Lefort V, Longueville JE, Gascuel O. SMS: smart model selection in PhyML. Mol. Biol. Evol., 34 (9) (2017 Sep 1), pp. 2422-2424

32. Lemecha H, Mulatu W, Hussein I, Rege E, Tekle T, Abdicho S, Ayalew W. Response of four indigenous cattle breeds to natural tsetse and trypanosomosis challenge in the Ghibe valley of Ethiopia. Veterinary Parasitology. 2006 Oct 10;141(1-2):165-76.

33. Lord JS, Torr SJ, Auty HK, Brock PM, Byamungu M, Hargrove JW, Morrison LJ, Mramba F, Vale GA, Stanton MC. Geostatistical models using remotely-sensed data predict savanna tsetse decline across the interface between protected and unprotected areas in Serengeti, Tanzania. Journal of Applied Ecology. 2018 Jul;55(4):1997-2007.

34. Malele I, Nyingilili H, Msangi A. Factors defining the distribution limit of tsetse infestation and the implication for livestock sector in Tanzania. African Journal of Agricultural Research. 2011 May 31;6(10):2341-7.

35. Mbahin N, Affognon H, Andoke J, Tiberius M, Mbuvi D, Otieno J, Muasa P, Saini RK. Parasitological prevalence of bovine trypanosomosis in Kubo division of Kwale county of coastal: baseline survey. Am J Anim Vet Sci. 2013 Jan 1;8(1):28-36.

36. Muriithi BW, Gathogo NG, Diiro GM, Kidoido MM, Okal MN, Masiga DK. Farmer perceptions and willingness to pay for novel livestock pest control technologies: A case of tsetse repellent collar in Kwale County in Kenya. PLoS Neglected Tropical Diseases. 2021 Aug 17;15(8):e0009663.

37. Murray M, Murray PK, McIntyre WI. An improved parasitological technique for the diagnosis of African trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1977 Jan 1;71(4):325-6.

38. Mweempwa C, Marcotty T, De Pus C, Penzhorn BL, Dicko AH, Bouyer J, De Deken R. Impact of habitat fragmentation on tsetse populations and trypanosomosis risk in Eastern Zambia. Parasites & Vectors. 2015 Dec;8(1):1-0.

39. Nasci RS. The size of emerging and host-seeking Aedes aegypti and the relation of size to blood-feeding success in the field. J Am Mosq Control Assoc. 1986 Mar 1;2(1):61-2.

40. Ngari NN, Gamba DO, Olet PA, Zhao W, Paone M, Cecchi G. Developing a national atlas to support the progressive control of tsetse-transmitted animal trypanosomosis in Kenya. Parasites & Vectors. 2020 Dec;13:1-2.

41. Njiru ZK, Constantine CC, Guya S, Crowther J, Kiragu JM, Thompson RC, Dávila AM. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitology Research. 2005 Feb;95(3):186-92.

42. Odongo S, Delespaux V, Ngotho M, Bekkele SM, Magez S. Comparative evaluation of the nested ITS PCR against the 18S PCR-RFLP in a survey of bovine trypanosomiasis in Kwale County, Kenya. Journal of Veterinary Diagnostic Investigation. 2016 Sep;28(5):589-94.

43. Omondi D, Masiga DK, Ajamma YU, Fielding BC, Njoroge L, Villinger J. Unraveling host-vector-arbovirus interactions by two-gene high resolution melting mosquito bloodmeal analysis in a Kenyan wildlife-livestock interface. PloS One. 2015 Jul 31;10(7):e0134375.

44. Ouma JO, Marquez JG, Krafsur ES. Macrogeographic population structure of the tsetse fly, *Glossina pallidipes* (Diptera: Glossinidae). Bulletin of Entomological Research. 2005 Oct;95(5):437-47.

45. Pollock JN, editor. Training manual for tsetse control personnel. Food and Agriculture Organization of the United Nations; 1982.

46. Reid RS, Kruska RL, Deichmann U, Thornton PK, Leak SG. Human population growth and the extinction of the tsetse fly. Agriculture, Ecosystems & Environment. 2000 Feb 1;77(3):227-36.

47. Saini RK, Orindi BO, Mbahin N, Andoke JA, Muasa PN, Mbuvi DM, Muya CM, Pickett JA, Borgemeister CW. Protecting cows in small holder farms in East Africa from tsetse flies by mimicking the odor profile of a non-host bovid. PLoS Neglected Tropical Diseases. 2017 Oct 17;11(10):e0005977.

48. Salekwa LP, Nnko HJ, Ngonyoka A, Estes AB, Agaba M, Gwakisa PS. Relative abundance of tsetse fly species and their infection rates in Simanjiro, Northern Tanzania. Livest Res Rural Dev. 2014 Dec 1;26:213.

49. Shaw AP, Cecchi G, Wint GR, Mattioli RC, Robinson TP. Mapping the economic benefits to livestock keepers from intervening against bovine trypanosomosis in Eastern Africa. Preventive Veterinary Medicine. 2014 Feb 1;113(2):197-210.

50. Vale GA, Hargrove JW, Solano P, Courtin F, Rayaisse JB, Lehane MJ, Esterhuizen J, Tirados I, Torr SJ. Explaining the host-finding behavior of blood-sucking insects: computerized simulation of the effects of habitat geometry on tsetse fly movement. PLoS Neglected Tropical Diseases. 2014 Jun 12;8(6):e2901.

51. Wamwiri FN, Alam U, Thande PC, Aksoy E, Ngure RM, Aksoy S, Ouma JO, Murilla GA. Wolbachia, Sodalis and trypanosome co-infections in natural populations of *Glossina austeni* and *Glossina pallidipes*. Parasites & Vectors. 2013 Dec;6(1):1-1.

CHAPTER 3

Tsetse Bloodmeal Analyses Incriminate the Common Warthog *Phacochoerus africanus* as an Important Cryptic Host of Animal Trypanosomes in Smallholder Cattle Farming Communities in Shimba Hills, Kenya

---

This chapter has been published as:

Faith I. Ebhodaghe, Michael N. Okal**,** Shewit Kalayou**,** Armanda D. S. Bastos and Daniel K. Masig**a** (2021). Tsetse Bloodmeal Analyses Incriminate the Common Warthog *Phacochoerus africanus* as an Important Cryptic Host of Animal Trypanosomes in Smallholder Cattle Farming Communities in Shimba Hills, Kenya. *Pathogens* ***10***: 1501.

https://doi.org/10.3390/pathogens10111501

3.1 Abstract

Trypanosomes are endemic and retard cattle health in Shimba Hills, Kenya. Wildlife in the area act as reservoirs of the parasites. However, wild animal species that harbor and expose cattle to tsetse-borne trypanosomes are not well known in Shimba Hills. Using xeno-monitoring surveillance to investigate wild animal reservoirs and sources of trypanosomes in Shimba Hills, I screened 696 trypanosome-infected and uninfected tsetse flies for vertebrate DNA using multiple-gene PCR-High Resolution Melting analysis and amplicon sequencing. Results revealed that tsetse flies fed on 13 mammalian species, preferentially *Phacochoerus africanus* (warthogs) (17.39%, 95% CI: 14.56–20.21) and *Bos taurus* (cattle) (11.35%, 95% CI: 8.99–13.71). Some tsetse flies showed positive cases of bloodmeals from multiple hosts (3.45%, 95% CI: 2.09–4.81), including warthog and cattle (0.57%, 95% CI: 0.01–1.14). Importantly, tsetse flies that took bloodmeals from warthog had significant risk of infections with *Trypanosoma vivax* (5.79%, 95% CI: 1.57–10.00), *T. congolense* (7.44%, 95% CI: 2.70–12.18), and *T. brucei sl* (2.48%, 95% CI: −0.33–5.29). These findings implicate warthogs as important reservoirs of tsetse-borne trypanosomes affecting cattle in Shimba Hills and provide valuable epidemiological insights to underpin the parasites targeted management in *Nagana* vector control programs in the area.

Keywords: Trypanosomiasis; nagana; epidemiology; pathogen; spill-over; reservoir; asymptomatic host; wildlife-livestock interface; Shimba Hills; Kenya

3.2 Introduction

Wildlife are reservoirs of a plethora of pathogens including parasites that are transmitted from wildlife to humans and livestock through habitat sharing or dissemination by haematophagous arthropod-vectors [1]. In sub-Saharan Africa, the tsetse-transmitted trypanosomes responsible for *Nagana* cattle disease and human sleeping sickness are examples of arthropod-borne parasites harbored by asymptomatic wildlife hosts [2–5]. However, most epidemiological studies on trypanosomes in Africa have focused on the tsetse vectors, human and livestock hosts and only rarely on wildlife reservoirs. In the Serengeti National Park (NP) in Tanzania, Kaare et al. [6] identified trypanosomes in different wild animal species, including warthogs which were the only wildlife shown to harbor *Trypanosoma brucei rhodesiense* the causative agent of acute human sleeping sickness in East Africa. Furthermore, warthogs harbored the widest diversity of animal trypanosomes and were thought to be the source of trypanosomes detected in cattle in the area. These results support suggestions that warthogs are among the wildlife species that contribute to maintaining endemicity and transmission of trypanosome infections in the Luangwa Valley, Zambia [2].

The findings in the Serengeti NP [6] and Luangwa Valley [2] and other wildlife areas in the continent [7–10] suggest that warthogs may be contributing to trypanosome transmission at the wildlife-livestock interface of the Shimba Hills National Reserve (NR) in Kenya following the wildlife species abundance in the area. Previous epidemiological studies in other wildlife areas in Kenya have identified African buffalo *Syncerus caffer*, giraffe *Giraffa camelopardalis*, African savannah elephants *Loxodonta africana*, and hippopotamus *Hippopotamus amphibious* as the dominant reservoirs of trypanosomes [8,11]. Muturi et al. [8] and Makhulu et al. [11] adopted an alternative strategy to wildlife examination based on xeno-monitoring to

characterize animal reservoirs of trypanosomes. Xeno-monitoring, a strategy which explores knowledge of the blood-feeding behaviour of tsetse flies to track animal reservoirs and sources of trypanosomes, provides otherwise inaccessible data on available fauna in and outside local sylvatic ecologies and is particularly convenient because of the difficulty in sampling wildlife directly. Xeno-monitoring also allows investigators to profile wildlife hosts of trypanosomes in real-time in high throughput analysis and over extensive landscapes including hard-to-reach locations in areas where capturing wildlife is both difficult and risky.

As part of a prior epidemiological survey [12], different animal species were described as providing bloodmeals for tsetse flies in Shimba Hills. But the relative contributions of these animals to trypanosome infections in cattle populations in smallholder farming systems in the wildlife-livestock interface remains poorly understood. Moreover, the epidemiological survey by Channumsin et al. [12] was restricted to just two locations (Buffalo Ridge and Zunguluka) and was conducted over a brief sample collection period (about four weeks) thus limiting full understanding of the range of animal bloodmeal hosts of tsetse flies in the area. A clear understanding of wildlife reservoirs of trypanosomes in Shimba Hills [13] will help identify areas where parasites spill-over from wildlife to livestock is highest and where cattle are at greatest risk to trypanosome infections. This would expedite a rational design and efficient implementation of targeted interventions against the tsetse-vectors, thereby alleviating *Nagana*'s adverse effects on cattle health and production and smallholder farmer livelihoods in the area.

In this study, I investigated bloodmeal sources of tsetse flies in Shimba Hills. To visualize the feeding behaviours of tsetse flies, I designed a bipartite interaction network used in epidemiological studies [14,15] to illustrate vector-host relationships and an *UpSet* plot to show frequencies of tsetse bloodmeals on multiple hosts. As

tsetse flies have an exclusively haematophagous diet and are exposed to trypanosomes only by feeding on infected animal hosts [3], knowledge of animal species from which the infected vectors obtain nourishment could provide insights into probable sources of infections. I therefore characterised vertebrate DNA in tsetse flies in an attempt to track the animal sources of trypanosomes in samples of the vectors from Shimba Hills using molecular tools to screen the vectors for bloodmeal hosts.

3.3.0 Materials and Methods

3.3.1 Ethical Consent

The study received ethical consent from Kenya's National Commission for Science, Technology, and Innovation (License No.: NACOSTI/P/20/7344) and the Pwani University Ethics Review (approval number ERC/EXT/002/2020). The study was conducted according to guidelines stipulated by the International Centre of Insect Physiology and Ecology *icipe* Kenya. Collections of tsetse flies were done in collaboration with local communities, the Kenya Tsetse and Trypanosomiasis Eradication Council (KENTTEC), and the Kenya Wildlife Service (KWS).

3.3.2 Study Area

The Shimba Hills NR is located in Kwale County in southeast Kenya (Figure 3.1). A major wildlife area in East Africa, the Reserve is just ~218 km^2 yet hosts Kenya's highest density of elephants (*Loxodonta africana*) [41]. Further, it is home to a wide biodiversity and accommodates important vertebrates including threatened and endangered animal species prominently the Roosevelt's sable antelope *Hippotragus niger* (Kenya Wildlife Service KWS 2021, **www.kws.go.ke**, assessed 03rd November 2021). Among animal species domiciled in the area are warthogs (*Phacochoerus africanus*), bushbuck (*Tragelaphus scriptus*), and buffalo (*Syncerus scaffer*). The Shimba Hills area is warm and moist, with average annual temperature and rainfall of ~24 °C and 1150 mm, respectively. The area experiences bimodal rainfall patterns characterise by long rains from March to May, sometimes extending to July, and short rains from October to December. Main economic activities in communities residing at the edge of the reserve are crop and livestock (mainly cattle) production. Vegetation is green year-round

hence encouraging intensive cropping activities and discouraging seasonal livestock migration.

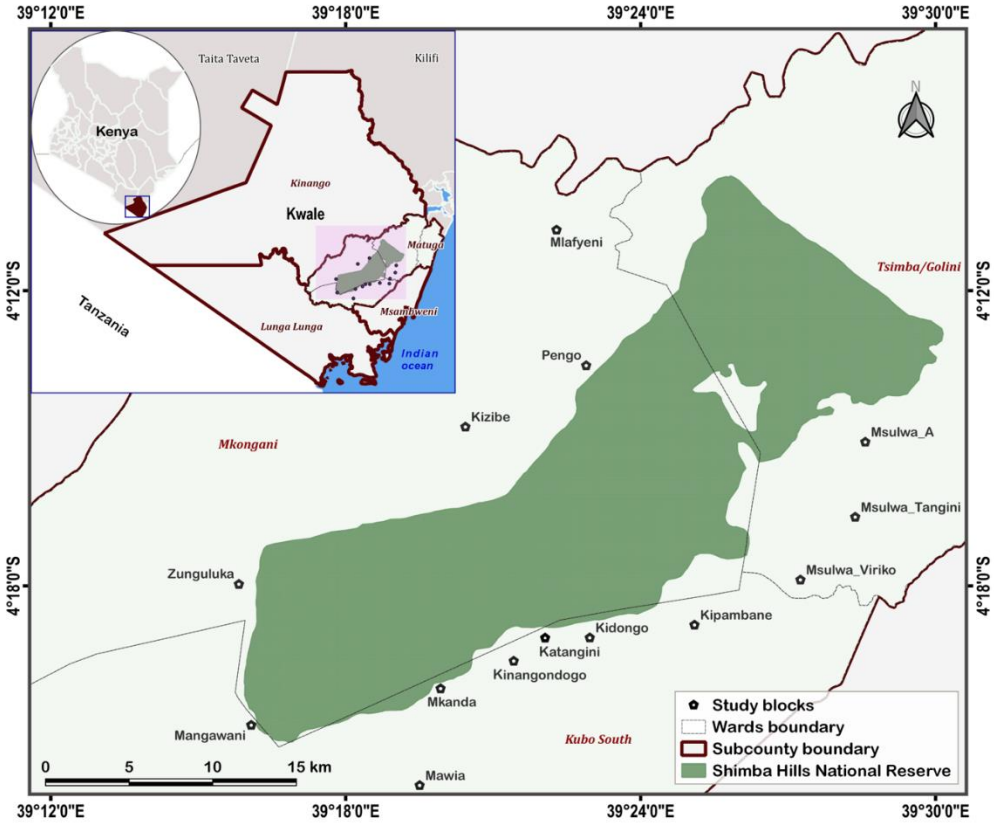


Figure 3. 1. Map of study locations in Shimba Hills in Kwale county, Kenya.

3.3.3 Tsetse Fly Collection and Characterisation

Samples of tsetse flies were collected over a 10-month period (November 2018 to September 2019) in the Shimba Hills wildlife-livestock interface. Biconical traps for tsetse collection [42] were baited with cow urine and acetone at respective release rates of 1000 mg/hr and 500 mg/hr and deployed at a density of 1 trap per km^2 within 5 km of the border of the reserve, over an area stretching ~230 km^2. Collections of tsetse flies were done bi-monthly throughout the sampling period,

across different vegetation landscapes, and in locations at varying proximities to the Shimba Hills NR. Tsetse flies were identified using established taxonomic keys [43], sorted according to sex and species, and subsequently stored in 95% ethanol. Each fly sample was later assessed for age based on the wing fray scoring technique developed by Jackson [44].

3.3.4 Identification of Vertebrate Bloodmeal Sources in Tsetse Flies

Tsetse flies were sterilized in alcohol, air-dried, and crushed using a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK, USA). This was followed by DNA extraction from crushed fly samples using Genomic DNA extraction kits (Bioloine, London, UK) according to the manufacturer's instructions for animal tissues. Two vertebrate mitochondrial genes were then targeted in separate Polymerase Chain Reactions (PCRs): the first, the *16S* ribosomal RNA gene was amplified with Vert *16S* For: 5′-GAGAAGACCCTRTGGARCTT-3′ and Vert *16S* Rev: 5′-CGCTGTTATCCCTAGGGTA-3′ primers which target a ~200 bp region [45] and the second, the cytochrome *b* gene was amplified with the Cyt *b* For: 5′-CCCCTCAGAATGATATTTGTCCTCA-3′ and Cyt *b* Rev: 5′-CATCCAACATCTCAGCATGATGAAA-3′ primers that target a ~383 bp region [46]. For each PCR-reaction, I used 0.5 μM of each Forward and Reverse primer (Macrogen, Europe, Amsterdam, The Netherlands) in a 10 μL reaction-volume comprising of 1 μL template DNA and 2 μL of pre-formulated 5X HOT FIREPol® EvaGreen® HRM Mix, (Solis BioDyne, Tartu, Estonia). DNA amplifications were carried out for *16S* ribosomal *RNA* and cytochrome *b* in a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) and QuantStudio 3 Real-Time PCR System thermal cycler (MicroAmp®; Applied Biosystems, Inc., Foster city, CA, USA), respectively with the following thermal cycling conditions: initial denaturation for 15min at 95 °C, followed by 40 cycles of denaturation at 95 °C

for 40 s, annealing at 56 °C for 20 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. High-Resolution Melting analysis of amplicons followed immediately with gradual melting from 75 °C to 95 °C. Non-template negative controls were included in the experiments to ascertain the success of each run. DNA extracted from cattle, sheep, donkey, giraffe, bushbuck, baboon, impala, hippopotamus, and human were used as positive controls, and tsetse bloodmeal sources were identified by inspecting HRM profile alignments with those of positive controls. Tsetse flies that fed on multiple hosts had HRM curves aligned with more than one positive controls. Melting profiles were analysed in the software Rotor-Gene Q v2.1 and QuantStudioTM Design & Analysis v1.5.1 depending on the machine used for PCR-HRM analysis. Where a profile was different from those of positive control and could not be clearly identified, samples were subjected to *CO1* gene amplification [47] and amplicon sequencing. PCR-reactions targeting a ~750 bp region of the *CO1* gene were carried out in a 15 µL reaction-volume containing 0.5 µM of each Forward and Reverse primer (Macrogen, Europe) (VF1d For: TCTCAACCAACCACAARGAYATYGG; VR1d Rev: TAGACTTCTGGGTGGCCRAARAAYCA) [47], 2 µL template DNA, 3 µL of 5X HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia) with the following cycling conditions: initial denaturation for 15 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 57 °C for 30 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 7 min. DNA amplification was ascertained by electrophoresis of PCR-products for 30 min in a 1.5% agarose-gel stained with 5 µg/mL ethidium bromide at 120 V. Unincorporated dNTPs and PCR primers were removed from amplicons using Exo-SAP (USB Corporation, Cleveland, OH, USA). Purified amplicons were then submitted for unidirectional Sanger sequencing at Macrogen in Europe.

3.3.5 Molecular Identification of Trypanosomes in Tsetse Flies

Detection of trypanosomes was done using the same crushed homogenates used for bloodmeal analysis. Amplification of trypanosome DNA was performed in a 10 µL reaction-volume comprising of 1 µL DNA template, 5 µL DreamTaq Master Mix (2X) (Thermo Scientific, UK), and 0.5 µL at 10 µM of each Forward and Reverse ITS-1 primers (CF: CCGGAAGTTCACCGATATTG, BR: TTGCTGCGTTCTTCAACGAA) [48]. Cycling conditions for DNA amplification were initial denaturation for 1 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 20 s, and extension at 72 °C, and a final extension at 72 °C for 7 min. PCR-products were visualized following 1.5% agarose-gel electrophoresis against a molecular weight marker (Gene-Ruler 100 bp DNA ladder, Thermo Scientific, Lithuania) and ethidium bromide staining (5 µg/mL). Where trypanosome infections were present, the parasite species were characterise by the following unique band sizes: *T. vivax* ~250 bp, *T. godfreyi* ~300 bp, *T. simiae* Tsavo ~370 bp, *T. simiae* ~400 bp, *Trypanozoon* (*T. brucei sp.*) ~480 bp, *T. congolense* Kilifi ~620 bp, and *T. congolense* Savannah/Forest ~700 bp [48]. To confirm trypanosome identity, amplicons were cleaned using Exo-SAP (USB Corporation, Cleveland OH) to remove unincorporated dNTPs and PCR primers and thereafter sent for Sanger sequencing of the ITS1gene [48].

3.3.6 Data Analyses

Returned vertebrate DNA sequences were inspected for quality based on their chromatograph profiles and edited in BioEdit *v7.2.5* [49]. Edited sequences were subjected to BLAST analysis for comparison to nucleotide sequences in the NCBI GeneBank-*nr* database (**https://blast.ncbi.nlm.nih.gov/Blast.cgi**) and a homology cut-off of 98.00% to 100.00% identity was used to infer vertebrate

species. The process of trypanosome DNA identification is reported in a parallel work.

Difference in proportions of bloodmeal-positive tsetse flies were tested for significance using the Binomial Generalized Linear Model [50]. *p*-values were significant if <0.05. Where significant differences were present, *Tukey's Post-Hoc* test was carried out in the '*multcomp' R* package [51] for pairwise comparisons. Next, Binomial Generalized Linear Mixed Model (B-GLMM) analyses with '*trap_ID*' as random factor were implemented in the *GlmmTMB R* package [52,53] to investigate associations between tsetse fly bloodmeals on animal hosts and the vector risk of exposure to trypanosome infections. Furthermore, I designed a bipartite interaction network in the *bipartite R* package [54] to visually depict animal blood-feeding behavior of tsetse fly species in Shimba Hills. Finally, I used an *UpSet* plot to show the number of tsetse flies that fed on particular animal species and the number of tsetse flies containing bloodmeals from one or multiple animal host species.

3.4.0 Results

3.4.1. Animal Bloodmeals in Tsetse Flies

Overall, 50.00% (348/696) (95% CI: 46.28–53.72) of tsetse flies screened for vertebrate DNA harbored animal bloodmeals. The proportion of trapped tsetse flies that had detectable bloodmeals was higher in females [54.80% (251/458) (95% CI: 50.23–59.38)] than males [40.76% (97/238) (95% CI: 34.47–47.04)] [Binomial-Generalized Linear Model (B-GLM]: $p < 0.05$) and in *Glossina pallidipes* [53.42% (281/526) (95% CI: 49.15–57.70)] and *G. austeni* [62.50% (25/40) (95% CI: 46.82–78.18)] than *G. brevipalpis* [32.31% (42/130) (95% CI: 24.16–40.45)] (B-GLM: $p < 0.05$) (Table 3.1). Proportions of bloodmeal-positive tsetse flies were similar between different age groups and collection sites (B-GLM: $p > 0.05$) (Tables 3.1 and 3.2).

Table 3. 1: Percentage of tsetse with bloodmeals in Shimba Hills according to intrinsic traits and collection sites.

	Number of Tsetse Flies Screened	% Feeding Rate	95% CI
Fly sex ‡			
Female	458	54.80 [a]	50.23–59.38
Male	238	40.76 [b]	34.47–47.04
Fly species ‡			
G. austeni	40	62.50 [b]	46.82–78.18
G. brevipalpis	130	32.31 [a]	24.16–40.45
G. pallidipes	526	53.42 [b]	49.15–57.70
Fly age †			
Juvenile	186	51.61 [a]	44.36–58.86
Old	155	55.48 [a]	47.57–63.40
Young	355	46.76 [a]	41.55–51.98
Landscape †			
Cultivated field	144	47.92 [a]	39.66–56.17
Forest	55	50.91 [a]	37.27–64.55
Fruit-Orchard	110	53.64 [a]	44.17–63.10
Grassland	161	50.93 [a]	43.13–58.74
Peridomicilliary	11	54.55 [a]	19.46–89.63
Shrubs	215	48.37 [a]	41.64–55.11
Distance from the SHNR †			
<1000 m	614	50.65 [a]	46.69–54.62
1000 to 1999 m	61	44.26 [a]	31.44–57.09
>2000 m	21	47.62 [a]	24.32–70.91

‡ Significant ($p < 0.05$); † Insignificant ($p > 0.05$); Letters in superscript have been used to indicate presence or absence of significant differences in pairwise comparisons between the numbers of tsetse flies positive for animal bloodmeals. Significantly

different pairs are denoted using different letters while insignificantly different pairs are indicated using same letters.

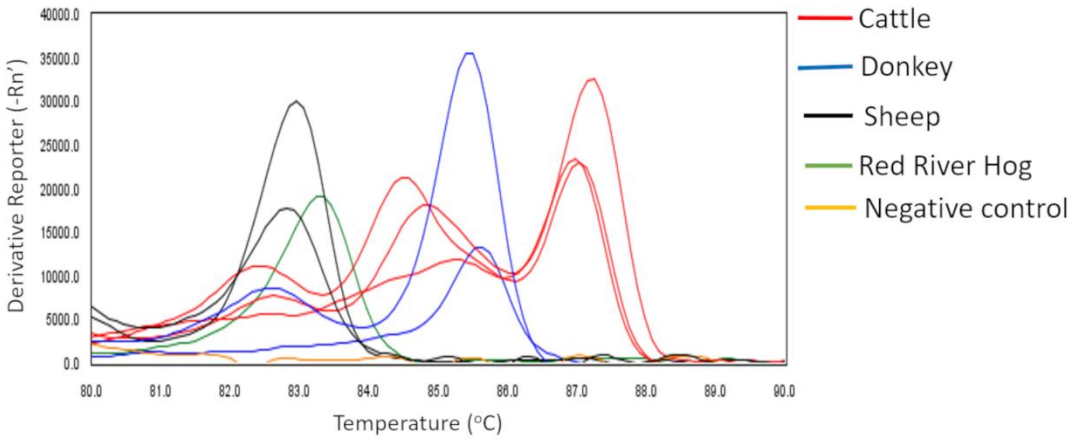
Table 3. 2. Percentage of tsetse with bloodmeals in Shimba Hills according to cluster-location.

	Number of Tsetse Flies Screened	% Feeding Rate	95% CI
Cluster †			
Katangini	25	44.00 [a]	23.09–64.91
Kidongo	12	50.00 [a]	16.82–83.18
Kinangondogo	17	47.06 [a]	20.61–73.51
Kipambane	15	53.33 [a]	24.74–81.93
Kizibe	134	47.76 [a]	39.19–56.33
Mangawani	36	52.78 [a]	35.65–69.91
Mawia	36	44.44 [a]	27.39–61.50
Mkanda	8	12.50 [a]	−17.06–42.06
Mlafyeni	160	55.00 [a]	47.21–62.79
Msulwa A	27	66.67 [a]	47.66–85.67
Msulwa Tangini	2	100.00 [a]	100.00–100.00
Msulwa Viriko	6	33.33 [a]	−20.86–87.53
Pengo	196	46.43 [a]	39.38–53.47
Zunguluka	22	63.64 [a]	41.81–85.47

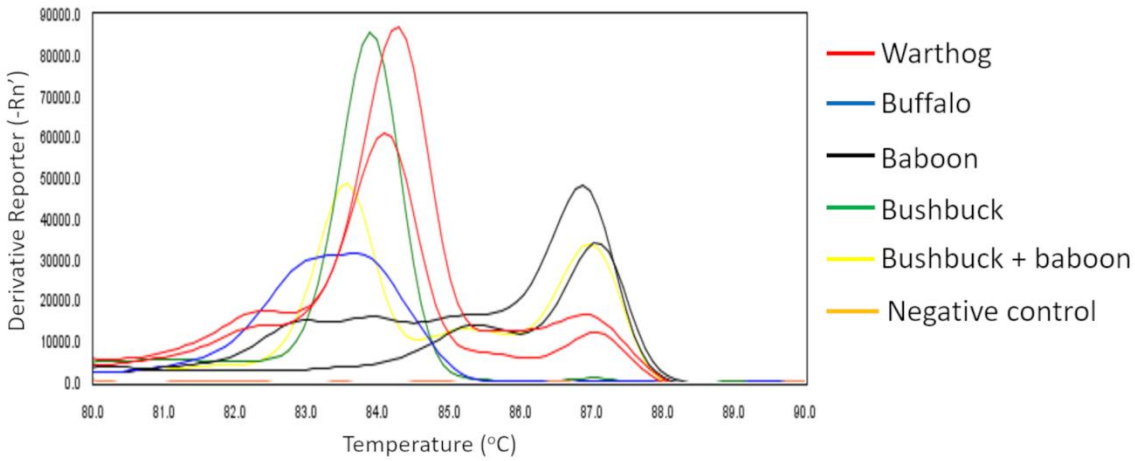
† Insignificant ($p > 0.05$). Letters in superscript are all same and indicate absence of significant differences in the number of tsetse flies positive for animal bloodmeals between pairs of cluster-locations

Tsetse flies were positive for bloodmeals of animals from 6 taxonomic families and 13 species (Figure 3.2, Table 3.3). These included two suid species *Phacochoerus africanus* (warthog) and *Potamochoerus porcus* (red riverhog) and seven bovid species *Bos taurus* (cattle), *Ovis aries* (sheep), *Syncerus caffer* (buffalo), *Capra hircus* (goat), *Tragelaphus scriptus* (bushbuck), *Neotragus moschatus* (suni) and *Aepyceros melampus* (impala). The other animal bloodmeals identified in tsetse flies were from *Papio anubis* (baboon) (Cercopithecidae), *Loxodonta africana* (elephant) (Elephantidae), *Equus asinus* (donkey) (Equidae) and *Homo sapiens* (human) (Hominidae).

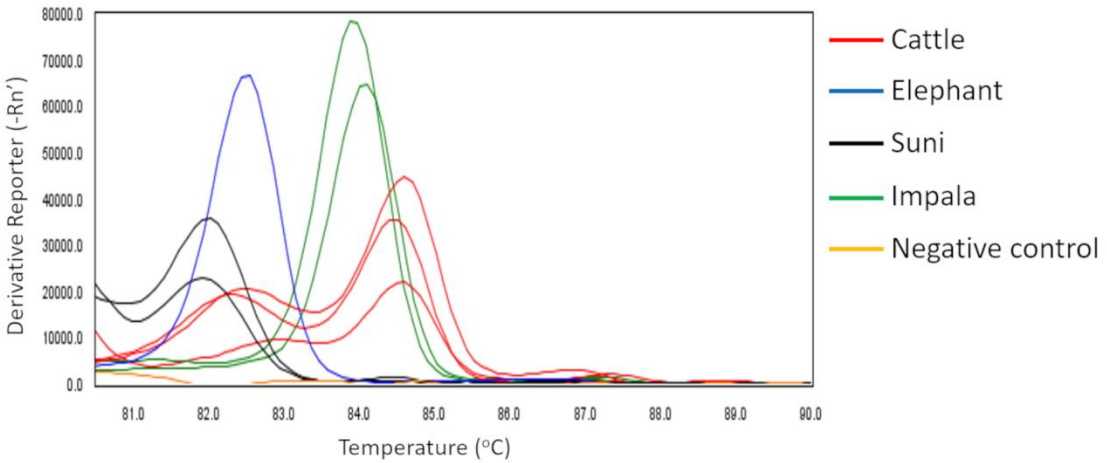
(A)


Derivative Reporter (-Rn')
Temperature (°C)
Cattle
Donkey
Sheep
Red River Hog
Negative control

(B)


Derivative Reporter (-Rn')
Temperature (°C)
Warthog
Buffalo
Baboon
Bushbuck
Bushbuck + baboon
Negative control

(C)


Derivative Reporter (-Rn')
Temperature (°C)
Cattle
Elephant
Suni
Impala
Negative control

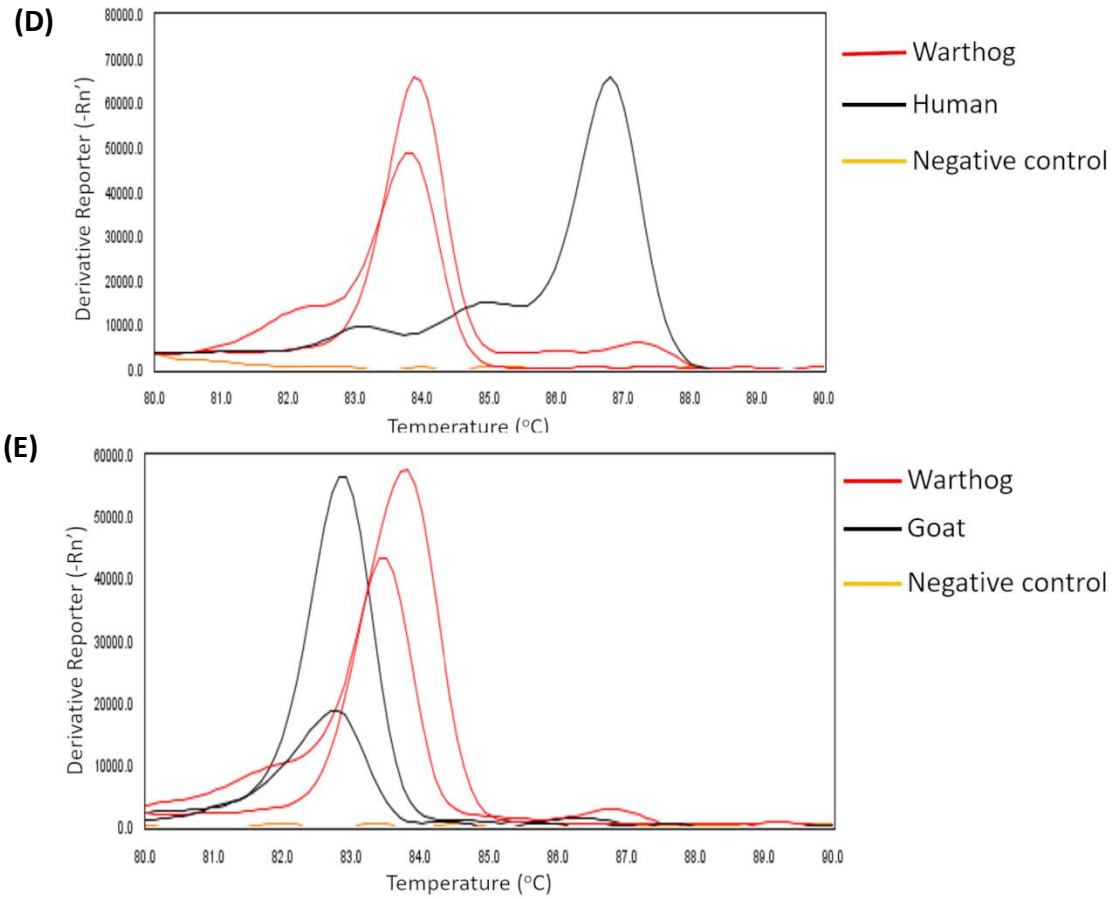


Figure 3. 2 (A to E). High-Resolution Melt profiles of vertebrate bloodmeals in tsetse flies. Profiles are distinguished using different colours to denote different vertebrate bloodmeal hosts. The identity of a vertebrate bloodmeal is shown on the right side of each graph.

Table 3. 3. Identification of nucleic acid sequences of vertebrate bloodmeals detected in tsetse flies from Shimba Hills.

Sample ID (GenBank Accession No.)	Block	Latitude	Longitude	Fly Species	Fly Sex	Sequence Length (bp)	Closest Match on GenBank (Location)	Aimal Host Species	Sequence Identity (%)
GP370 (MZ816958)	Mlafyeni	−4.17453	39.39222	*G. pallidipes*	F	667	DQ409327 (Africa)	*Phacochoerus africanus*	99.55
GP536 (MZ816959)	Mlafyeni	−4.20606	39.40222	*G. pallidipes*	F	595	MN124266 (Kenya)	*Phacochoerus africanus*	100.00
GP411 (MZ816967)	Pengo	−4.20742	39.37234	*G. pallidipes*	M	607	MN124266 (Kenya)	*Potamochoerus porcus*	99.34
GB412 (MZ816968)	Pengo	−4.25076	39.36938	*G. brevipalpis*	F	607	MN124266 (Kenya)	*Potamochoerus porcus*	99.01
GP425 (MZ816969)	Pengo	−4.25076	39.36938	*G. pallidipes*	F	607	MN124266 (Kenya)	*Potamochoerus porcus*	99.38
GB762 (MZ816966)	Pengo	−4.22782	39.37926	*G. brevipalpis*	F	607	MN124266 (Kenya)	*Potamochoerus porcus*	99.34
GP362 (MZ816960)	Mlafyeni	−4.25085	39.36904	*G. pallidipes*	F	652	MN124245 (Kenya)	*Bos taurus*	99.85
GP89 (MZ816962)	Mangawani	−4.3584	39.27996	*G. pallidipes*	F	652	MT576844 (China)	*Bos taurus*	100.00
GP888 (MZ816961)	Mangawani	−4.3584	39.27996	*G. pallidipes*	F	652	MT576844 (China)	*Bos taurus*	100.00
GB349 (MZ816970)	kinangondogo	−4.33653	39.34352	*G. brevipalpis*	F	396	MN124271 (Kenya)	*Loxodonta africana*	98.99
GA379 (MZ816964)	katangini	−4.33402	39.35677	*G. austeni*	F	638	JN645581 (Gabon)	*Neotragus moschatus*	99.84
GB545 (MZ816965)	Kizibe	−4.27812	39.31002	*G. brevipalpis*	F	538	MF437212 (UAE)	*Homo sapiens*	100.00
GP665 (MZ816963)	Pengo	−4.28013	39.35485	*G. pallidipes*	F	662	MN124246 (Kenya)	*Capra hircus*	100.00
GP344 (MZ816971)	Katangini	−4.31766	39.36762	*G. pallidipes*	F	470	MN124256 (Kenya)	*Syncerus caffer*	98.94

Fly sex: M: Male; F: Female.

Table 3.4 shows the number of tsetse fly species that fed on the different animal hosts. This information is visually depicted in the bipartite interaction network in Figure 3.3. The top and bottom bars on the bipartite network respectively represent animal hosts of tsetse flies and tsetse fly species that fed on these hosts. The size of a bar reflects the number of blood-fed tsetse flies (if it is a bottom bar) or the number of the vector that took bloodmeal from a mammalian species (if it is a top bar). The lines are used to show interactions between tsetse flies and animal bloodmeal hosts. The size of a line is proportional to the number of tsetse flies that took bloodmeals from the mammalian host to which it is connected to. The thick lines between *G. pallidipes* and warthog, cattle, baboon and sheep indicate that the tsetse fly species, more than the other fly species, fed intensely on these animals (Table 3.4).

Table 3. 4. Rate of tsetse bloodmeals on animal species according to tsetse fly species.

	G. austeni (*n* = 40) †			*G. brevipalpis* (*n* = 130) †			*G. pallidipes* (*n* = 526) ‡		
	No	**%**	**95% CI**	**No**	**%**	**95% CI**	**No**	**%**	**95% CI**
Baboon	4	10 [a]	0.28–19.72	4	3.08 [a]	0.07–6.09	54	10.27 [c]	7.66–12.87
Buffalo	2	5 [a]	−2.06–12.06	0	0	NA	6	1.14 [a]	0.23–2.05
Bushbuck	0	0	NA	0	0	NA	4	0.76 [a]	0.02–1.51
Cattle	7	17.5 [a]	5.19–21.81	14	10.77 [a]	5.37–16.17	58	11.03 [c]	8.34–13.71
Donkey	1	2.5 [a]	−2.56–7.56	0	0	NA	2	0.38 [a]	−0.15–0.91
Elephant	0	0	NA	3	2.31 [a]	−0.31–4.92	2	0.38 [a]	−0.15–0.91
Goat	0	0	NA	1	0.77 [a]	−0.75–2.29	3	0.57 [a]	−0.08–1.22
Human	3	7.5 [a]	−1.03–16.03	6	4.62 [a]	0.96–8.27	17	3.23 [ab]	1.72–4.75
Impala	0	0	NA	0	0	NA	3	0.57 [a]	−0.08–1.22
Red Riverhog	1	2.5 [a]	−2.56–7.56	4	3.08 [a]	0.07–6.09	8	1.52 [a]	0.47–2.57
Sheep	0	0	NA	5	6.65 [a]	0.50–7.20	35	6.65 [bc]	4.52–8.79
Suni	4	10 [a]	0.28–19.72	0	0	NA	0	0	NA
Warthog	6	15 [a]	3.43–26.57	7	5.38 [a]	1.45–9.32	108	20.53 [d]	17.07–23.10

NA: Not Available. ‡ Significant ($p < 0.05$); † Insignificant ($p > 0.05$). Letters in superscript have been used to indicate presence or absence of significant difference in pairwise comparisons between animal hosts regarding the numbers of tsetse flies that fed on them. Significantly different pairs are denoted using different letters while insignificantly different pairs are indicated using same letters.

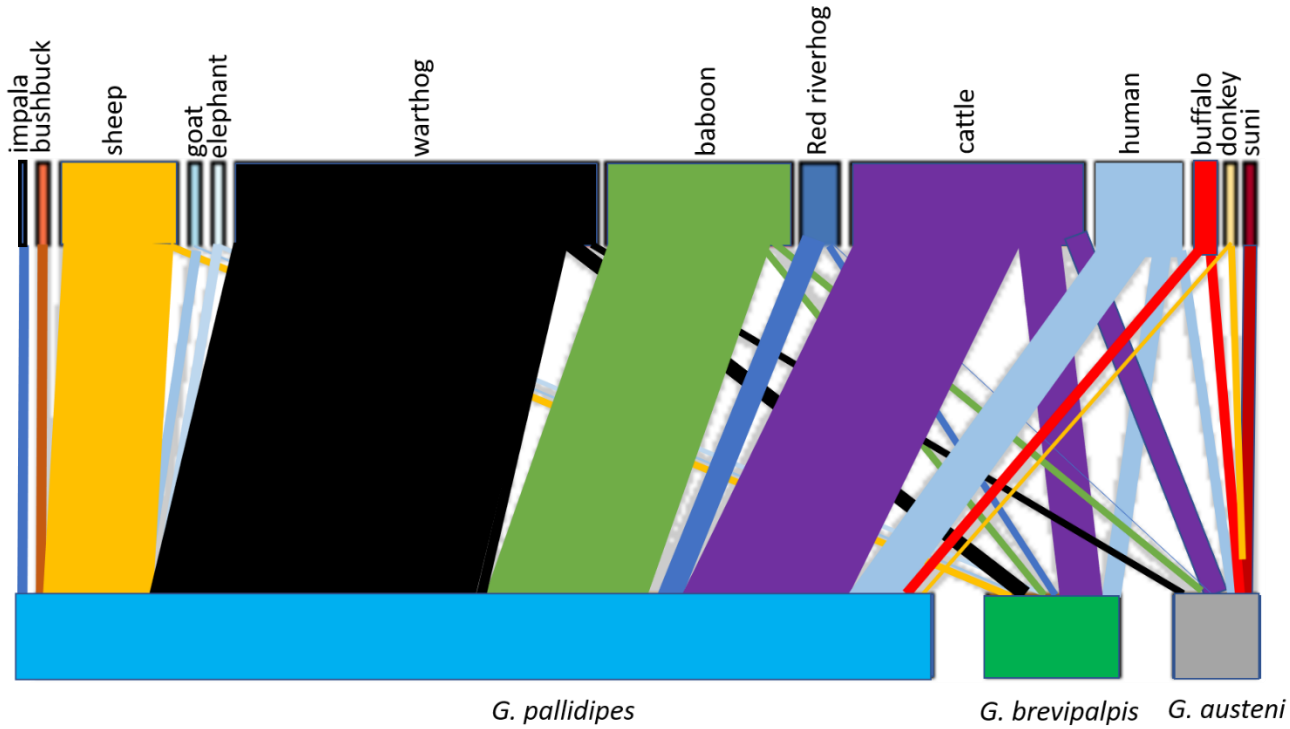


Figure 3. 3. A bipartite network showing interactions between tsetse flies and animal bloodmeal hosts in Shimba Hills, Kenya.

G. pallidipes being the dominant tsetse flies in Shimba Hills made up 75.57% (526/696) of the total fly individuals screened for vertebrate bloodmeals and thus contributed the highest number of tsetse flies with bloodmeals [80.75% (281/348)]. Over half (59.07%, 166/281) the animal bloodmeals in *G. pallidipes* were from warthog and cattle with 38.43% (108/281) of the fly species bloodmeals from warthog. The proportions of warthog bloodmeals in tsetse flies were similar between blocks though highest in Mlafyeni (Supplementary file S1) and significantly different between Mlafyeni and Pengo (B-GLM: $p < 0.05$). Furthermore, tsetse flies in Kinangondogo had the highest rate of cattle bloodmeals but proportions of cattle bloodmeals were insignificantly different between cluster-locations (B-GLM: $p > 0.05$). For all tsetse fly species, B-GLM

analyses with pairwise comparisons revealed significantly higher proportion of tsetse flies positive for warthog bloodmeal than other animal bloodmeal ($p < 0.001$) except cattle bloodmeal ($p > 0.05$) (Table 3.5). Tsetse flies were also more likely to feed on suids ($p < 0.0001$) and bovids ($p < 0.0001$) than other animal hosts (Table 3.5).

Table 3. 5. Rate of tsetse bloodmeals on animal hosts according to animal family and species.

Host Family	No. of tsetse flies	Feeding rate (%)	95% CI
Bovidae	142	20.40 [a]	17.40-23.40
Suidae	134	19.25 [a]	16.32-22.19
Cercopithecidae	62	8.91 [b]	6.79-11.03
Hominidae	26	3.74 [c]	2.32-5.15
Elephantidae	5	0.72 [d]	0.09-1.35
Equidae	3	0.43 [d]	-0.06-0.92
Host Species			
Warthog	121	17.39 [f]	14.56-20.21
Cattle	79	11.35 [ef]	8.99-13.71
Baboon	62	8.91 [de]	6.79-11.03
Sheep	40	5.75 [cd]	4.01-7.48
Human	26	3.74 [bc]	2.32-5.15
Red River Hog	13	1.87 [ab]	0.86-2.88
Buffalo	8	1.15 [ab]	0.36-1.94
Elephant	5	0.72 [a]	0.09-1.35
Bushbuck	4	0.57 [a]	0.01-1.14
Goat	4	0.57 [a]	0.01-1.14
Suni	4	0.57 [a]	0.01-1.14
Donkey	3	0.43 [a]	-0.06-0.92
Impala	3	0.43 [a]	-0.06-0.92

Letters in superscript have been used to indicate presence or absence of significant difference in pairwise comparisons between animal hosts in the numbers of tsetse flies that fed on them. Significantly different pairs are denoted using different letters while insignificantly different pairs are indicated using same letters.

The *UpSet* plot in Figure 3.4 presents the frequency of tsetse bloodmeals on single and double animal species. Warthogs 16.38% (114/696) (95% CI: 13.62–19.14) and cattle 10.63% (74/696) (95% CI: 8.34–12.93) bloodmeals were the most frequently detected in tsetse flies that took bloodmeals from single host species, and baboons plus sheep 1.15% (8/696) (95% CI: 0.36–1.94) and warthogs plus cattle 0.57% (4/696) (95% CI: 0.01–1.14) in the vectors that fed on multiple host species.

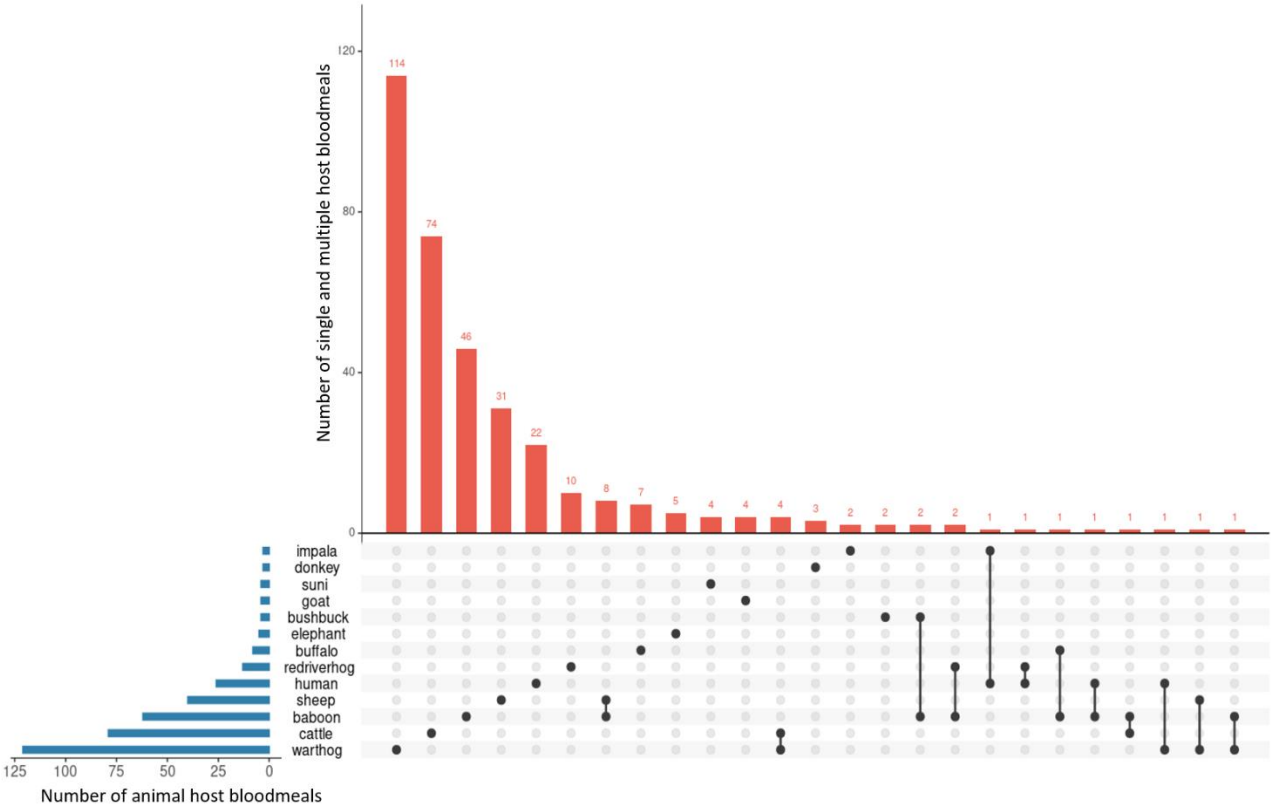


Figure 3. 4. UpSet plot showing the frequency of tsetse bloodmeals on single and double animal species in Shimba Hills, Kenya.

3.4.2. Trypanosome Infections in Blood-Fed Tsetse Flies

Overall, 10.92% (38/348) (95% CI: 7.63–14.21) of blood-fed tsetse flies that harbored trypanosome infections had bloodmeals from 10 of the 13 animal species identified. Trypanosomes were not detected in tsetse flies that had fed on impala, goat, and bushbuck.

Trypanosomes in tsetse flies comprised of the livestock pathogens: *T. vivax*, *T. congolense* Kilifi, *T. congolense* Savannah, *T. simiae* Tsavo, *T. simiae*, *T. godfreyi* and *T. brucei sl*. Tsetse flies positive for warthog bloodmeals harbored all seven species and subspecies of trypanosomes. Tsetse flies that fed on cattle were positive for all trypanosomes except *T. congolense* Kilifi and *T. brucei sl*. For the other tsetse flies that fed on animal species other than warthogs and cattle, trypanosome infections were comprised of either one or two species but not more.

Tsetse flies that fed on warthogs were significantly exposed to *T. vivax* (Binomial-Generalized Linear Mixed Model [B-GLMM]: $p < 0.05$), *T. congolense* (B-GLMM: $p < 0.05$) and *T. brucei sl* (B-GLMM: $p < 0.05$) infection risk (Table 3.6). I also observed significant risk of trypanosome infection (*T. godfreyi*) (B-GLMM: $p < 0.05$) in tsetse flies that took bloodmeals from red riverhog. The only other species of animal bloodmeal outside the Suidae family for which I noted a significant risk of trypanosome infection (*T. congolense*) was suni (Table 3.6).

Table 3. 6: Risk of trypanosome infection in tsetse flies that obtained bloodmeals from different animal species.

	Bloodmeal-positive tsetse flies	*T. vivax*		*T. simiae* Tsavo		*T. simiae*	
		% (95% CI)	*p*-value	% (95% CI)	*p*-value	% (95% CI)	*p*-value
Bovidae	142	2.82 (0.06-5.57)	0.746	1.41 (-0.55-3.37)	0.844	0.70 (-0.69-2.10)	0.982
Suidae ‡	134	5.22 (1.41-9.04)	**0.027**	1.49 (-0.59-3.57)	0.479	1.49 (-0.59-3.57)	0.259
Cercopithecidae	62	1.61 (-1.61-4.84)	0.660	1.61 (-1.61-4.84)	0.886	NA	-
Hominidae	26	3.85 (-4.08-11.77)	0.640	3.85 (-4.08-11.77)	0.093	NA	-
Elephantidae ‡	5	NA	-	NA	-	NA	-
Equidae	3	NA	-	NA	-	NA	-
Warthog ‡	121	5.79 (1.57-10.00)	**0.014**	0.83 (-0.81-2.46)	0.865	1.65 (-0.65-3.96)	0.205
Cattle	79	1.27 (-1.25-3.79)	0.482	2.53 (-1.01-6.07)	0.313	1.27 (-1.25-3.79)	0.548
Baboon	62	1.61 (-1.61-4.84)	0.660	1.61 (-1.61-4.84)	0.886	NA	-
Sheep	40	5.00 (-2.06-12.06)	0.294	NA	-	NA	-
Human	26	3.85 (-4.08-11.77)	0.640	3.85 (-4.08-11.77)	0.093	NA	-
Red River Hog	13	NA	-	7.69 (-9.07-9.07)	0.084	NA	-
Buffalo	8	12.50 (-17.06-42.06)	0.103	NA	-	NA	-
Elephant	5	NA	-	NA	-	NA	-
Bushbuck	4	NA	-	NA	-	NA	-
Goat	4	NA	-	NA	-	NA	-
Suni	4	NA	-	NA	-	NA	-
Donkey	3	NA	-	NA	-	NA	-

Impala	3	NA	-	NA	-	NA	-

	Bloodmeal-positive tsetse flies	***T. godfreyi***		***T. congolense*** ‖		***T. brucei sl***	
		% (95% CI)	***p*-value**	**% (95% CI)**	***p*-value**	**% (95% CI)**	***p*-value**
Bovidae	142	0.70 (-0.69-2.10)	0.689	4.93 (-1.33-8.53)	0.345	NA	-
Suidae ‡	134	1.49 (-0.59-3.57)	0.535	6.72 (-2.42-11.01)	0.060	2.24 (-0.30-4.78)	**0.043**
Cercopithecidae	62	NA	-	NA	-	NA	-
Hominidae	26	NA	-	NA	-	NA	-
Elephantidae	5	NA	-	NA	-	NA	-
Equidae	3	NA	-	NA	-	NA	-
Warthog ‡	121	0.83 (-0.81-2.46)	0.828	7.44 (2.70-12.18)	**0.033**	2.48 (-0.33-5.29)	**0.031**
Cattle	79	1.27 (-1.25-3.79)	0.806	3.80 (-0.51-8.11)	0.994	NA	-
Baboon	62	NA	-	NA	-	NA	-
Sheep	40	NA	-	2.50 (-2.56-7.56)	0.785	NA	-
Human	26	NA	-	NA	-	NA	-
Red River Hog ‡	13	7.69 (-9.07-9.07)	**0.046**	NA	-	NA	-
Buffalo	8	NA	-	NA	-	NA	-
Elephant	5	NA	-	NA	-	NA	-
Bushbuck	4	NA	-	NA	-	NA	-
Goat	4	NA	-	NA	-	NA	-
Suni ‡	4	NA	-	75.00 (4.56-154.56)	**0.0004**	NA	-

Donkey	3	NA	-	NA	-	NA	-
Impala	3	NA	-	NA	-	NA	-

‖**:** *T. congolense* comprising of both the Kilifi and the Savannah strains; **‡ Significant** ($P < 0.05$); **NA**: Small sample size, or too few number or absence of infection cases

3.5 Discussion

Tsetse flies in Shimba Hills fed preferentially on suids (19.25%) and bovids (20.40%). Importantly, the vectors took bloodmeals from mostly warthogs (17.39%) and cattle (11.35%) among the 13 animal species whose bloodmeals I detected in samples of tsetse flies. These findings support previous observations of tsetse preference for cattle bloodmeals [16] and preferential selection of warthogs among wild animals in sylvatic ecologies, including areas where warthogs are sparse in relation to other animal species [17–20]. In Tanganyika, for example, warthogs made up <3.00% of the total population of wild mammals but >75.00% of the bloodmeals of tsetse flies [19].

My data show that tsetse flies in Shimba Hills feed preferentially on warthog bloodmeals. However, the underlining reasons for this are not well understood. In a previous study, the mosquito *Anopheles stephensi* preferred to feed on rabbits than guineapigs because the blood from rabbits was of higher nutritional quality and easier to digest [21]. Some experiments confirmed high dietary quality of porcine blood, hence making it the bloodmeal of choice for mass-rearing of tsetse colonies [22]. In one study, it was discovered that warthog skin and urine odours increased catches of tsetse flies [23]. These findings and tsetse disposition for feeding on warthogs in Shimba Hills suggest that further investigation of warthog-based tsetse-attractant semio-chemicals could enhance the toolbox of odour-attractants applied in tsetse surveillance and control in sub-Saharan Africa.

Cattle emit large amounts of tsetse attractant-odours through their urine. This underpins the rationale for urine adoption for tsetse attraction in entomological surveillance and control [24–26]. Furthermore, *G. pallidipes* which in Shimba Hills is the dominant tsetse fly species, have an intrinsic predisposition towards

bovids, including cattle [16]. It was therefore not surprising that tsetse flies in Shimba Hills were found to have fed frequently on cattle. A contrary finding by Channumsin et al. [12] in Shimba Hills of absence of cattle bloodmeals in tsetse flies may be the result of sampling bias occasioned by the short sampling time (of less than five weeks) reported in that study.

The frequent detection of trypanosomes in tsetse flies positive for warthog and cattle bloodmeals indicates that trypanosomes may move between the sylvatic and domestic cycles. This may explain why high prevalence of trypanosomes in cattle is common in this area, with reports confirming infections in nearly half of cattle livestock assessed during high transmission season [13]. Based on my observations of high diversity and rate of trypanosomes in tsetse flies positive for warthog bloodmeals, it is likely that warthogs play an important role as cryptic reservoirs and epidemiological drivers of cattle trypanosome parasites responsible for *Nagana* disease in smallholder farming systems in the wildlife interface of Shimba Hills.

Tourism, cattle herding, and land cultivation at the Shimba Hills Wildlife Reserve boundary are important factors that expose humans to attacks by tsetse flies. However, I could only detect human DNA in a few tsetse flies, possibly because the savannah tsetse fly species endemic in Shimba Hills are generally averse to feeding on humans [27]. The case is different for riverine tsetse flies, for example *G. fuscipes fuscipes* which feed frequently on humans [16] and in the process transmit *T. b. rhodesiense* responsible for the human sleeping sickness disease in Kenya and other East African countries except for northwest Uganda [4]. In Kenya, sleeping sickness is presently only endemic to the western region bordering Uganda but absent in the coastal area where Shimba Hills is located

[28]; Kenya Tsetse and Trypanosomiasis Eradication Council KENTTEC, 2019 www.kenttec.go.ke, assessed on 03rd November 2021).

Some animal species were detected infrequently in tsetse fly bloodmeals, probably because of their sparse presence in Shimba Hills. However, infrequent detections of Suni antelope, goat, and impala in Shimba Hills could be explained by the defensive behaviours of these animals against tsetse flies during attempts by the vectors to feed [3]. For sheep, the body covering by thick-wool makes it difficult for tsetse flies to obtain bloodmeals. Elephants have a non-uniform spatial distribution in Shimba Hills being mainly found in areas around Mlafyeni in proximity to the Mwalunganje Elephant Sanctuary [20]. Aside from Mlafyeni and the nearby Pengo and Kizibe, the only other location where I detected elephant bloodmeals in tsetse was in Kinangondogo. Still, the finding was made in a single *G. brevipalpis*, which according to Weitz [16] prefers elephants. This may also be due to the preference of *G. brevipalpis* for forested areas, where elephants in the Shimba Hills National Reserve are frequently found.

Allomonal volatile emissions may explain the absence in tsetse flies of bloodmeals from zebra [29] and waterbuck [13,30], both of which are present in Shimba Hills (Kenya Wildlife Service KWS 2021, www.kws.go.ke/content/shimba-hills-national-reserve, assessed 03rd November 2021). However, skin coloration patterns in zebra are believed to confuse tsetse flies and discourage vector attacks [31–33]. Even though I did not detect bloodmeals of zebra, waterbuck, and several other animal species (e.g., giraffe and monitor lizard) previously shown to be fed on by tsetse flies [3], bloodmeal host diversity in tsetse flies was high in Shimba Hills in comparison to reports from some similar ecologies, for example, the Kafue National Park

Zambia and Hurungwe Game Reserve Zimbabwe [34]. Large fauna community, extensive spatio-temporal samplings, and adoption of multiple gene-markers to segregate DNA of vertebrates in a high-throughput analysis using the sensitive PCR-HRM technique [35] are important factors which contributed to the wide diversity of tsetse fly bloodmeal hosts in Shimba Hills.

Multiple-host feeding by tsetse flies was presumably the result of certain animals' anti-feeding behaviours to discourage the vectors from biting attacks. Baboons, like goats and impala, display defensive behaviors against tsetse flies, hence it was not surprising that seven of the twelve sets of multiple hosts involved baboons. Disruption of tsetse-feeding before repletion on a host causes the vectors to switch to other hosts to continue feeding, thus allowing trypanosome-dissemination among and between wildlife and livestock [3,12]. In Shimba Hills, over half the cases of multiple-host feeding involved wildlife and livestock, prominently baboons and sheep, and warthogs and cattle. The finding of warthog and cattle bloodmeals in individual tsetse flies is further evidence that cattle in Shimba Hills are exposed to trypanosomes from warthogs.

Warthog and cattle multiple bloodmeals were detected in *G. pallidipes* and *G. austeni* and in male and female tsetse flies. However, *G. pallidipes* and female tsetse flies have a higher potential for trypanosome transmission in Shimba Hills because they make up >90.00% of the tsetse flies in Shimba Hills, outlive their male counterparts [36,37], and have relatively high rates of displacement which allows them to feed on and distribute infections among a large repertoire of animal species [38]. True to this, the rate of trypanosome infection was higher in older tsetse flies and in female tsetse flies. The wider host range in young flies

(data not shown) may be because they have a much greater quest for bloodmeals and consequently are more elastic in choice of hosts.

Stationary-baits for tsetse control in Shimba Hills should ideally target *G. pallidipes* because of the fly species high feeding rate on warthogs and cattle with deployment of the control tools prioritized to areas where warthogs are abundant and co-exist with cattle. It might, however, be more effective to integrate stationary-baits with live-baits (or synthetic tsetse repellent odour-treatment of cattle [13]) since tsetse flies in Shimba Hills also feed abundantly on cattle. The live-bait technique in Shimba Hills would have an added advantage of also controlling for ticks, which in the area transmit a large variety of pathogens [39], including *Theileria parva* responsible for the East Coast Fever and which in an epidemiological survey was detected in warthogs in the area [40].

Supplementary Materials: The following are available online at **https://www.mdpi.com/article/10.3390/pathogens10111501/s1**, Table S1: Data on tsetse fly bloodmeal hosts and trypanosome infections in the different study-blocks in Shimba Hills, Kenya.

References

1. Hassell, J.M.; Begon, M.; Ward, M.J.; Fèvre, E.M. Urbanization and disease emergence: Dynamics at the wildlife–livestock–human interface. *Trends Ecol. Evol.* 2017, *32*, 55–67.

2. Anderson, N.E.; Mubanga, J.; Fevre, E.M.; Picozzi, K.; Eisler, M.C.; Thomas, R.; Welburn, S.C. Characterisation of the wildlife reservoir community for human and animal trypanosomiasis in the Luangwa Valley, Zambia. *PLoS Negl. Trop. Dis.* 2011, *5*, e1211.

3. Auty, H.; Morrison, L.J.; Torr, S.J.; Lord, J. Transmission dynamics of Rhodesian sleeping sickness at the interface of wildlife and livestock areas. *Trends Parasitol.* 2016, *32*, 608–621.

4. Büscher, P.; Cecchi, G.; Jamonneau, V.; Priotto, G. Human african trypanosomiasis. *Lancet* 2017, *390*, 2397–2409.

5. Diall, O.; Cecchi, G.; Wanda, G.; Argilés-Herrero, R.; Vreysen, M.J.; Cattoli, G.; Viljoen, G.J.; Mattioli, R.; Bouyer, J. Developing a progressive control pathway for African animal trypanosomosis. *Trends Parasitol.* 2017, *33*, 499–509.

6. Kaare, M.T.; Picozzi, K.; Mlengeya, T.; Fèvre, E.M.; Mellau, L.S.; Mtambo, M.M.; Cleaveland, S.; Welburn, S.C. Sleeping sickness—A re-emerging disease in the Serengeti? *Travel Med. Infect. Dis.* 2007, *5*, 117–124.

7. Claxton, J.R.; Faye, J.A.; Rawlings, P. Trypanosome infections in warthogs (*Phacochoerus aethiopicus*) in the Gambia. *Vet. Parasitol.* 1992, *41*, 179–187.

8. Muturi, C.N.; Ouma, J.O.; Malele, I.I.; Ngure, R.M.; Rutto, J.J.; Mithöfer, K.M.; Enyaru, J.; Masiga, D.K. Tracking the feeding patterns of tsetse flies (*Glossina*

genus) by analysis of bloodmeals using mitochondrial cytochromes genes. *PloS One* 2011, *6*, e17284.

9. Munang'andu, H.M.; Siamudaala, V.; Munyeme, M.; Nalubamba, K.S. A review of ecological factors associated with the epidemiology of wildlife trypanosomiasis in the Luangwa and Zambezi valley ecosystems of Zambia. *Interdiscip. Perspect. Infect. Dis.* 2012, *2012*, 372523.

10. Squarre, D.; Hayashida, K.; Gaithuma, A.; Chambaro, H.; Kawai, N.; Moonga, L.; Namangala, B.; Sugimoto, C.; Yamagishi, J. Diversity of trypanosomes in wildlife of the Kafue ecosystem, Zambia. *Int. J. Parasitol. Parasites Wildl.* 2020, *12*, 34–41.

11. Makhulu, E.E.; Villinger, J.; Adunga, V.O.; Jeneby, M.M.; Kimathi, E.M.; Mararo, E.; Oundo, J.W.; Musa, A.A.; Wambua, L. Tsetse blood-meal sources, endosymbionts and trypanosome-associations in the Maasai Mara National Reserve, a wildlife-human-livestock interface. *PLoS Negl. Trop. Dis.* 2021, *15*, e0008267.

12. Channumsin, M.; Ciosi, M.; Masiga, D.; Auty, H.; Turner, C.M.; Kilbride, E.; Mable, B.K. Blood meal analysis of tsetse flies (*Glossina pallidipes*: Glossinidae) reveals higher host fidelity on wild compared with domestic hosts. *Wellcome Open Res.* 2021, *6*, 213.

13. Saini, R.K.; Orindi, B.O.; Mbahin, N.; Andoke, J.A.; Muasa, P.N.; Mbuvi, D.M.; Muya, C.M.; Pickett, J.A.; Borgemeister, C.W. Protecting cows in small holder farms in East Africa from tsetse flies by mimicking the odor profile of a non-host bovid. *PLoS Negl. Trop. Dis.* 2017, *11*, e0005977.

14. Navia-Gine, W.G.; Loaiza, J.R.; Miller, M.J. Mosquito-host interactions during and after an outbreak of equine viral encephalitis in eastern Panama. *PloS One* 2013, *8*, e81788.

15. Escobar, D.; Ascencio, K.; Ortiz, A.; Palma, A.; Sánchez, A.; Fontecha, G. Blood meal sources of Anopheles spp. in malaria endemic areas of Honduras. *Insects* 2020, *11*, 450.

16. Weitz, B. The feeding habits of *Glossina*. *Bull. World Health Organ.* 1963, *28*, 711.

17. Weitz, B.; Glasgow, J.P. The natural hosts of some species of *Glossina* in East Africa. *Trans. R. Soc. Trop. Med. Hyg.* 1956, *50*, 593–612.

18. Auty, H.; Cleaveland, S.; Malele, I.; Masoy, J.; Lembo, T.; Bessell, P.; Torr, S.; Picozzi, K.; Welburn, S.C. Quantifying heterogeneity in host-vector contact: Tsetse (*Glossina swynnertoni* and *G. pallidipes*) host choice in Serengeti National Park, Tanzania. *PloS One* 2016, *11*, e0161291.

19. Lamprey, H.F.; Glasgow, J.P.; Lee-Jones, F.; Weitz, B. A simultaneous census of the potential and actual food sources of the tsetse fly *Glossina swynnertoni* Austen. *J. Anim. Ecol.* 1962, *31*, 151–156.

20. Ngene, S.M.; Mukeka, J. *Aerial Census of Elephants and Other Mammals in Shimba Hills, Mkongani, and Mwaluganje Conservation areas*; Kenya Wildlife Service: Nairobi, Kenya, 2012.

21. Glasgow, J.P.; Weitz, B. The natural hosts of some species of Glossina in East Africa. *Trans. R. Soc. Trop. Med. Hyg.* 1956, *50*, 593–612.

22. De Beer, C.J.; Venter, G.J.; Potgieter, F.T. *Developing Quality Control Procedures to Sustain a Supply of High Quality Blood for Mass Rearing Tsetse Flies*; IAEA: Vienna, Austria, 2012.

23. Späth, J. Natural host odours as possible attractants for *Glossina tachinoides* and *G. longipalpis* (Diptera: Glossinidae). *Acta Trop.* 1997, *68*, 149–158.

24. Hargrove, J.W.; Vale, G.A. The effect of host odour concentration on catches of tsetse flies (Glossinidae) and other Diptera in the field. *Bull. Entomol. Res.* 1978, *68*, 607–612.

25. Brightwell, B.; Dransfield, B.; Maudlin, I.; Stevenson, P.; Shaw, A. Reality vs. rhetoric–a survey and evaluation of tsetse control in East Africa. *Agric. Hum. Values* 2001, *18*, 219–233.

26. Leak, S.G.; Ejigu, D.; Vreysen, M.J. *Collection of Entomological Baseline Data for Tsetse Area-Wide Integrated Pest Management Programmes*; Food and Agriculture Organization of the United Nations (FAO): Rome, Italy, 2008.

27. Vale, G.A. The responses of tsetse flies (Diptera, Glossinidae) to mobile and stationary baits. *Bull. Entomol. Res.* 1974, *64*, 545–588.

28. Rutto, J.J.; Karuga, J.W. Temporal and spatial epidemiology of sleeping sickness and use of geographical information system (GIS) in Kenya. *J. Vector Borne Dis.* 2009, *46*, 18.

29. Olaide, O.Y.; Tchouassi, D.P.; Yusuf, A.A.; Pirk, C.W.; Masiga, D.K.; Saini, R.K.; Torto, B. Zebra skin odor repels the savannah tsetse fly, *Glossina pallidipes* (Diptera: Glossinidae). *PLoS Negl. Trop. Dis.* 2019, *13*, e0007460.

30. Bett, M.K.; Saini, R.K.; Hassanali, A. Repellency of tsetse-refractory waterbuck (*Kobus defassa*) body odour to *Glossina pallidipes* (Diptera: Glossinidae):

Assessment of relative contribution of different classes and individual constituents. *Acta Trop.* 2015, *146*, 17–24.

31. Waage, J.K. How the zebra got its stripes-biting flies as selective agents in the evolution of zebra coloration. *J. Entomol. Soc. South. Afr.* 1981, *44*, 351–358.

32. Gibson, G. Do tsetse flies 'see' zebras? A field study of the visual response of tsetse to striped targets. *Physiol. Entomol.* 1992, *17*, 141–147.

33. Caro, T.; Izzo, A.; Reiner, R.C.; Walker, H.; Stankowich, T. The function of zebra stripes. *Nat. Commun.* 2014, *5*, 1–10.

34. Gaithuma, A.; Yamagishi, J.; Hayashida, K.; Kawai, N.; Namangala, B.; Sugimoto, C. Blood meal sources and bacterial microbiome diversity in wild-caught tsetse flies. *Sci. Rep.* 2020, *10*, 1–10.

35. Ouso, D.O.; Otiende, M.Y.; Jeneby, M.M.; Oundo, J.W.; Bargul, J.L.; Miller, S.E.; Wambua, L.; Villinger, J. Three-gene PCR and high-resolution melting analysis for differentiating vertebrate species mitochondrial DNA for biodiversity research and complementing forensic surveillance. *Sci. Rep.* 2020, *10*, 1–13.

36. Wamwiri, F.N.; Alam, U.; Thande, P.C.; Aksoy, E.; Ngure, R.M.; Aksoy, S.; Ouma, J.O.; Murilla, G.A. Wolbachia, Sodalis and trypanosome co-infections in natural populations of *Glossina austeni* and *Glossina pallidipes*. *Parasites vectors* 2013, *6*, 1–11.

37. Kubi, C.; Billiouw, M.; Van den Bossche, P. Age prevalence of trypanosomal infections in female Glossina morsitans morsitans (Diptera: Glossinidae) on the plateau area of eastern Zambia. *Onderstepoort J. Vet. Res.* 2007, *74*, 223–229.

38. Vale, G.A.; Hargrove, J.W.; Solano, P.; Courtin, F.; Rayaisse, J.B.; Lehane, M.J.; Esterhuizen, J.; Tirados, I.; Torr, S.J. Explaining the host-finding behavior of blood-sucking insects: Computerized simulation of the effects of habitat geometry on tsetse fly movement. *PLoS Negl. Trop. Dis.* 2014, *8*, e2901.

39. Mwamuye, M.M.; Kariuki, E.; Omondi, D.; Kabii, J.; Odongo, D.; Masiga, D.; Villinger, J. Novel Rickettsia and emergent tick-borne pathogens: A molecular survey of ticks and tick-borne pathogens in Shimba Hills National Reserve, Kenya. *Ticks tick-borne Dis.* 2017, *8*, 208–218.

40. Jagero, G.O. Molecular Evidence of Arthropod-Borne Pathogens in Wildlife and Livestock in Shimba Hills National Reserve (SHNR), Kwale County, Kenya. Master's Dissertation, The University of Edinburgh, Edinburgh, UK, 2015.

41. Knickerbocker, T.J.; Waithaka, J. People and elephants in the Shimba Hills, Kenya. *Conserv. Biol. Ser. -Camb.* 2005, *9*, 224.

42. Challier, A.; Laveissiere, C. A new trap for catching *Glossina*: Description and field trials. *Cah. ORSTOM Ser. Entomol. Med et Parasitol.* 1973, *11*, 251–262.

43. Pollock, J.N. (Ed.) *Training Manual for Tsetse Control Personnel*; Food and Agriculture Organization of the United Nations: Rome, Italy, 1982.

44. Jackson, C.H. An artificially isolated generation of tsetse flies (Diptera). *Bull. Entomol. Res.* 1946, *37*, 291–299.

45. Omondi, D.; Masiga, D.K.; Ajamma, Y.U.; Fielding, B.C.; Njoroge, L.; Villinger, J. Unraveling host-vector-arbovirus interactions by two-gene high resolution melting mosquito bloodmeal analysis in a Kenyan wildlife-livestock interface. *PloS One* 2015, *10*, e0134375.

46. Boakye, D.A.; Tang, J.; Truc, P.; Merriweather, A.; Unnasch, T.R. Identification of bloodmeals in haematophagous Diptera by cytochrome B heteroduplex analysis. *Med. Vet. Entomol.* 1999, *13*, 282–287.

47. Ivanova, N.V.; Zemlak, T.S.; Hanner, R.H.; Hebert, P.D. Universal primer cocktails for fish DNA barcoding. *Mol. Ecol. Notes* 2007, *7*, 544–548.

48. Njiru, Z.K.; Constantine, C.C.; Guya, S.; Crowther, J.; Kiragu, J.M.; Thompson, R.C.; Dávila, A.M. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. *Parasitol. Res.* 2005, *95*, 186–192.

49. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids Symp. Ser.* 1999, *41*, 95–98.

50. Dunn, P.K.; Smyth, G.K. Models for Proportions: Binomial GLMs. In *Generalized Linear Models With Examples in R*; Springer: New York, NY, USA, 2018; pp. 333–369.

51. Hothorn, T.; Bretz, F.; Westfall, P.; Heiberger, R.M.; Schuetzenmeister, A.; Scheibe, S.; Hothorn, M.T.; Package 'multcomp'. *Simultaneous Inference in General Parametric Models*; Project for Statistical Computing: Vienna, Austria, 2016.

52. Brooks, M.E.; Kristensen, K.; Van Benthem, K.J.; Magnusson, A.; Berg, C.W.; Nielsen, A.; Skaug, H.J.; Machler, M.; Bolker, B.M. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R J.* 2017, *9*, 378–400.

53. Team RC. *R: A Language and Environment for Statistical Computing*; https://www.R-project.org/Vienna, Austria, 2013.

54. Dormann, C.F.; Gruber, B.; Fründ, J. Introducing the bipartite package: Analysing ecological networks. *Interaction* 2008, *8*, 8–11

CHAPTER 4

Molecular characterisation of *Trypanosoma vivax* in tsetse flies confirms the presence of the virulent *Tvv4* genotype in Kenya: Potential implications for the control of trypanosomiasis in Shimba Hills

---

This chapter has been published as:

Faith I. Ebhodaghe, Armanda D. S. Bastos, Kevin O. Kidambasi, Shewit Kalayou**,** Daniel K. Masig**a** & Michael N. Okal (2021). Molecular characterisation of *Trypanosoma vivax* in tsetse flies confirms the presence of the virulent *Tvv4* genotype in Kenya: Potential implications for the control of trypanosomiasis in Shimba Hills. *Infection, Genetics & Evolution* **93**: 104953.

https://www.sciencedirect.com/science/article/pii/S1567134821002501

4.1 Abstract

Trypanosoma vivax is a vector-borne protozoan parasite of livestock endemic to Africa and South America. To date, fifteen genotypes of the parasite have been described in vertebrate and insect hosts in East Africa. However, information regarding *T. vivax* diversity remains limited in many endemic countries in the sub-region, including Kenya. Such information could deepen insight into the local epidemiology of animal trypanosomiasis in Shimba Hills, a wildlife area in southeast Kenya where *T. vivax* is endemic and infects livestock. I employed two-gene conventional-PCR-sequencing and phylogenetic analysis to characterize *T. vivax* genotypes in tsetse flies collected between November 2018 and September 2019 in the wildlife-livestock interface of the Shimba Hills National Reserve. Phylogenetic analysis of Internal Transcribed Spacer-1 (ITS-1) sequences of *T. vivax* isolates confirmed the presence of two *T. vivax* genotypes in Shimba Hills of which >80% of *T. vivax* isolates from tsetse flies clustered within the virulent *Tvv4*-genotype clade. Tsetse infections with the *Tvv4* genotype were also confirmed based on 18S rRNA gene sequencing. Expanded gene characterisation identified three closely related haplotypes within the *Tvv4*-clade. The *Tvv4*-isolates were detected in male and female *Glossina pallidipes* tsetse flies, most of which were collected from grasslands and within two kilometres of the Shimba Hills National Reserve boundary. Considering that *T. vivax* is the most common trypanosome in the Shimba Hills area and causes severe clinical conditions in livestock, the *Tvv4* genotype reported here for the first time in Kenya contributes to our understanding of these pathologies. The effectiveness of trypanocidal drugs in the management of *Tvv4* is presently not clearly understood. Therefore, the parasite management

in Shimba Hills should focus on vector control to reduce the density of *G. pallidipes*, especially in grasslands near the wildlife protectorate.

Keywords: *Trypanosoma vivax; Tvv4;* Kenya; Tsetse; Wildlife-livestock interface

Abbreviations: SHNR: Shimba Hills National Reserve; KWS: Kenya Wildlife Service; KENTTEC: Kenya Tsetse and Trypanosomiasis Eradication Council; DNA: Deoxy-ribo Nucleic Acid; PCR: Polymerase Chain Reaction; *icipe*: International Centre of Insect Physiology and Ecology.

4.2 Introduction

Trypanosoma vivax is a pathogenic tsetse-transmitted trypanosome parasite of livestock in sub-Saharan Africa. The parasite is also transmitted by other biting flies such as *Stomoxys* sp. and tabanids. This has enabled its sustained transmission outside the tsetse-belt of sub-Saharan Africa (Jones and Dávila, 2001). In sub-Saharan Africa, *T. vivax*, *T. congolense* and *T. brucei sl* account for the major trypanosome burden on livestock health and production in agro-pastoralist communities, many of which are located along the interface of wildlife reserves (Auty et al., 2016; Anderson et al., 2011; Squarre et al., 2020; Lord et al., 2018; Lord et al., 2020; Auty et al., 2012; Votýpka et al., 2015). Other trypanosomes, including *T. simiae*, *T. simiae* Tsavo, and *T. godfreyi* have also been described to cause pathology in livestock though mainly in suids (Auty et al., 2012; Votýpka et al., 2015; Adams et al., 2006; Garcia et al., 2018; Njiru et al., 2005; Hamilton et al., 2008; Gaithuma et al., 2019; Ng'ayo et al., 2005; Njiru et al., 2004; Von Wissmann et al., 2011).

Trypanosoma vivax is genetically diverse and different genotypes show subtle differences in transmission patterns and variable responses to chemotherapy with trypanocides. These genotypes also exhibit non-uniform virulence in infected animal hosts (Rodrigues et al., 2008; Rodrigues et al., 2017; Fasogbon et al., 1990; Giordani et al., 2016). It is believed that *T. vivax* isolates from East Africa are less virulent than isolates from West Africa (Losos and Ikede, 1972). However, some acute outbreaks and severe hemorrhagic cases have been reported in cattle in Kenya and Uganda following infections with *T. vivax* (Gardiner et al., 1989; Magona et al., 2008). In Mozambique, a *T. vivax* isolate from nyala was reportedly highly virulent with severe conditions observed in an experimentally infected goat less than three weeks after inoculation

(Rodrigues et al., 2008). Using molecular techniques for trypanosome examination, Rodrigues et al. (Rodrigues et al., 2008) generated DNA sequences of the nyala-derived trypanosome. A subsequent taxonomic revision, reporting novel and highly diverse *T. vivax* genotypes from Mozambique (Rodrigues et al., 2017), placed the isolate within the *Tvv4*-genotype of *T. vivax* (Rodrigues et al., 2017). To date, reports are scanty regarding the distribution of this highly virulent genotype.

In Kenya, epidemiological studies showed the genotype diversity of *T. brucei* and *T. evansi* (Echodu et al., 2015; Kamidi et al., 2017). However, relatively little is known about the molecular diversity of *T. vivax*. A prior study using isoenzyme polymorphisms confirmed that at least two genotypes occur in Kenya (Fasogbon et al., 1990). A recent review of *T. vivax* genotype distribution in Africa reported one genotype in Kenya based on the ITS rDNA gene (Rodrigues et al., 2017). However, in Ethiopia and Tanzania, four and three genotypes were reported, respectively. These results contrast with findings in Mozambique, where most studies have been conducted, and ten *T. vivax* genotypes are described (Rodrigues et al., 2017).

I used the conventional-Polymerase Chain Reaction (PCR) technique, molecular sequencing, and phylogenetic analysis to describe genotypes of *T. vivax* in tsetse flies from the wildlife-livestock interface of the Shimba Hills National Reserve on the coast of Kenya. *Trypanosoma vivax* is known to be the main trypanosome circulating in populations of tsetse flies in Shimba Hills, where high parasitological rates of trypanosome infections in cattle are associated with anaemic conditions (Ebhodaghe F, unpublished). Currently, there is no clear understanding of *T. vivax* genetic diversity in Shimba Hills. A study that

addresses this knowledge gap can help explain trypanosome pathologies in cattle and guide interventions against animal trypanosomiasis.

4.3 Materials and methods

4.3.1 Ethical clearance

The study was done according to guidelines stipulated by the International Centre of Insect Physiology and Ecology *icipe* Nairobi, Kenya. Tsetse fly samples were collected in collaboration with the Kenya Wildlife Service (KWS) and the Kenya Tsetse and Trypanosomiasis Eradication Council (KENTTEC).

4.3.2 Study setting and tsetse flies sampling

The Shimba Hills National Reserve (SHNR) in Kwale County on the coast of Kenya is one of East Africa's biodiversity hotspots (Malonza et al., 2018). Extending over 200 km2, the SHNR accommodates a large community of wildlife species, including the critically endangered sable antelope (*Hippotragus niger*), warthog (*Phacochoerus africanus*), buffalo (*Syncerus scaffer*), and bushbuck (*Tragelaphus scriptus*) and is remarkable for its high elephant (*Loxodonta africana*) density. Historically, conflicts between wildlife and humans/livestock were common around the SHNR. Such conflicts still arise, albeit not physically but due to disease spillover. Pathogens from wildlife in Shimba Hills that impact the health of livestock include trypanosomes transmitted by tsetse flies. In the present study, tsetse flies were collected in bimonthly entomological surveys between November 2018 and September 2019 in the SHNR (Fig. 4.1). Briefly, tsetse fly samples were collected using cow urine and acetone-baited biconical traps deployed at a density of one per km grid-cell over a ~ 230 km2 area stratified into 14 blocks along the wildlife-livestock interface of the SHNR. Trapped flies were collected after 48 h of trap deployment and identified using established taxonomic keys (Pollock, 1982). All tsetse fly samples were sorted according to sex and species and then preserved in 95% ethanol until further analysis. Tsetse flies identified as male and female *Glossina pallidipes* Austen,

1903, *G. brevipalpis* Newstead, 1910, and *G. austeni* Newstead, 1912 were selected from traps deployed across the 14 study blocks and screened for *T. vivax* infection. Overall, 696 tsetse flies from 113 traps were randomly selected for screening. The number of fly samples from each trap ranged from 1 to 45 (average: 6 flies per trap).

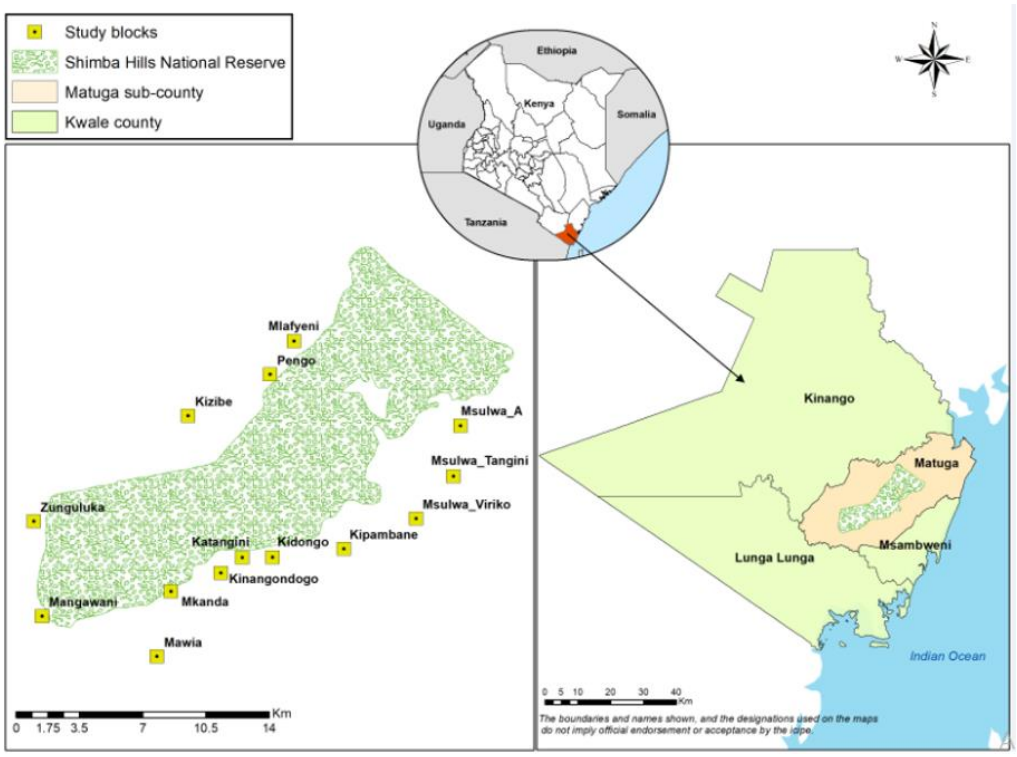


Figure 4. 1. Map of Kenya showing Kwale county. Right: Map of Kwale County showing Matuga district and location of Shimba Hills National Reserve. Left: Map of Shimba Hills National Reserve showing block locations where sampling was done.

4.3.3 DNA extraction and trypanosome detection and identification

DNA was extracted from dry, crushed homogenates using the Bioline Genomic DNA extraction kits (London, UK) following the manufacturer's instructions for

animal tissues. Briefly, individual tsetse flies were removed from alcohol and allowed to air-dry on paper towels and afterwards crushed in a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK, USA). DNA amplification was conducted in a 10 µl reaction volume comprising of 0.5 µl (concentration: 10 µM) each of Forward and Reverse ITS-1 primers (CF: CCGGAAGTTCACCGATATTG, BR: TTGCTGCGTTCTTCAACGAA) (Njiru et al., 2005), 3 µl nuclease-free water, 5 µl DreamTaq, and 1 µl DNA template with the following cycling conditions: initial denaturation for 1 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 20 s, and extension at 72 °C, followed by a final extension at 72 °C for 7 min. PCR products were run against a Gene-Ruler 100 bp DNA ladder (Thermo Scientific, Lithuania) on a 1.5% agarose gel stained with 5 µg/ml ethidium bromide. Tsetse fly extracts that produced ~250 bp band were scored as positive for *T. vivax* infection (Njiru et al., 2005). Positive samples were subjected to 18S rRNA amplification using published primers and thermal cycling conditions (Maslov et al., 1996).

4.3.4 Nucleotide sequencing

I conducted unidirectional sequencing of ITS-1 amplicons with the ITS1 CF (Njiru et al., 2005) primer to confirm the identity of trypanosomes detected. Similarly, unidirectional sequencing of the ~200 bp 18S rRNA amplicons was carried out using 18S rRNA primer GACCRTTGTAGTCCACACTG (Maslov et al., 1996) to confirm *T. vivax* genotype identity. Amplicons were cleaned using EXO-SAP to remove unincorporated dNTPs and primers. Alternatively, DNA bands were excised from gels using clean scalpel blades under blue light illumination and purified using Qiagen Gel Extraction Kits following the

manufacturer's instructions. Sanger sequencing was outsourced to Macrogen (Macrogen Europe B.V., The Netherlands).

4.3.5 Phylogenetic analysis

DNA sequences were inspected for quality based on chromatograph profiles and edited using the BioEdit software v7.2.5 (Hall, 1999). BLAST searches were conducted against the GenBank-nr database to identify sequences with the highest levels of sequence identity using the NCBI BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignments were undertaken using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The dataset was complemented with reference sequences from Kenya, Nigeria and Burkina Faso, and closely related sequences before removing end-unaligned sequences and regions corresponding to primer-binding sites. Maximum-Likelihood trees were inferred in the Molecular Evolution and Genetic Analysis MEGA-X software (Kumar et al., 2018) using the Hasegawa-Kishino-Yano HKY model of sequence evolution (Hasegawa et al., 1985), selected as the best-fit model using the Smart Model Selection in PhyMl (Lefort et al., 2017). The Nearest Neighbour Interchange was used to estimate tree topologies and nodal support was estimated from 1000 bootstrap replications. The kinetoplastid Bodo caudatus in the Bodonidae family (GenBank accession number: AY028450) was selected as out-group to root trees.

4.3.6 DNA sequence and haplotype analysis of *T. vivax* isolates

I estimated *T. vivax* population diversity indices [haplotype diversity (Hd), polymorphic (or segregating) sites (S), parsimony-informative sites, and nucleotide diversity (π)] in DnaSP v6 (Rozas et al., 2017). Median-joining networks (Bandelt et al., 1999) were constructed in the PopART software (Leigh

and Bryant, 2015) to visually explore relationships among haplotypes from Shimba Hills and available closely related sequences in the GenBank database.

4.4.0 Results

Overall, 2.44% (17/696) of tsetse flies were positive for *Trypanosoma vivax* based on ITS-PCR. Infections were detected in one female *Glossina brevipalpis* (0.77%, 1/130) and the other cases in males and females of *G. pallidipes* (3.04%, 16/526). All *T. vivax*-positive tsetse flies were collected in traps within 3 km of the Shimba Hills National Reserve border.

4.4.1.0 Genotypes of *T. vivax* in Shimba Hills and Kenya

4.4.1.1 Based on ITS-1 DNA

A total of ten *T. vivax* isolates were sequenced based on the ITS1 gene and confirmed by BLAST analysis to be *T. vivax*. However, I excluded three of these isolates from the study due to the poor quality of sequences revealed by their chromatographs, thus leaving us with 7 ITS1 sequences (Table 4.1).

Table 4. 1. Identification of nucleic acid sequences of *T. vivax* detected in tsetse flies from Shimba Hills (2018 to 2019).

Sample ID	Sequence length (bp)	Closest match on GenBank (host, country)	Sequence identity (%)
ITS-1			
DNA			
GP464	221	KX584844 (Tsetse fly, Mozambique)	100
GP49	209	KX584844 (Tsetse fly, Mozambique)	99.42
GP586	208	KX584844 (Tsetse fly, Mozambique)	100
GP105	215	KM391825 (Cattle, Ethiopia)	98.58
GP306	210	KX584844 (Tsetse fly, Mozambique)	100
GP870	210	KX584844 (Tsetse fly, Mozambique)	98.82
GP525	203	KX584844 (Tsetse fly, Mozambique)	95.86
18S *rRNA*			
GP49	163	EU477537 (Nyala, Mozambique)	99.38
GP306	176	EU477537 (Nyala, Mozambique)	97.74
GP586	169	EU477537 (Nyala, Mozambique)	98.21
GP599	213	EU477537 (Nyala, Mozambique)	98.27
GP788	173	EU477537 (Nyala, Mozambique)	98.28

Trypanosoma vivax ITS1 phylogeny revealed three major genotype clades for the parasite in Kenya, with the first and second clades present in the Shimba Hills National Reserve area (Fig. 4.2, S1). The first clade consists of Kenyan isolates (GenBank Accession Nos.: DQ316042, DQ316043, DQ316040, DQ316039, DQ316038, DQ316037, DQ316044, and DQ316041) from cattle and an isolate GP105 from my study (Fig. 4.2) (GenBank Accession Number: MW689621).

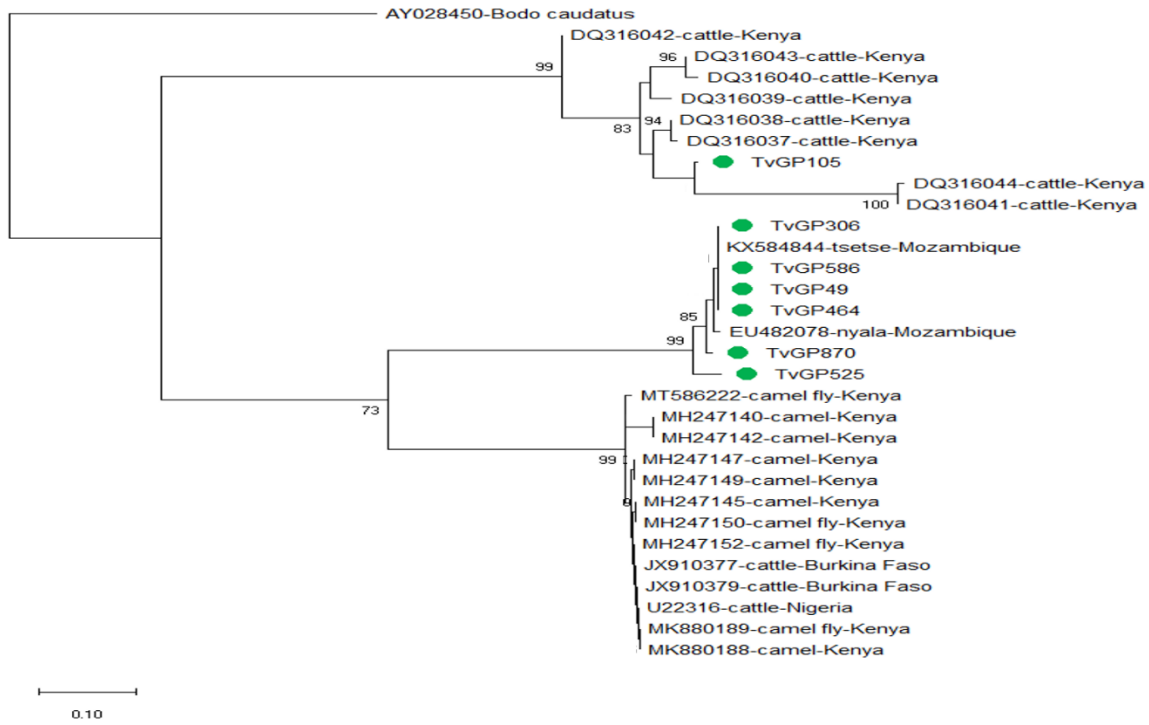


Figure 4. 2. Maximum-Likelihood phylogenetic tree inferred using partial ITS-1 *T. vivax* sequences (220 base-pairs) from tsetse flies collected in Shimba Hills, Kenya. Sequences from this study are bulleted in green and 'Tv' captioned before the sample ID to indicate that they are *T. vivax* species. Other sequences were obtained from GenBank. 'Host' and 'country' of isolation are added to each of the GenBank sequences. *Bodo caudatus* is designated as the outgroup. Nodal support values >70% based on 1000 bootstrap replicates are indicated next to the relevant nodes. The branch length represents substitution per site.

The second clade comprises of *T. vivax Tvv4* genotype first identified from a wild-caught nyala antelope in the Sofala Province in Mozambique (GenBank Accession No.: EU482078) and a Morsitans tsetse fly from the Gorongosa National Park in the Central region of Mozambique (GenBank Accession No.:

KX584844), as well as samples from this study (GP49, GP306, GP464, GP586, GP870 and GP525) (GenBank Accession Number: MW689622- MW689627).

The third clade is made up of isolates from Nigeria and Burkina Faso in West Africa and from Kenya in East Africa. Isolates from Kenya were obtained from camel flies (GenBank Accession Nos.: MK880189, MH247152, MH247150, and MT586222) and dromedary camels (GenBank Accession Nos.: MK880188, MH247145, MH247149, MH247147, MH247140, and MH247142) in the tsetse-free region of northern Kenya. The isolates from Nigeria (GenBank Accession No.: U22316) and Burkina Faso (GenBank Accession No.: JX910379, JX910377) were obtained from cattle.

4.4.1.2 18S rRNA gene phylogeny

I selected the isolates GP49, GP306, GP464, GP586, and GP525 (shown on the ITS1 phylogeny as *Tvv4*) and 2 other isolates GP599 and GP788 for 18S rRNA gene amplification and sequencing. Nucleotide BLAST searches performed with each of the resulting sequencing revealed that each of the 7 18S rRNA *T. vivax* isolates (GenBank Accession No.: MW686915-MW686917, MW812256-MW812259) are closest in identity to a sequence for a nyala antelope-derived *T. vivax* isolate in Mozambique (GenBank Accession No.: EU477537) (Table 4.1).

Two major genotype clades emerged on the 18S rRNA phylogeny (Fig. 4.3, S2). Isolates GP49, GP306, and GP586 (GenBank Accession Number: MW686915-MW686917) selected to represent the *Tvv4* genotype (as shown on the ITS1 phylogeny) clustered within the first clade. This first clade also features an isolate from a nyala antelope in Mozambique (GenBank Accession No.: EU477537).

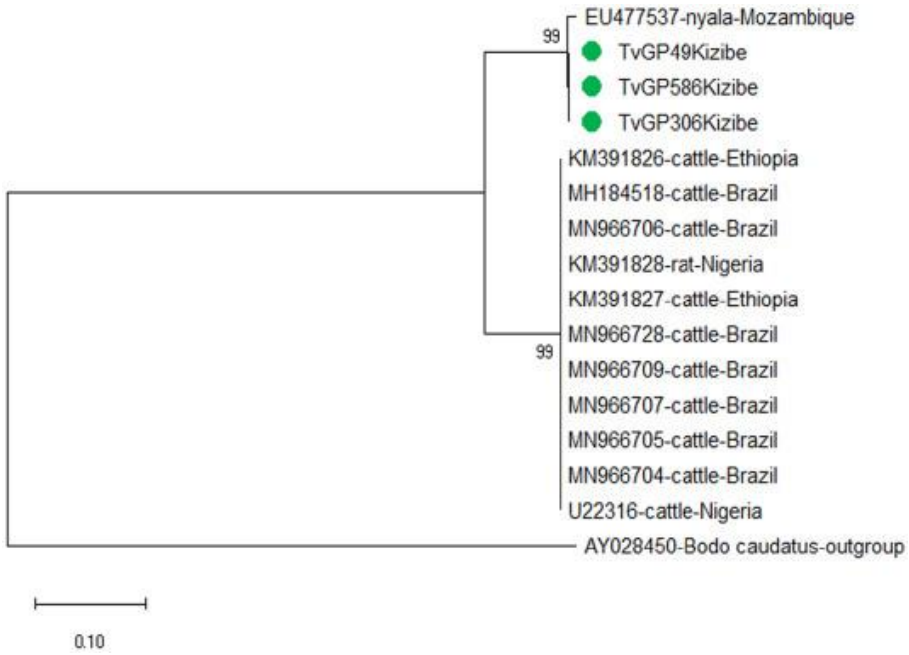


Figure 4. 3. Maximum-Likelihood phylogenetic tree inferred using partial 18S rRNA *T. vivax* sequences (159 base-pair) from tsetse flies collected in Shimba Hills, Kenya. Sequences from this study are bulleted in green and 'Tv' captioned before the sample ID to indicate that they are *T. vivax* species. Other sequences without the green bullets are from the GenBank database. 'Host' and 'country' of isolation are added to each of the GenBank sequences. *Bodo caudatus* is designated as an out-group. Nodal support >70% based on 1000 bootstrap replicates is indicated next to the relevant nodes. The branch length represents substitution per site.

The second genotype group consists of isolates from Africa and the Americas. Isolates from Nigeria obtained from a rat (GenBank Accession No.: KM391828) and cattle (GenBank Accession No.: U22316) clustered with those from cattle in Ethiopia (GenBank Accession No.: KM391826 and KM391827) in East Africa and Brazil (GenBank Accession Nos.: MH184518, MN966706, MN966728, MN966709, MN966707, MN966705, MN966704) in South America.

4.4.2 Variation among DNA sequences of *T. vivax* isolates from Shimba Hills

Haplotype and nucleotide diversity were respectively 0.714 and 0.123 for ITS-1 sequences (174 base-pair long) for seven *T. vivax* isolates from Shimba Hills. I noted a total of 68 polymorphic sites in the set of aligned sequences with 5.88% (4/68) of these sites being parsimony informative (S3).

Haplotype diversity for the seven 18S rRNA isolate sequences (165 base-pair) was 0.714, and nucleotide diversity 0.058. The entire set of aligned sequences had 28 polymorphic sites with 21.43% (6/28) appearing as parsimony informative sites (Table 4.1, S3).

4.4.3 Median-joining haplotype network analysis of *T. vivax* isolates from Shimba Hills and other locations in sub-Saharan Africa and Brazil in South America

Three major groups emerged from my median-joining network analysis based on ITS-1 DNA sequences (Fig. 4.4). The first group was the *Tvv4* comprising of GP49, GP306, GP464, GP525, GP586, and GP870 from Shimba Hills and the nyala trypanosome isolate from Mozambique (EU482078). The *Tvv4* group had four distinct haplotypes, the main haplotype comprising of GP49, GP306, GP464, and GP525 differing from the nyala isolate from Mozambique by a single mutation. GP870 and GP 525 differed from the main haplotype by two and seven mutations, respectively. The second group (*TvL1-G*) contained the sample from Shimba Hills (GP105) and the IL3905 strain (DQ316041) from Kenya, which differed by five nucleotide substitutions and a single nucleotide indel from each other. Finally, the third group was made up of isolates from Kenya (MH247149), Nigeria (U22316), and Burkina Faso (JX910379), all sharing a common haplotype

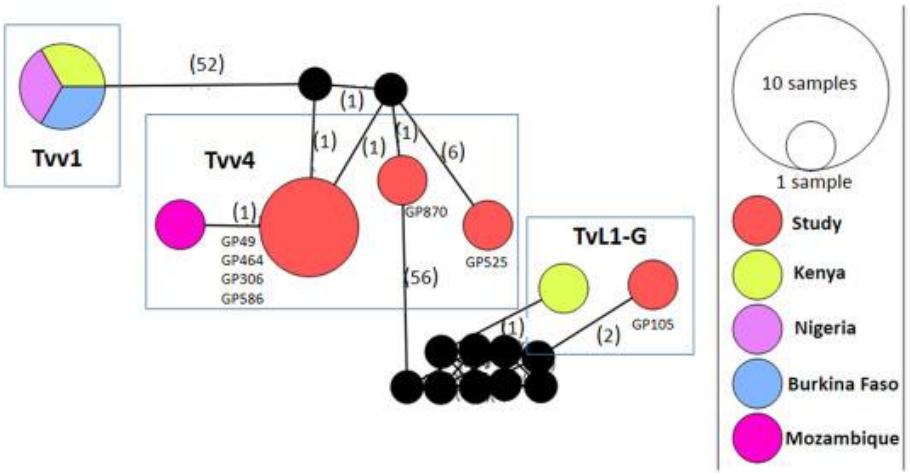


Figure 4. 4. Median-joining haplotype network of *T. vivax* isolates from Shimba Hills and different African countries, including Kenya, based on the ITS-1 gene sequences. Unique colours denote the haplotypes according to the location of origin. The black circles are median vectors (i.e. hypothetical haplotypes). Circle sizes are proportional to the haplotype frequencies. The number in parentheses on branches indicates the number of mutation changes segregating haplotypes. *TvL1-G*: Study (Shimba Hills) (GP105) and Kenya (GenBank Accession No.: DQ316041); *Tvv1*: Nigeria (GenBank Accession No.: U22316), Burkina Faso (GenBank Accession No.: JX910379) and Kenya (GenBank Accession No.: MH247149); *Tvv4*: Mozambique (GenBank Accession No.: EU482078) and Study (Shimba Hills) (GP49, GP306, GP464, GP525, GP586, and GP870).

When analysed based on the 18S rRNA sequences, *T. vivax* isolates GP49, GP306 and GP586 from Shimba Hills shared same haplotype, differing from the nyala isolate from Mozambique (EU477537) by a single mutation (Fig. 4.5). Isolates from Brazil (MN966728), Ethiopia (KM391827), and Nigeria (U22316) belonged to the same *Tvv1* group having a common haplotype.

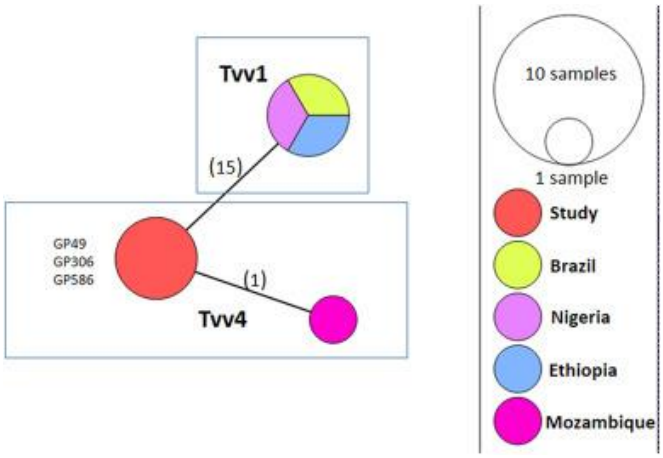


Figure 4. 5. Median-joining haplotype network of *T. vivax* isolates from Shimba Hills and different African countries including Kenya, and from Brazil in South America based on the 18S rRNA gene sequences. The haplotypes are denoted by unique colours according to the location of origin. The black circles are median vectors (i.e. hypothetical haplotypes). Circle sizes are proportional to the haplotype frequencies. The number in parentheses on branches indicates the number of mutation changes segregating haplotypes. *Tvv1*: Nigeria (GenBank Accession No.: U22316), Brazil (GenBank Accession No.: MN966728), and Ethiopia (GenBank Accession No.: KM391827); *Tvv4*: Study (GP49, GP306 and GP586) and Mozambique (GenBank Accession No.: EU477537).

4.4.4 *T. vivax* diversity in relation to tsetse fly and location in Shimba Hills

The *T. vivaxTvv4* genotype was detected in both male and female tsetse flies (Table 4.2), while the single *TvL1-G* genotype was found in a female tsetse fly. Both genotypes were observed in *Glossina pallidipes*. The tsetse fly positive for the *TvL1-G* genotype was collected from a fruit orchard three kilometres from the boundary of the Shimba Hills National Reserve in Msulwa A (Fig. 4.1 and Table 4.2. S4). Tsetse flies positive for the *Tvv4* were collectedly mostly in grasslands. However, some were also found in fruit orchards, shrubland and cultivated fields within two kilometres from the fence of the Shimba Hills National Reserve in Pengo, Kizibe, and Mlafyeni (Fig.4.1).

Table 4. 2. Details about tsetse flies from which T. vivax isolates were recovered.

Sample ID	Genotype	Block	latitude	longitude	Fly species	Sex	Fly collection date	Vegetation landscape	Elevation (m)	Distance from the SHNR (m)
GP464	*Tvv4*	Pengo	-4.24723	39.36326	*G. pallidipes*	M	February 2019	Grassland	178.00	775.00
GP49	*Tvv4*	Kizibe	-4.27402	39.30951	*G. pallidipes*	F	December 2018	Grassland	181.50	557.08
GP586	*Tvv4*	Kizibe	-4.2715603	39.33925	*G. pallidipes*	M	April 2019	Grassland	178.10	228.81
GP105	*TvL1-G*	Msulwa A	-4.26385	39.46818	*G. pallidipes*	F	June 2019	Fruit orchard	126.00	2292.57
GP306	*Tvv4*	Kizibe	-4.28925	39.27263	*G. pallidipes*	M	September 2019	Fruit orchard	170.30	0.00
GP870	*Tvv4*	Mlafyeni	-4.1903429	39.3789	*G. pallidipes*	F	June 2019	Grassland	121.60	243.00
GP525	*Tvv4*	Mlafyeni	-4.21615	39.39431	*G. pallidipes*	F	February 2019	Cultivated field	171.00	149.48
GP599	*Tvv4*	Pengo	-4.2427876	39.37366	*G. pallidipes*	F	April 2019	Shrub land	206.00	117.70
GP788	*Tvv4*	Mlafyeni	-4.1903355	39.37814	*G. pallidipes*	F	December 2018	Cultivated field	122.80	1679.37

M: male. F: female

*Elevation: metre above sea level

SHNR: Shimba Hills National Reserve

4.5 Discussion

My study in Shimba Hills, Kenya, characterised *T. vivax* infections in tsetse flies, a major vector for trypanosomiasis in the area. Importantly, the study provided valuable insights into the parasite diversity in Shimba Hills and reported the presence of two *T. vivax* genotypes, one of which is here reported for the first time in Kenya. Using molecular markers common to previous studies, it was possible to evaluate this diversity in a regional and global context.

Trypanosoma vivax is ubiquitous in sub-Saharan Africa: The pathogen occurs in wildernesses and livestock-producing areas where tsetse flies and biting flies such as *Stomoxys* sp. and tabanids are present and act as vectors. My detection of *T. vivax* in tsetse flies from Shimba Hills shows that the area provides suitable conditions to sustain the parasite. That I detected infections in tsetse flies at a rate higher than reported in some similar ecologies such as the Msubugwe and Tarangire conservation areas in Tanzania adds evidence to this (Adams et al., 2010). The higher rate of *T. vivax* in Shimba Hills than in Msubugwe and Tarangire could also have been because investigators in Msubugwe and Tarangire used the relatively less sensitive microscopy methods. In contrast, I used sensitive molecular tools to screen tsetse flies for trypanosomes. Nonetheless, my results re-affirm *T. vivax* endemicity in Shimba Hills (Channumsin et al., 2018), with potential for transmission to cattle in smallholder agropastoral communities along the wildlife-livestock interface where I collected the tsetse flies.

The two genotypes *Tvv4* and *TvL1-G* described in Shimba Hills comprised of four haplotypes, three haplotypes for *Tvv4* and a single haplotype for *TvL1-G*. Haplotypes for the *Tvv4* genotype had eight within-genotype polymorphic sites but 65 polymorphic sites when considered together with the single haplotype

for the *TvL1-G* genotype. This reveals a wide genetic variation between both genotypes in Shimba Hills. It also shows the close genetic relationship existing among all the three haplotypes for the *Tvv4* genotype and support the haplotypes' assignment into a single phylogenetic clade named by Rodrigues et al. (2017) as *Tvv4*.

Trypanosomes belonging to the *Tvv4* genotype clade were originally isolated from a wild-caught nyala antelope in the Sofala Province of Mozambique (Rodrigues et al., 2008). Subsequent studies in wildernesses in Mozambique detected the *Tvv4* trypanosome infections in cattle and tsetse flies (Rodrigues et al., 2017). Nakamura et al. (2021) also recently detected the *Tvv4* (reported as TviCatL7 based on the Cathepsin L-like cysteine protease gene sequence) in cattle in Kafue, Zambia's largest and oldest National Park. These results and those from my study suggest that the *Tvv4* genotype is widely distributed over East to Southern Africa.

The study by Nakamura et al. (2021)) reported anaemia in cattle positive for different *T. vivax* genotypes, including the TviCatL7 (*Tvv4*). The *Tvv4*, following its first detection in Nyala, was inoculated into a goat (Rodrigues et al., 2008). The goat developed a range of pathological conditions –severe anaemia, high parasitaemia, fever, and emaciation. The findings by Rodrigues et al. (2008) showed that the *Tvv4* is highly virulent in livestock hence may be a major cause for pathology in cattle in Shimba Hills especially given the high rates of *T. vivax* infections in tsetse flies and livestock in Shimba Hills (unpublished) and the *Tvv4* dominance among isolates in my study. Additionally, a high proportion of *Glossina pallidipes* feeds are from bovids (including cattle) (Weitz, 1963) and the *Tvv4*-infected fly individuals were mostly collected from grasslands where cattle grazing activities are ongoing.

All the *G. pallidipes* found positive for the *Tvv4* trypanosome were collected within the two-kilometre interface area outside of the Shimba Hills National Reserve. This closeness to wildlife suggests that wildlife could potentially be the reservoir and source of infection especially since the *Tvv4* pathogens has to date only been detected in wilderness areas (Rodrigues et al., 2008; Rodrigues et al., 2017; Adams et al., 2010). *Tvv4* detection in wildlife, cattle, and tsetse flies in Mozambique (Rodrigues et al., 2017) adds evidence that tsetse flies, after acquiring the parasite from wildlife, are likely to transmit infections to livestock. In a separate unpublished study that analysed the blood meal sources of tsetse flies, I found evidence of ongoing trypanosome transmission between wildlife and livestock in Shimba Hills and showed that individual tsetse flies with *T. vivax* isolates GP49 and GP586 had respectively fed on warthog and cattle. This finding further strengthens the argument of wildlife as reservoirs for the genotype and with the possibility of transmission to livestock.

The *TvL1-G* genotype comprised of the *T. vivax* IL3905 strain from cattle in Kenya. The genotype infectivity to cattle (Nakamura et al., 2021) demonstrates its epidemiological importance and its detection in my study, portraying it as a potential threat to livestock production along the wildlife interface in Shimba Hills. However, the characterisation of the *TvL1-G* genotype infection in Shimba Hills was based on a single tsetse fly, suggesting this genotype may be less common than the *Tvv4* genotype. The TS06009 isolate that also clustered within the *TvL1-G* clade was from buffalo in the Serengeti National Park in Tanzania (Auty et al., 2012). This lends support to the likely interplay between wildlife and livestock in the circulation of *T. vivax*. Wildlife may be playing a pivotal role as a source of infections to livestock in the Shimba Hills area (Channumsin et al., 2019), similar to what has been documented in the Luangwa Valley of

Zambia (Anderson et al., 2011) among other wildernesses in sub-Saharan Africa (Fetene et al., 2021).

Sequences of *T. vivax* isolates from the tsetse-free camel-keeping areas in the northern region of Kenya segregated into an entirely different *Tvv1* genotype clade, thus bringing the total number of *T. vivax* genotypes identified based on ITS-1 sequences studied in Kenya to three (*Tvv4*, *TvL1-G*, and *Tvv1*). These isolates were supported in the same clade with those from cattle in Nigeria and Burkina Faso. A similar observation has been made in Ethiopia, where *T. vivax* isolates from a tsetse-free region clustered with West African isolates (Fikru et al., 2016). Thus, my study re-affirms the genetic relatedness of *T. vivax* isolates from East and West Africa (Fikru et al., 2016).

A major limitation in my study was that tsetse flies from within the Shimba Hills National Reserve were not included in my sampling. Findings from wilderness areas in Mozambique (Rodrigues et al., 2008; Rodrigues et al., 2017), Kenya (Majiwa et al., 1993), Tanzania (Malele et al., 2003), Central Africa Republic (Votýpka et al., 2015), and recently Zambia (Nakamura et al., 2021) uncovered novel genotypes of trypanosomes, unveiling the potential of sylvatic ecologies to host a wide range of genotypes. My finding in the Shimba Hills wildlife-livestock interface of the *Tvv4* genotype in Kenya is further evidence of this. Thus, I may expect that analysis of tsetse flies from within the Shimba Hills Wildlife Reserve will in subsequent studies, uncover additional genotypes of *T. vivax*. Such studies should in addition to the ITS-1 DNA and 18S rRNA in the current work consider targeting other gene markers for example the glyceraldehyde 3-phosphate dehydrogenase and Cathepsin L-like cysteine protease genes. Consideration of isoenzymes will also be important as it will allow for comparisons with the findings by Fasogbon et al (1990) in coastal

Kenya of a *T. vivax* stock (MID 627) with unique enzyme banding patterns distinct from those of West and other East African stocks.

4.5.1 Conclusion

This is the first report of the *Tvv4* genotype of *T. vivax* in Kenya to the best of my knowledge. The *Tvv4* genotype is dominant in Shimba Hills, a wilderness area where livestock experience severe pathological conditions due to animal trypanosomiasis. The genotypes of *T. vivax* in Shimba Hills may vary in key phenotypes, such as response to available trypanocidal drugs. Since there are currently no clear guidelines for treating *Tvv4* genotype infections with trypanocides, I recommend vector control particularly targeting *G. pallidipes* within 2-3kms of the SHNR boundary to prevent *T. vivax* infections in the Shimba Hills area. More studies will be required to further describe the diversity and pathogenicity of *T. vivax* in Shimba Hills, and the role of wildlife species in the transmission thereof.

Supplementary data

Available online at **https://doi.org/10.1016/j/meegid.2021.104953**

References

1. E.R. Adams, I.I. Malele, A.R. Msangi, W.C. Gibson. Trypanosome identification in wild tsetse populations in Tanzania using generic primers to amplify the ribosomal RNA ITS-1 region. Acta Trop., 100 (1–2) (2006 Nov 1), pp. 103-109
2. E.R. Adams, P.B. Hamilton, A.C. Rodrigues, I.I. Malele, V. Delespaux, M.M. Teixeira, W. Gibson. New Trypanosoma (Duttonella) vivax genotypes from tsetse flies in East Africa. Parasitology., 137 (4) (2010 Apr 1), p. 641
3. N.E. Anderson, J. Mubanga, E.M. Fevre, K. Picozzi, M.C. Eisler, R. Thomas, S.C. Welburn. Characterisation of the wildlife reservoir community for human and animal trypanosomiasis in the Luangwa Valley, Zambia. PLoS Negl. Trop. Dis., 5 (6) (2011 Jun 21), Article e1211
4. H. Auty, N.E. Anderson, K. Picozzi, T. Lembo, J. Mubanga, R. Hoare, R.D. Fyumagwa, B. Mable, L. Hamill, S. Cleaveland, S.C. Welburn. Trypanosome diversity in wildlife species from the Serengeti and Luangwa Valley ecosystems. PLoS Negl. Trop. Dis., 6 (10) (2012 Oct 18), Article e1828
5. H. Auty, L.J. Morrison, S.J. Torr, J. Lord. Transmission dynamics of Rhodesian sleeping sickness at the interface of wildlife and livestock areas. Trends Parasitol., 32 (8) (2016 Aug 1), pp. 608-621
6. H.J. Bandelt, P. Forster, A. Röhl. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol., 16 (1) (1999 Jan 1), pp. 37-48
7. M. Channumsin, M. Ciosi, D. Masiga, C.M. Turner, B.K. Mable. Sodalis glossinidius presence in wild tsetse is only associated with presence of

trypanosomes in complex interactions with other tsetse-specific factors. BMC Microbiol., 18 (1) (2018 Nov), pp. 69-82

8. M. Channumsin, M. Ciosi, D. Masiga, H. Auty, C.M. Turner, B.K. Mable. Blood meal analysis of tsetse flies (*Glossina pallidipes*: Glossinidae) reveals a reduction in host fidelity when feeding on domestic compared to wild hosts. BioRxiv., 1 (2019 Jan), p. 692053
9. R. Echodu, M. Sistrom, R. Bateta, G. Murilla, L. Okedi, S. Aksoy, C. Enyioha, J. Enyaru, E. Opiyo, W. Gibson, A. Caccone. Genetic diversity and population structure of Trypanosoma brucei in Uganda: implications for the epidemiology of sleeping sickness and Nagana. PLoS Negl. Trop. Dis., 9 (2) (2015 Feb 19), Article e0003353
10. A.I. Fasogbon, G. Knowles, P.R. Gardiner. A comparison of the isoenzymes of Trypanosoma (Duttonella) vivax isolates from east and West Africa. Int. J. Parasitol., 20 (3) (1990 May 1), pp. 389-394
11. E. Fetene, S. Leta, F. Regassa, P. Büscher. Global distribution, host range and prevalence of *Trypanosoma vivax*: a systematic review and meta-analysis. Parasit. Vectors, 14 (1) (2021 Dec), pp. 1-20
12. R. Fikru, I. Matetovici, S. Rogé, B. Merga, B.M. Goddeeris, P. Büscher, N. Van Reet. Ribosomal DNA analysis of tsetse and non tsetse transmitted Ethiopian *Trypanosoma vivax* strains in view of improved molecular diagnosis. Vet. Parasitol., 220 (2016 Apr 15), pp. 15-22
13. A.K. Gaithuma, J. Yamagishi, A. Martinelli, K. Hayashida, N. Kawai, M. Marsela, C. Sugimoto. A single test approach for accurate and sensitive detection and taxonomic characterisation of trypanosomes by comprehensive analysis of internal transcribed spacer 1 amplicons. PLoS Negl. Trop. Dis., 13 (2) (2019 Feb 25), Article e0006842

14. H.A. Garcia, C.M. Rodrigues, A.C. Rodrigues, D.L. Pereira, C.L. Pereira, E.P. Camargo, P.B. Hamilton, M.M. Teixeira. Remarkable richness of trypanosomes in tsetse flies (Glossina morsitans morsitans and *Glossina pallidipes*) from the Gorongosa National Park and Niassa National Reserve of Mozambique revealed by fluorescent fragment length barcoding (FFLB). Infect. Genet. Evol., 63 (2018 Sep 1), pp. 370-379
15. P.R. Gardiner, R.K. Assoku, D.D. Whitelaw, M. Murray. Haemorrhagic lesions resulting from *Trypanosoma vivax* infection in Ayrshire cattle. Vet. Parasitol., 31 (3–4) (1989 Jun 1), pp. 187-197
16. F. Giordani, L.J. Morrison, T.G. Rowan, H.P. De Koning, M.P. Barrett. The animal trypanosomiases and their chemotherapy: a review. Parasitology., 143 (14) (2016 Dec), pp. 1862-1889 Hall, 1999
17. T.A. Hall. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series 1999 Jan 1 (Vol. 41, No. 41, pp. 95–98). [London]: Information Retrieval Ltd., c1979-c2000 (1999)
18. P.B. Hamilton, E.R. Adams, I.I. Malele, W.C. Gibson. A novel, high-throughput technique for species identification reveals a new species of tsetse-transmitted trypanosome related to the Trypanosoma brucei subgenus, Trypanozoon. Infect. Genet. Evol., 8 (1) (2008 Jan 1), pp. 26-33
19. M. Hasegawa, H. Kishino, T.A. Yano. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol., 22 (2) (1985 Oct 1), pp. 160-174
20. T.W. Jones, A.M. Dávila. *Trypanosoma vivax*–out of Africa. Trends Parasitol., 17 (2) (2001 Feb 1), pp. 99-101

21. C.M. Kamidi, N.P. Saarman, K. Dion, P.O. Mireji, C. Ouma, G. Murilla, S. Aksoy, A. Schnaufer, A. Caccone. Multiple evolutionary origins of Trypanosoma evansi in Kenya. PLoS Negl. Trop. Dis., 11 (9) (2017 Sep 7), Article e0005895
22. S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol., 35 (6) (2018 Jun 1), pp. 1547-1549
23. V. Lefort, J.E. Longueville, O. Gascuel. SMS: smart model selection in PhyML. Mol. Biol. Evol., 34 (9) (2017 Sep 1), pp. 2422-2424
24. J.W. Leigh, D. Bryant. POPART: full-feature software for haplotype network construction. Methods Ecol. Evol., 6 (9) (2015 Sep 1), pp. 1110-1116
25. J.S. Lord, S.J. Torr, H.K. Auty, P.M. Brock, M. Byamungu, J.W. Hargrove, L.J. Morrison, F. Mramba, G.A. Vale, M.C. Stanton. Geostatistical models using remotely-sensed data predict savanna tsetse decline across the interface between protected and unprotected areas in Serengeti, Tanzania. J. Appl. Ecol., 55 (4) (2018 Jul), pp. 1997-2007
26. J.S. Lord, R.S. Lea, F.K. Allan, M. Byamungu, D.R. Hall, J. Lingley, F. Mramba, E. Paxton, G.A. Vale, J.W. Hargrove, L.J. Morrison. Assessing the effect of insecticide-treated cattle on tsetse abundance and trypanosome transmission at the wildlife-livestock interface in Serengeti, Tanzania. PLoS Negl. Trop. Dis., 14 (8) (2020 Aug 25), Article e0008288
27. G.J. Losos, B.O. Ikede. Review of pathology of diseases in domestic and laboratory animals caused by Trypanosoma congolense, *T. vivax*, *T. brucei*, T. rhodesiense and T. gambiense. Vet. Pathol., 9 (1_suppl) (1972 Jan), pp. 1-79

28. J.W. Magona, J. Walubengo, J.T. Odimin. Acute haemorrhagic syndrome of bovine trypanosomosis in Uganda. Acta Trop., 107 (2) (2008 Aug 1), pp. 186-191
29. P.A. Majiwa, M. Maina, J.N. Waitumbi, S. Mihok, E. Zweygarth. Trypanosoma (Nannomonas) congolense: molecular characterisation of a new genotype from Tsavo, Kenya. Parasitology, 106 (2) (1993 Feb), pp. 151-162
30. Malele, L. Craske, C. Knight, V. Ferris, Z. Njiru, P. Hamilton, S. Lehane, M. Lehane, W. Gibson. The use of specific and generic primers to identify trypanosome infections of wild tsetse flies in Tanzania by PCR. Infect. Genet. Evol., 3 (4) (2003 Nov 1), pp. 271-279
31. P.K. Malonza, D.M. Mulwa, J.O. Nyamache, G. Jones. Biogeography of the Shimba Hills ecosystem herpetofauna in Kenya. Zool. Res., 39 (2) (2018 Mar 18), p. 97
32. D.A. Maslov, J. Lukeš, M. Jirku, L. Simpson. Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. Mol. Biochem. Parasitol., 75 (2) (1996 Jan 1), pp. 197-205
33. Y. Nakamura, K. Hayashida, V. Delesalle, Y. Qiu, R. Omori, M. Simuunza, C. Sugimoto, B. Namangala, J. Yamagishi. Genetic diversity of African trypanosomes in tsetse flies and cattle from the Kafue ecosystem. Front. Vet. Sci., 8 (2021 Jan 27), p. 5
34. M.O. Ng'ayo, Z.K. Njiru, E.U. Kenya, G.M. Muluvi, E.O. Osir, D.K. Masiga. Detection of trypanosomes in small ruminants and pigs in western Kenya: important reservoirs in the epidemiology of sleeping sickness?. Kinetoplastid Biol. Dis., 4 (1) (2005 Dec), pp. 1-7

35. Z.K. Njiru, C.C. Constantine, J.M. Ndung'u, I. Robertson, S. Okaye, R.C. Thompson, S.A. Reid. Detection of Trypanosoma evansi in camels using PCR and CATT/T. evansi tests in Kenya. Vet. Parasitol., 124 (3–4) (2004 Oct 5), pp. 187-199
36. Z.K. Njiru, C.C. Constantine, S. Guya, J. Crowther, J.M. Kiragu, R.C. Thompson, A.M. Dávila. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol. Res., 95 (3) (2005 Feb 1), pp. 186-192
37. J.N. Pollock (Ed.), Training Manual for Tsetse Control Personnel, FAO, Rome (1982)
38. A.C. Rodrigues, L. Neves, H.A. Garcia, L.B. Viola, A. Marcili, F.M. Da Silva, I. Sigauque, J.S. Batista, F. Paiva, M.M. Teixeira. Phylogenetic analysis of *Trypanosoma vivax* supports the separation of South American/West African from East African isolates and a new *T. vivax*-like genotype infecting a nyala antelope from Mozambique. Parasitology, 135 (11) (2008 Sep 1), p. 1317
39. C.M. Rodrigues, H.A. Garcia, A.C. Rodrigues, A.G. Costa-Martins, C.L. Pereira, D.L. Pereira, Z. Bengaly, L. Neves, E.P. Camargo, P.B. Hamilton, M.M. Teixeira. New insights from Gorongosa National Park and Niassa National Reserve of Mozambique increasing the genetic diversity of *Trypanosoma vivax* and *Trypanosoma vivax*-like in tsetse flies, wild ungulates and livestock from East Africa. Parasit. Vectors, 10 (1) (2017 Dec 1), p. 337
40. J. Rozas, A. Ferrer-Mata, J.C. Sánchez-DelBarrio, S. Guirao-Rico, P. Librado, S.E. Ramos-Onsins, A. Sánchez-Gracia. DnaSP 6: DNA sequence

polymorphism analysis of large data sets. Mol. Biol. Evol., 34 (12) (2017 Dec 1), pp. 3299-3302

41. D. Squarre, K. Hayashida, A. Gaithuma, H. Chambaro, N. Kawai, L. Moonga, B. Namangala, C. Sugimoto, J. Yamagishi. Diversity of Trypanosomes in Wildlife of the Kafue Ecosystem Zambia. International Journal for Parasitology, Parasites and Wildlife (2020 Apr 23)
42. B. Von Wissmann, N. Machila, K. Picozzi, E.M. Fèvre, M. Barend, I.G. Handel, S.C. Welburn. Factors associated with acquisition of human infective and animal infective trypanosome infections in domestic livestock in western Kenya. PLoS Negl. Trop. Dis., 5 (1) (2011 Jan 18), Article e941
43. J. Votýpka, J. Rádrová, T. Skalický, M. Jirků, D. Jirsová, A.D. Mihalca, G. D'Amico, K.J. Petrželková, D. Modrý, J. Lukeš. A tsetse and tabanid fly survey of African great apes habitats reveals the presence of a novel trypanosome lineage but the absence of Trypanosoma brucei. Int. J. Parasitol., 45 (12) (2015 Oct 1), pp. 741-748
44. B. Weitz. The feeding habits of Glossina. Bull. World Health Organ., 28 (5–6) (1963), p. 711

CHAPTER 5

Characterising the epidemiological effect of tsetse control in trypanosome hotspots in Shimba Hills, Kenya: A cluster randomised controlled trial using insecticide-treated fabrics *with* and *without* tsetse-repellent odours on cattle

---

This chapter has been prepared for submission to *PLoS Neglected Tropical Diseases*:

Faith I. Ebhodaghe, Armanda D. S. Bastos, Shewit Kalayou**,** Daniel K. Masig**a** & Michael N. Okal (2022). Characterising the epidemiological effect of tsetse control in trypanosome hotspots in Shimba Hills, Kenya: A cluster randomised controlled trial using insecticide-treated fabrics *with* and *without* tsetse-repellent odours on cattle. Manuscript prepared for submission to *PLoS Neglected Tropical Diseases*.

5.1 Abstract

Tailoring disease-vector control to epidemiological hotspots could potentially accelerate reductions in pathogen risk in human and animal populations. Efficacy of interventions against pathogens may be further increased when insecticide-treated materials used to manage arthropod-vectors in disease control programmes are combined with repellent odours that reduce vector-host contacts. This cluster randomised controlled trial conducted in the Shimba Hills wildlife-livestock interface in Kenya deployed insecticide-treated fabrics (ITFs) to tsetse-borne trypanosome hotspots within one thousand metres from the Shimba Hills National Reserve. In addition, a synthetic blend of waterbuck-mimicking tsetse-repellent odours was applied on cattle in the intervention arm but not in the control arm of the trial. ITFs were allocated to both arms, each of which required 32 clusters to detect an intervention-effect of 70.00% at 80.00% power and 95.00% confidence level assuming 5.00% attrition rate and Design-Effect of 2. Cattle trypanosome infections were diagnosed using the buffy-coat technique. Five months into the trial, trypanosome risk reduced significantly with a >50.00% decline in cattle infection rate. Within this period, significant reductions in risks were observed for *T. congolense* in both treatment arms and for *T. vivax* in the intervention arm but never in the control arm, where *Stomoxys* abundance was significantly higher. Combining tsetse-repellent odours to ITFs further reduced incidences of cattle infections but this occurred below the 70.00% threshold specified *a priori*. Unadjusted and adjusted odds ratios based on *as-treated, intention-to-treat* and *per-protocol* analyses indicated that trypanosome risks were not significantly different between treatment arms. These results indicate that ITFs targeted to trypanosome hotspots are able to effectively reduce cattle infections in Shimba Hills and may not require integration with tsetse-repellent odours on

cattle. However, further studies are needed to unravel *Stomoxys* involvement in *T. vivax* epidemiology in Shimba Hills.

5.2 Author summary

Targeting insecticide-treated blue-black fabrics to trypanosome hotspots in interventions that use repellent odours to minimize tsetse-host contacts could accelerate reductions in the incidence of tsetse-borne trypanosomiasis. A cluster randomised controlled trial (RCT) in Shimba Hills (Kenya) revealed significant reductions in cattle trypanosome infections five months after deployment of insecticide-treated fabrics to the parasite hotspots. Reductions were more apparent for the trypanosome *Trypanosoma congolense* (transmitted by tsetse flies) than *T. vivax* (transmitted by both tsetse flies and mechanical vectors). Combining insecticide-treated fabrics with synthetic tsetse-repellent odour application on cattle further reduced trypanosome incidence but below the expected level. The cluster RCT confirmed that hotspot-targeted insecticide-treated fabrics are effective for trypanosome tsetse-vector control in Shimba Hills and may not necessarily require integration with tsetse-repellent odours. Further studies at the human-wildlife-livestock interface in Shimba Hills are, however, required to unravel the role of mechanical vectors in *T. vivax* epidemiology in the area.

5.3 Introduction

Disease-vector control programmes are implemented to interrupt transmission, reduce incidences, and alleviate the burden of human and animal pathogens (Wilson et al. 2020). Most of these programmes are led by resource-constrained communities thus require cost-effective and locally adaptable vector control tools (Pérez et al. 2021).

Insecticide-treated nets that reduce vector-host contacts and fabrics that visually attract vectors to insecticide-treated surfaces are examples of vector control tools used in community-led disease interventions. The low-cost and ease-of-use of insecticide-treated nets and fabrics and the fact that they are non-hazardous have encouraged resource-limited communities in and outside sub-Saharan Africa to widely engage these tools in the control of arthropods that transmit economically important diseases known to retard rural development and constrain smallholder farmer livelihoods (Wilson et al. 2014; Han et al. 2020).

Trypanosomiasis is among important public health and economic constraints in under-developed communities. Chemotherapy is extensively adopted as the mainstay of disease management (Brun et al. 2010; Giordani et al. 2016). However, chemotherapeutic interventions against tsetse-borne trypanosome parasites responsible for human and animal trypanosomiases in sub-Saharan Africa are confronted by a plethora of limitations. These include problems of trypano-resistance (Mungube et al. 2012; Sow et al. 2012) and re-infection of treated humans and livestock especially in sylvatic environments in East and Southern Africa where wildlife harbour and act as cryptic sources of tsetse-borne trypanosomes (Anderson et al. 2011; Auty et al. 2012; Büscher et al. 2018; Kasozi et al. 2021).

Aerial-wide control of tsetse flies in sub-Saharan Africa have historically significantly reduced trypanosome transmission and incidence in human and animal populations, but interventions were not sustainable due to high cost of interventions, adverse effects of insecticides on non-target organisms, and reinvasion of controlled areas by remnant tsetse flies (Torr et al. 2005). However, many of these challenges can be overcome by community-led intervention programmes that restrict insecticide applications to locally constructed stationary baits, comprising of traps and pieces of fabrics called targets (Barrett & Okali, 1998; Kuzoe & Schofield, 2005; Meyer et al. 2016).

Tsetse-borne trypanosome transmission risks are spatially heterogeneous in local ecologies where human and animal trypanosomiases are endemic (Stone & Chitnis 2015). Stationary bait technologies are cost-effective for control of tsetse flies and may have greater impact in reducing trypanosome incidence when assigned to parasite epidemiological hotspots. However, data describing trypanosome transmission patterns and hotspot locations remain sparse in many tsetse infested areas particularly sylvatic landscapes (Auty et al. 2016). Consequently, intervention programmes that use insecticide-treated traps and targets are unable to rationally deploy these tools in a manner that optimises outcomes.

In a study conducted in Ghana, Bauer et al. (2011) noted that insecticide-treated nets that protect livestock from tsetse infectious bites provide an affordable option for management of animal trypanosomiasis. They observed a ~90% reduction in infection rates in intervention areas versus 17% in non-intervention areas, after six months of field-trailing insecticide-treated nets. Despite their epidemiological impact in reducing the incidence of trypanosome infections, insecticide-treated nets protect livestock primarily in zero-grazing systems hence are less useful in areas like Shimba

Hills in southeast Kenya where farmers adopt the semi-extensive grazing system for livestock production.

Results from field trials with tsetse-repellent technologies in Kenya have been contradictory. In a field trial conducted in Shimba Hills (Saini et al. 2017) cattle were protected from tsetse infectious bites using novel semio-chemical based devices called the waterbuck repellent technology comprising of a 4-component blend of synthetic tsetse-repellent odours mimicking allomonal volatiles emitted by waterbuck. These results contrast with previous findings in Nkuruman and Nkineji in southwest Kenya where tsetse-repellent 2-methoxy 4-methyl phenol was shown to be ineffective in protecting cattle from tsetse infectious bites (Bett et al. 2010). The waterbuck (WB) repellents used in the Shimba Hills study were discharged in controlled releases from fabricated metallic dispensers tied to collars (henceforth WB collars) worn around the necks of cattle. These WB collars reduced trypanosome rate in cattle by >80.00% within 24 months (Saini et al. 2017). The intervention also assessed the epidemiological effect of insecticide-treated fabrics, but the outcome was lower compared to the effect of the WB collars, possibly because ITFs were deployed to random rather than targeted sites.

Prior modelling studies indicate higher intervention-effect in control programmes that combine insecticide-treated stationary baits with tsetse-repellent odours on cattle (Bett et al. 2003). This is not unconnected to the fact that both tools work synergistically with the repellent odours pushing tsetse flies from feeding on treated cattle and the stationary baits pulling the vectors to killing points where they pick up lethal chemical doses. However, Saini et al. (2017) were unable to demonstrate a clear benefit when combining insecticide-treated fabrics (ITFs) and WB collars, likely due to random deployment of ITFs.

In Chapter 2, I identified trypanosome hotspot locations in Shimba Hills where cattle are exposed to high risk of parasite infections from tsetse flies. This Cluster Randomised Controlled Trial (RCT) seeks to address limitations in the intervention by Saini et al. (2017) in Shimba Hills regarding deployment of ITFs for control of tsetse flies by assigning ITFs to trypanosome hotpots in the Shimba Hills wildlife-livestock interface. The ITFs used in the present study have been demonstrated to effectively control savannah tsetse flies (Byamungu et al. 2018), including *Glossina pallidipes* which in Shimba Hills are the most widely distributed and highly abundant tsetse flies (Chapter 2) with evidence from preliminary surveys (Chapter 3) showing that the vector species feed copiously on cattle and are possibly infecting livestock in the area with pathogenic and virulent trypanosomes acquired from wildlife reservoirs, particularly warthogs. This study main objective was to assess the effect of hotspot-targeted treated fabrics on animal trypanosome risk in areas where cattle are treated with tsetse repellent odors compared to other areas where cattle are without the tsetse repellent odors. I hypothesized that there would be a significant increase in ITF-effect on trypanosome incidence where ITFs strategically deployed to the parasite hotspots are complemented with WB collars on cattle. Finally, I collected entomological data on the abundance of tsetse flies and other dipterans (stable flies and horse flies) and compared results to findings on parasitological rates of cattle infections.

5.4.0 Methods

The trial is reported according to guidelines outlined in The Consolidated Standards of Reporting Randomised Trials (CONSORT) statement (Moher et al. 2001).

5.4.1 Ethical consent

Permission for the study was granted by the Kenyan National Commission for Science, Technology, and Innovation (License No.: NACOSTI/P/20/7344). Farmers in Shimba Hills gave verbal consent for recruitment of their cattle into the study and permission to screen animals for trypanosome infections. Entomological surveys were carried out in conjunction with the Kenya Wildlife Service (KWS), the Kenya Tsetse and Trypanosomiasis Eradication Council (KENTTEC) and local communities in Shimba Hills.

5.4.2 Setting

The study was conducted in local communities in the wildlife-livestock interface of the Shimba Hills National Reserve (Fig. 5.1), an East African biodiversity hotspot located in the southeast coastal region of Kenya (Malonza et al. 2018; Ochieng et al. 2020). The study communities in Shimba Hills extend across Kubo South, Mkongani and Tsimba/Golini administrative wards in the Matuga sub-County in Kwale County (Government of Kenya, 2018). Kwale County is ~8,000 km square with warm-moist climate (annual average temperature and rainfall amount being respectively ~24 degree Celsius and 1150 mm), bimodal rainfall pattern (long rains from March to July and Short rains from October to December), and vegetation comprising of forests, savannah grassland, woodland and shrubland. The human population size in Kwale is ~9,000 people, many of whom are farmers cultivating crops and raising livestock especially cattle —whose production in the area is constrained by infectious diseases, mainly tsetse-borne trypanosomiasis (Saini et al. 2017; Muriithi et al. 2021).

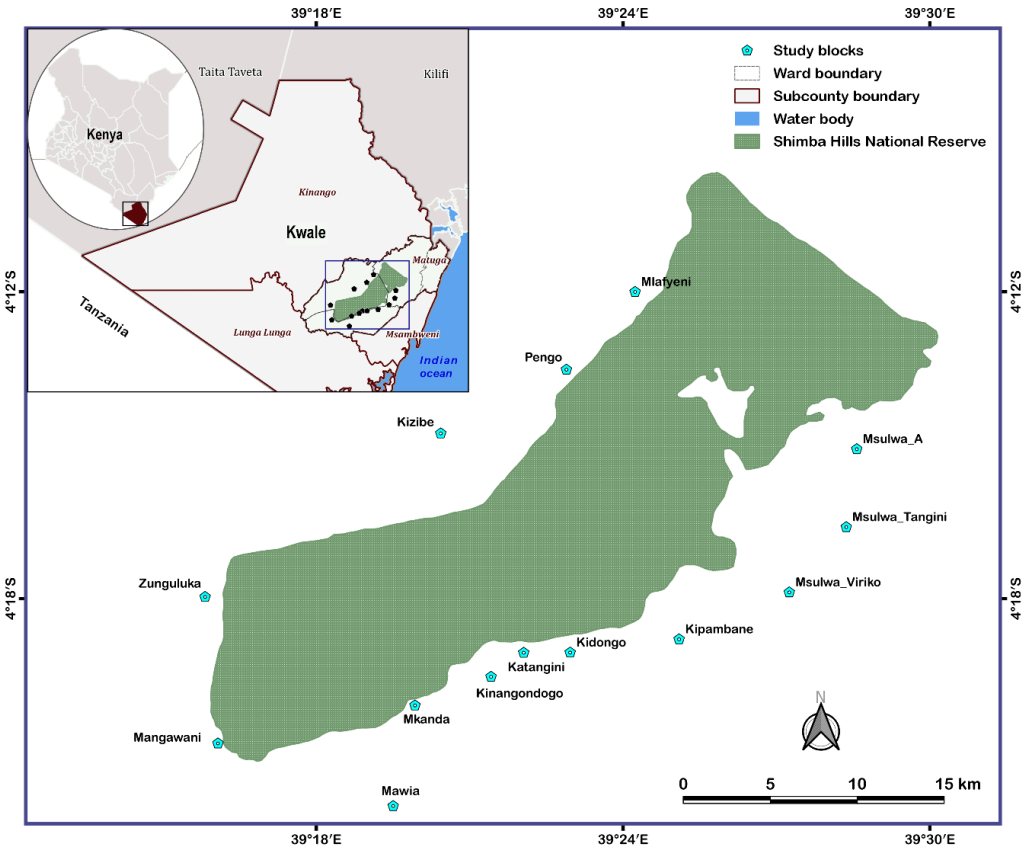


Figure 5. 1: Map of Kenya showing Kwale county (top left) and the 13 study locations in Shimba Hills (green)

5.4.3 Participants and baseline survey

Cattle were recruited from geographically segregated blocks into the parallel cluster randomised controlled trial (RCT). Preliminary epidemiological surveys in Shimba Hills had partitioned the area into 14 blocks. For a block to be considered for inclusion in the study, herds within it were to have complete or zero repellent coverage of all cattle individuals. Kizibe block (between Pengo and Zunguluka, Fig 5.1) was excluded as repellent treatments covered only a proportion of cattle individuals in certain herds within the block. Hence, the study recruited cattle from only 13 blocks instead of 14 blocks. Eligibility criteria for cattle inclusion in the trial were that

animals were domiciled in Shimba Hills, raised in semi-extensive grazing systems, and owned by smallholder farmers, resident in one of the study-blocks in Shimba Hills and willing to participate in the study. For the baseline survey, 1,032 cattle were sampled in November 2020 based on the purposive method and screened by a team of trained and experienced technical field staff for trypanosome infections. Blood aliquots were collected from cattle assembled in central crush-pens within blocks, following the procedures described previously (Chapter 2). Data were also collected on cattle heart girth measurement and packed cell volume (PCV).

5.4.4 Sample size

The WB tsetse-repellent collars alone had in a prior field trial in Shimba Hills reduced cattle trypanosome infection rates by >80.00% (Saini et al. 2017). The present Cluster RCT, therefore, hypothesized that the tsetse-repellent collars could have an additional intervention-effect of 70.00% in reducing infection rate in Shimba Hills when used in combination with ITFs. Consequently, I anticipated a post-intervention AAT rate of 1.89% given a cattle infection rate of 6.30% obtained in the November 2020 baseline parasitological survey in Shimba Hills. It was determined that a minimum sample size of 1,030 individual cattle with 32 herd-clusters per treatment group at 80.00% power, 95.00% significance level and Design-Effect of 2 was required to detect a 70.00% intervention effect. Cattle herds in the intervention arm and control arm that had fewer cattle than required for a cluster were merged with other small herds from the same block. A Coefficient of Variation (*CV*) of 0.51 was estimated from baseline data and considered in sample size calculation. Intra-cluster Correlation Coefficient (*k*) of 0.03 was derived from baseline data using the *ICCbin* package (Chakraborty & Hossain, 2018) in the *R* statistical environment (Team RC, 2013). The *k* together with the *CV*, an average cluster-size (*m*) of 16 cattle and assumed attrition

rate of 5.00% resulted in the Design-Effect of 2 [= 1 + {(CV^2 + 1) * (m – 1)} * k] (Eldridge et al. 2006) used to calculate sample size.

5.4.5 Intervention (December 2020 to August 2021)

Cattle randomised to the intervention arm received one WB tsetse-repellent collar each while those in the control arm were without the collar. The tsetse-repellent collar and the chemical composition thereof have been previously described (Bett et al. 2015; Saini et al. 2017). However, the present study used a modified version of the collar that releases repellent odours from an absorbent material instead of liquid formulation, which had the limitation of repellent leakage. All cattle in herds in the intervention arm were assigned repellent collars since treatment effects could spillover from treated to untreated cattle within close proximity to each other. The study-blocks in Shimba Hills are geographically apart and cattle grazing activities are mostly limited to within blocks.

Mkanda, one of the 13 study-blocks, had very few cattle. As a result, Mkanda was merged with an adjacent block (Kinangondogo; Fig. 5.1) thus resulting in a total of 12 study-blocks for the cluster RCT. The 12 blocks were partitioned into 6 zones, each comprising of 2 adjacent blocks. Each of the 6 zones received both treatments. WB collars were randomly assigned to cattle herds in one block within a zone while animals in cattle herds in the other block within the same zone were without collars.

I designed the experiment and analysed the data collected by the team of field staff. The field staff collected and delivered the data to me without concealing which treatment group a herd belonged to. Deltamethrin insecticide-treated fabrics (ITFs) made of blue-black fabrics were deployed to the intervention arm and control arm after the baseline parasitological survey (conducted in November 2020). Therefore, the intervention arm had the WB tsetse-repellent collars plus ITFs and the control

arm had ITFs without the WB tsetse-repellent collars. The control devices were deployed throughout the Shimba Hills wildlife-livestock interface at a density of 4 ITFs per km square in sites within one thousand metres from the Shimba Hills National Reserve.

5.4.6 Follow-up

Trypanosome infections were monitored in cattle in five bimonthly parasitological surveys from December 2020 to August 2021, first in December 2020, and subsequently in January-February, March-April, May-June and July-August 2021. Cattle were screened for *Trypanosoma* species using microscopy specifically the buffy-coat technique to increase sensitivity. Blood samples were collected in the mornings when trypanosomes are easy to detect in cattle peripheral blood. Cattle, after they were assessed for heart girth sizes using specially calibrated weighting bands (Rondo, UK), were pricked on their ear veins using sterilised lancets. Blood samples were collected into capillary tubes, centrifuged, assessed for packed cell volume using a microhaematocrit reader (Hawksley Ltd, UK) and screened for trypanosomes morphologically identified as *T. congolense* or *T. vivax*. Trypanosome-infected cattle and the livestock with PCV below 20 were administered Diminazene aceturate (Veriben® manufactured in France by Ceva Sante Animale) free of charge to farmers at a dose of 3 mg/kg.

The field staff during follow-up surveys monitored compliance by inspecting cattle in the intervention arm for tsetse-repellent collars with records taken of cattle lost to follow-up. Collars were recharged once every month throughout the period of the intervention. Further, cow urine and acetone baited biconical traps were deployed to monitor abundance of tsetse flies (*Glossina* species) in entomological surveillance which also collected stable flies (*Stomoxys* species) and horse flies (*Tabanus* species).

Traps were deployed at a density of one trap per km square throughout the Shimba Hills wildlife-livestock interface over an area ~230 km sq within 5 km from the Shimba Hills National Reserve. However, entomological surveys, due to logistic challenges, did not begin until February 2021 after an initial survey in June-July 2020 and lasted until June 2021. Fly collections in traps were morphologically identified and sorted into species and sex (Leak et al. 2008).

5.4.7 Statistical analyses

Poisson-Generalized Linear Model (P-GLM) analysis was used to compare the number of cattle between treatment groups at baseline. Chi-square tests were performed to assess models for goodness-of-fit by comparing the residual deviance and degrees of freedom. Furthermore, trypanosome risk differences between treatment groups and the effects of cattle sex, study-block, girth measurements and PCV on the same were assessed in Binomial-GLMs. Cattle girth measurements and PCV were significantly different from normal distribution ($P < 0.05$) using the Shapiro-Wilk's test thus were assessed for variations between treatment groups using the unpaired Wilcoxon-Mann-Whitney test.

Using data sets that considered all cattle individuals screened for infections in bimonthly surveys (*as-treated* analysis), originally randomised to treatment groups whether or not they have been lost to follow-up (*intention-to-treat* analysis) and that completed the trial without being lost to follow-up (*per-protocol* analysis), B-GLMMs with cattle individuals as random-effect were used to compare cattle infection risk between sampling months within treatment groups. Furthermore, odds ratios unadjusted and adjusted for baseline imbalances were calculated to assess for significant differences in cattle infections between treatment groups using *as-treated*, *intention-to-treat* and *per-protocol* analyses.

Finally, Negative Binomial GLMMs with '*Trap_ID*' as random effect were fitted to compare abundance of tsetse flies, *Stomoxys* species and *Tabanus* species between sampling months within treatment groups and throughout the study between treatment groups. All analyses were carried out in the *R* statistical environment (Team 2013) and GLMs and GLMMs respectively in the *MASS* (Ripley et al. 2013) and GLMMTMB (Brooks et al. 2017) packages. *Alpha*-level was 0.05 and pairwise comparisons for significant GLMMs implemented in the '*multcomp*' package (Hothorn et al. 2016) using the *Tukey's post-hoc* test.

5.5.0 Results

5.5.1 Baseline data (November 2020)

Cattle sample sizes of 562 (54.46%) in the intervention arm and 470 (45.54%) in the control arm were well balanced between the two treatment groups. The proportions of female cattle (57.47% and 58.94% respectively in the intervention arm and control arm) and male cattle (42.53% and 41.06% respectively in the intervention arm and control arm) were also well balanced between the treatment groups.

Cattle sex had no significant effect on cattle infection risk. However, cattle in the control arm had significantly higher risk of *Trypanosoma* species and *T. vivax* infections. Meanwhile, risk of *T. congolense* infection was similar between the control arm and the intervention arm. Furthermore, study-block had a significant effect on the likelihood of *Trypanosoma* species (*T. vivax* but not *T. congolense)* infection in cattle. Cattle girth measurements and anaemia scores were similar between the intervention arm and the control arm. Anaemia score but not girth measurement had a significant association with the presence of *Trypanosoma* species, *T. congolense* and *T. vivax* infections in cattle.

The study accounted for baseline variations in cattle risk of infections. Specifically, baseline differences in *Trypanosoma* species and *T. vivax* were respectively adjusted for in odds ratio analyses assessing for intervention-effects on *Trypanosoma* species and *T. vivax* incidences. Odds ratio analyses also accounted for baseline variations in anaemia score (for *Trypanosoma* species, *T. vivax* and *T. congolense* infections) and the possible confounding effect of study-block (for *Trypanosoma* species and *T. vivax* infections).

Table 5. 1: Baseline characteristics of cattle in the cluster RCT in Shimba Hills (November 2020)

	Control	Intervention
Sex		
Female	277 (58.94%)	323 (57.47%)
Male	193 (41.06%)	239 (42.53%)
Block		
Katangini	—	76 (13.52%)
Kidongo	29 (6.17%)	—
Kinango Ndogo	34 (7.23%)	—
Kipambane	47 (10.00%)	—
Mangawani	116 (24.68%)	—
Mawia	—	100 (17.79%)
Mkanda	22 (4.68%)	—
Mlafyeni	—	35 (6.23%)
Msulwa_A	124 (26.38%)	—
Msulawa_Tangini	—	219 (38.97%)
Msulwa_Viriko	—	88 (15.66%)
Pengo	98 (20.85%)	—

Zunguluka	—	44 (7.83%)
Girth Measurement	161.19 cm (95% CI: 155.87—166.52)	157.54 cm (95% CI: 152.78—162.30)
Packed Cell Volume	27.56 (95% CI: 27.17—27.95)	27.56 (95% CI: 27.20—27.93)
Infection Status		
Trypanosoma species	8.51% (95% CI: 5.98—11.04)	4.45% (95% CI: 2.74—6.16)
Trypanosoma congolense	5.32% (95% CI: 3.28—7.36)	3.74% (95% CI: 2.16—5.31)
Trypanosoma vivax	3.19% (95% CI: 1.60—4.79)	0.89% (95% CI: 0.11—1.67)

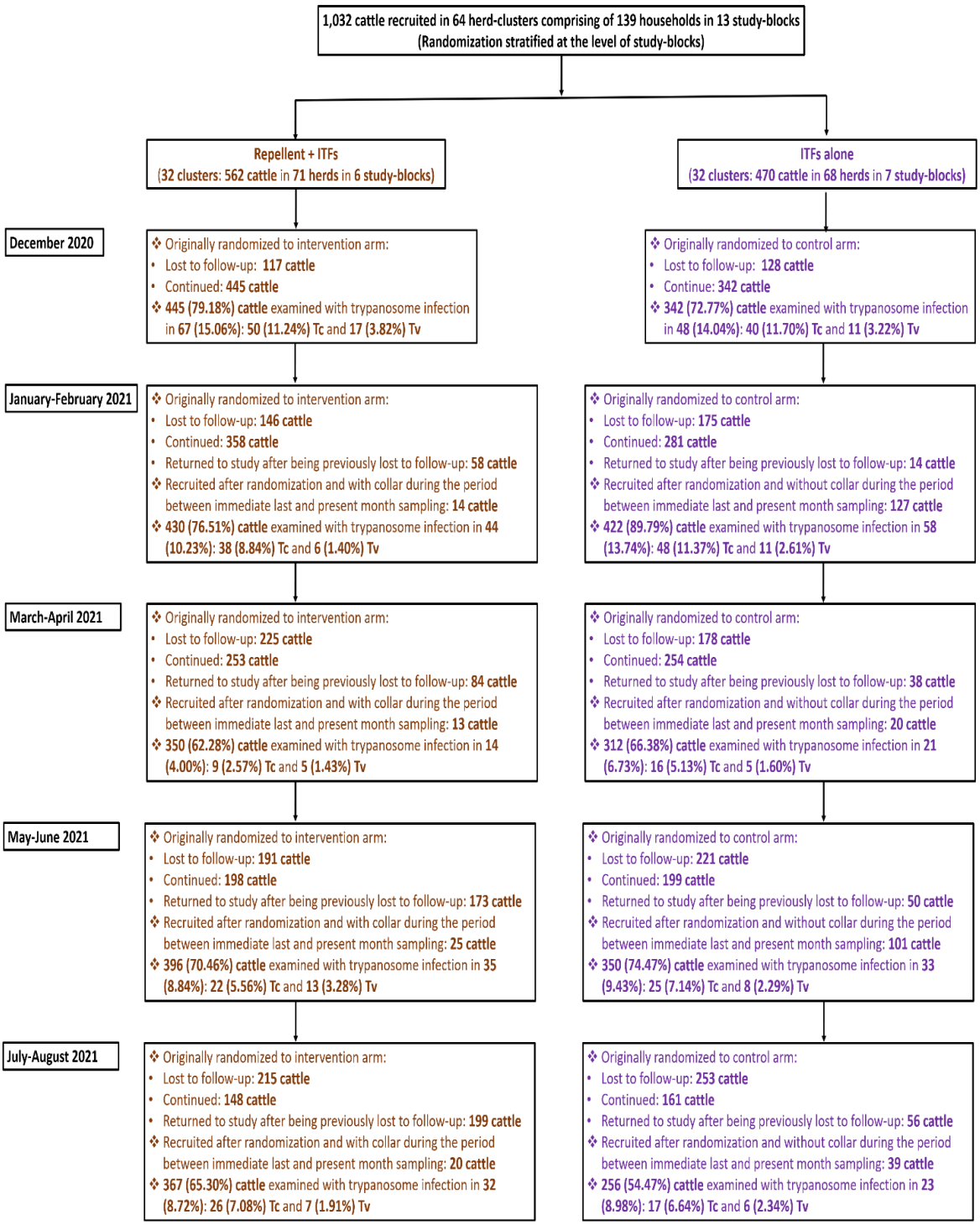


Figure 5. 2: A flow chart showing the number of cattle screened for trypanosomes on five occasions, and the number of cattle lost to follow-up during the cluster RCT in Shimba Hills

5.5.2 Participant flow

Overall, 1,032 cattle in 139 households spread out across 13 study-blocks in Shimba Hills were randomized to 2 treatment arms in November 2020 (Fig. 2). This comprised of 562 cattle in 71 households in the intervention arm and 470 cattle in 68 households in the control arm. Overall number of herd-clusters was 64 (32 clusters per treatment arm) after combining households that had small herd sizes within study-blocks.

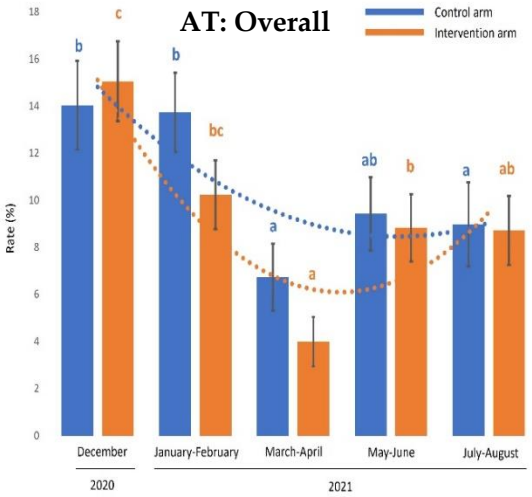
A total of 723 cattle out of the 1,032 cattle recruited into the study were lost to follow-up at least once during bimonthly parasitological surveys from December 2020 to August 2021 (Fig. 2). Cattle were lost to follow-up due to farmer-relocation outside the study area in Shimba Hills and animal offtake for slaughter. There were also cases of farmers who took their cattle out for grazing in the field at the time of parasitological surveys thus these animals could not be screened for infections. However, some of these animals lost to follow-up due to grazing were presented for trypanosome screening in subsequent surveys. The study also recruited other cattle aside from those enrolled at baseline in November 2020. Cattle later recruited in bimonthly surveys were assigned repellent collars if in the intervention arm and went without collars if in the control arm and were screened for trypanosome infections in successive parasitological samplings.

5.5.3 Trial outcome

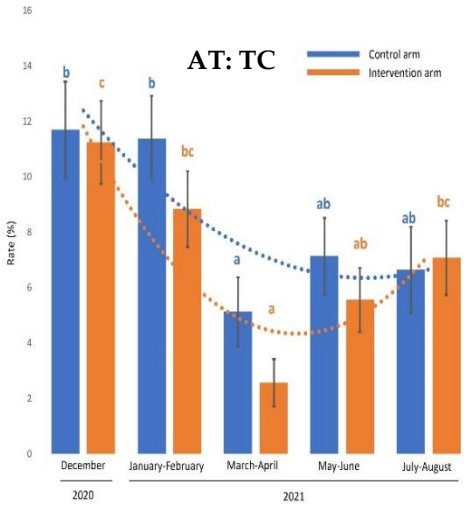
The initial cross-sectional cattle infection rate of 14.04% in the control arm and 15.06% in the intervention arm in December 2020 was reduced to 8.98% and 8.72% in the respective arms in August 2021 (Fig. 5.2). Significant reductions in *Trypanosoma* species and *T. congolense* risk were achieved within the arms in March-April 2021 (five months into the study) (Fig 5.3). *Trupanosoma vivax* risk was also significantly reduced in the intervention arm but the reduction in the control arm was not significant within this period.

Different intervention-effects on cattle infection were obtained for *Trypanosoma* species, *T. congolense* and *T. vivax* using *as-treated*, *intention-to-treat* and *per-protocol* analyses and was generally below 12.00%. The only exception was for *T. vivax* for which I obtained an intervention-effect of 50.00% using the *per-protocol* analysis. Further analyses based on unadjusted and adjusted Odds Ratio revealed that intervention-effects were not significant irrespective of trypanosome infection and analytical method used (Tables 5.2 to 5.8).

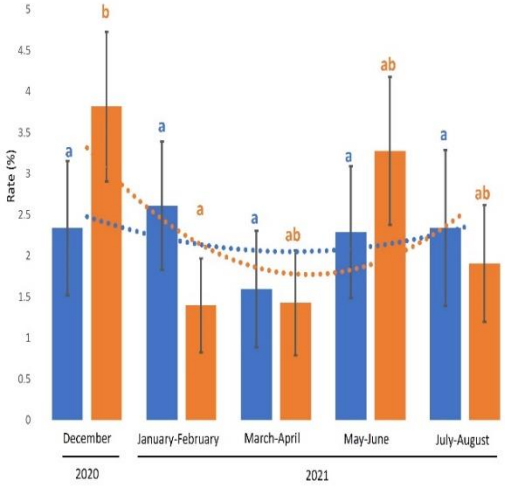
AT: Overall



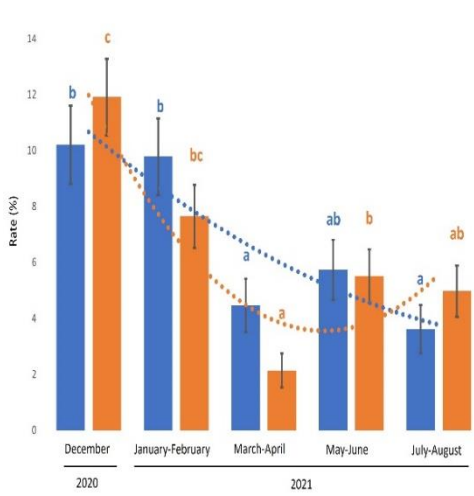
AT: TC



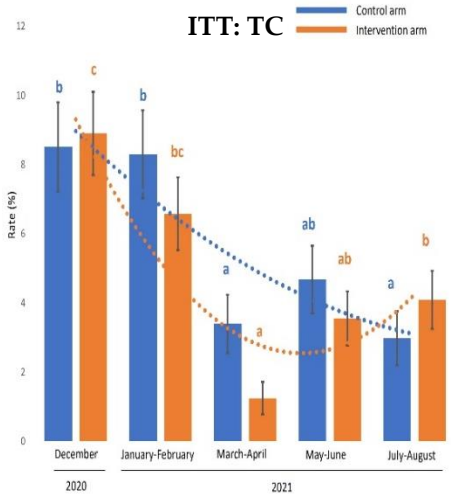
AT: TV



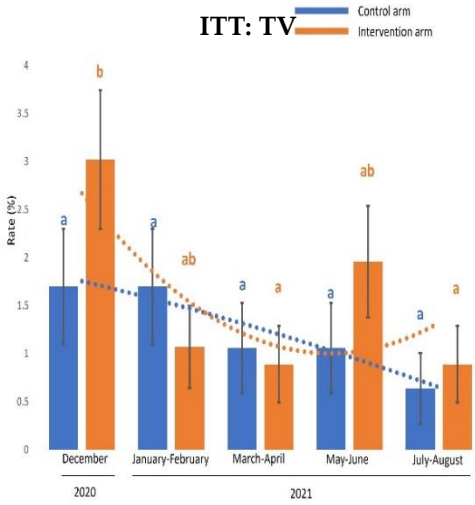
ITT: Overall



ITT: TC



ITT: TV



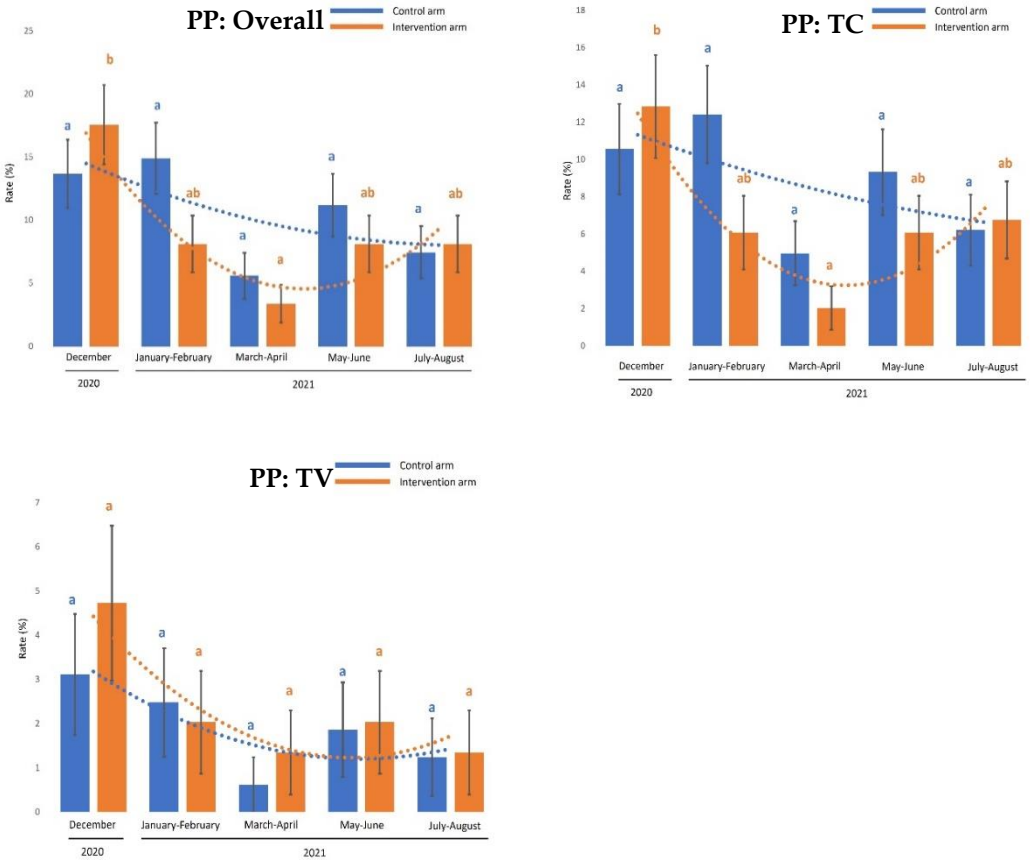


Figure 5. 3: Bar Charts with polynomial curves showing proportion of cattle infections during the cluster RCT in Shimba Hills. AT (As-Treated analysis). ITT (Intention-To-Treat analysis). PP (Per-Protocol analysis). Overall (*T. congolense* + *T. vivax*). TC (*T. congolense*). TV (*T. vivax*).

Table 5. 2: *Trypanosoma* species (*As-treated* analysis)

	No. examined (control/intervention)	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value
Survey 1	342/445	1.09 (0.73 – 1.63)	0.69	0.46 (0.06 – 2.15)	0.19
Survey 2	422/430	0.72 (0.41 – 1.08)	0.12	0.55 (0.14 – 2.07)	0.73
Survey 3	312/350	0.58 (0.28 – 1.15)	0.12	0.61 (0.15 – 2.19)	1.00
Survey 4	350/396	0.93 (0.56 – 1.54)	0.78	0.64 (0.14 – 2.79)	0.33
Survey 5	256/367	0.97 (0.55 – 1.71)	0.91	0.42 (0.08 – 1.95)	0.86

Table 5. 3: *Trypanosoma congolense* (*As-treated* analysis)

	No. examined (control/intervention)	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value
Survey 1	342/445	0.96 (0.62 – 1.49)	0.84	0.53 (0.07 – 2.50)	0.23
Survey 2	422/430	0.76 (0.48 – 1.18)	0.22	0.54 (0.13 – 2.19)	0.79
Survey 3	312/350	0.49 (0.20 – 1.10)	0.09	0.42 (0.05 – 0.91)	1.00
Survey 4	350/396	0.76 (0.42 – 1.38)	0.37	0.43 (0.08 – 0.86)	1.00
Survey 5	256/367	1.07 (0.57 – 2.05)	0.83	0.35 (0.04 – 0.85)	1.00

Table 5. 4: *Trypanosoma vivax* (*As-treated* analysis)

	No. examined (control/intervention)	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value
Survey 1	342/445	1.66 (0.73 – 4.12)	0.24	NA	-
Survey 2	422/430	0.53 (0.18 – 1.40)	0.21	0.75 (0.04 – 2.95)	1.00
Survey 3	312/350	0.89 (0.25 – 3.23)	0.85	1.15 (0.16 – 8.55)	1.00
Survey 4	350/396	1.45 (0.60 – 3.70)	0.41	NA	-
Survey 5	256/367	0.81 (0.27 – 2.54)	0.71	0.96 (0.03 – 2.51)	1.00

Table 5. 5: *Trypanosoma* species (*Intention-to-treat* analysis)

	No. examined (control/intervention)	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value
Survey 1	470/562	1.19 (0.81 – 1.77)	0.39	0.33 (0.05 – 1.44)	0.37
Survey 2	470/562	0.77 (0.51 – 1.16)	0.21	1.01 (0.31 – 3.18)	0.38
Survey 3	470/562	0.56 (0.27 – 1.10)	0.10	0.67 (0.17 – 2.22)	0.99
Survey 4	470/562	1.03 (0.63 – 1.69)	0.89	0.77 (0.18 – 3.07)	0.29
Survey 5	470/562	1.23 (0.71 – 2.15)	0.46	0.55 (0.11 – 2.19)	0.79

Table 5. 6: *T. congolense* (*Intention-to-treat* analysis)

	No. examined (control/intervention)	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value
Survey 1	470/562	1.05 (0.68 – 1.63)	0.83	0.34 (0.05 – 1.47)	0.51
Survey 2	470/562	0.81 (0.52 – 1.25)	0.34	0.99 (0.27 – 3.43)	1.00
Survey 3	470/562	0.47 (0.20 – 1.05)	0.07	0.22 (0.01 – 1.37)	1.00
Survey 4	470/562	0.85 (0.47 – 1.53)	0.59	0.40 (0.06 – 1.94)	1.00
Survey 5	470/562	1.35 (0.73 – 2.57)	0.34	0.46 (0.06 – 2.21)	1.00

Table 5. 7: *T. vivax* (*Intention-to-treat* analysis)

	No. examined (control/intervention)	OR (95% CI)	*P*-value	aOR (95% CI)	*P*-value
Survey 1	470/562	1.80 (0.79 – 4.45)	0.17	NA	-
Survey 2	470/562	0.56 (0.19 – 1.48)	0.26	1.17 (0.05 – 3.00)	1.00
Survey 3	470/562	0.85 (0.24 – 3.08)	0.80	1.19 (0.15 – 7.94)	1.00
Survey 4	470/562	1.59 (0.67 – 4.05)	0.30	NA	-
Survey 5	470/562	1.02 (0.34 – 3.19)	0.97	NA	-

Table 5. 8: Overall and trypanosome species-specific (*Per-protocol* analysis)

	No. examined (control/ intervention)	**OR (95% CI)**	***P*-value**	**aOR (95% CI)**	***P*-value**
Trypanosoma spp	161/148	0.80 (0.52 – 1.24)	0.32	NA	-
Trypanosoma congolense	161/148	0.73 (0.46 – 1.16)	0.19	NA	-
Trypanosoma vivax	161/148	1.20 (0.29 – 4.87)	0.80	NA	-

5.5.4 Entomological survey

A total of 9,614 *Glossina* species (2,569 males and 7,045 females) were collected in field surveys in Shimba Hills. Species comparison based on morphological identification was 8,833 *G. pallidipes*, 538 *G. austeni* and 243 *G. brevipalpis.* Also collected during the surveys were 1,241 *Stomoxys* species (421 males and 820 females) and 78 *Tabanus* species (2 males and 76 females).

Glossina abundance reduced significantly within treatment groups between February 2021 (control arm: 1.16 ftd, 95% CI: 0.84-1.48. intervention arm: 4.55 ftd, 95% CI: 0.01-0.26) and April 2021 (control arm: 0.46 ftd, 95% CI: 0.30-0.62. intervention arm: 0.80 ftd, 95% CI: 0.38-1.23) (Fig. A). *Stomoxys* abundance in treatment groups was also reduced significantly from 0.55 ftd (95% CI: 0.14-0.95) in the control arm and 0.13 ftd (95% CI: 0.01-0.26) in the intervention arm in February 2021 to 0.02 ftd (95% CI: 0.00-0.03) in the control arm and 0.00 ftd (95% CI: 0.00-0.01) in the intervention arm in April 2021 (Fig. B). *Tabanus* abundance in February 2021 was 0.00 ftd (95% CI: 0.00-0.01) in the control arm and 0.00 ftd (95% CI: 0.00-0.01) in the intervention arm and remained unchanged in April 2021 (Fig. C).

In GLMM analyses that adjusted for possible effects of sampling month and random effect of TrapID, abundance was not significantly different between treatment groups for *Glossina* (control: 0.85 ftd, 95% CI: 0.68-1.01; intervention: 2.13 ftd, 95% CI: 1.41-2.86. p = 0.2758) and *Tabanus* (control: 0.01 ftd, 95% CI: 0.01-0.02; intervention: 0.01 ftd, 95% CI: 0.01-0.02. p = 0.7255) but abundance was significantly higher in the control arm than the intervention arm for *Stomoxys* (control: 0.33 ftd, 95% CI: 0.20-0.47; intervention: 0.05 ftd, 95% CI: 0.02-0.09. p = <0.0001).

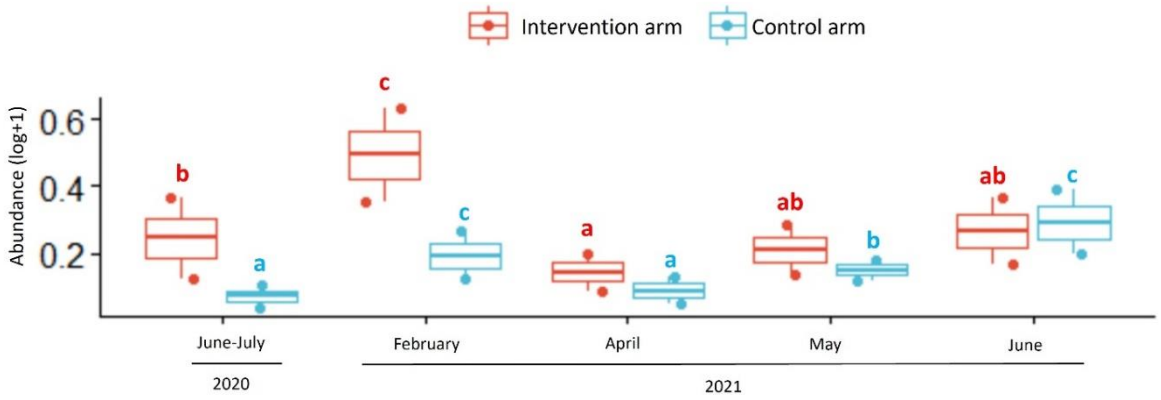


Figure 5. 4: Boxplots showing the abundance of Glossina tsetse flies during the cluster RCT

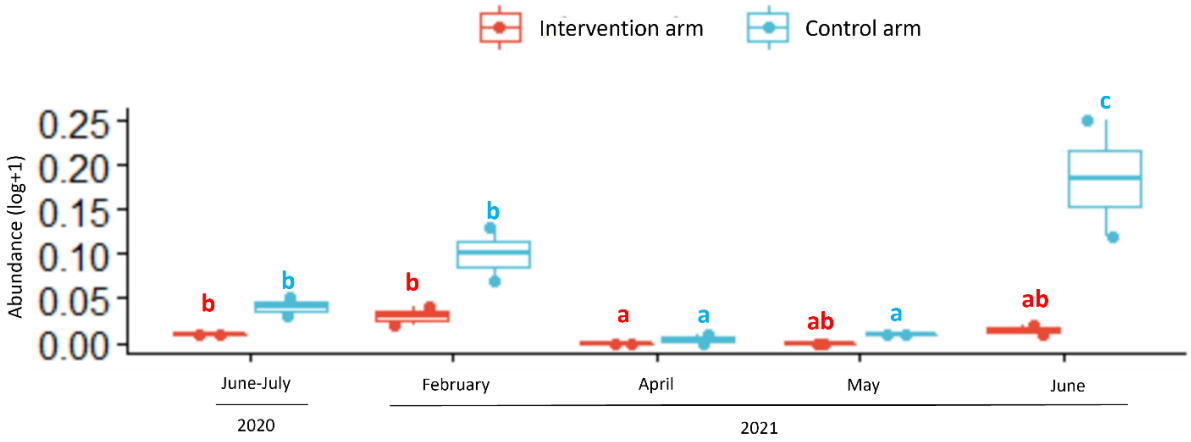


Figure 5. 5: Boxplots showing the abundance of Stable flies (*Stomoxys*) during the cluster RCT

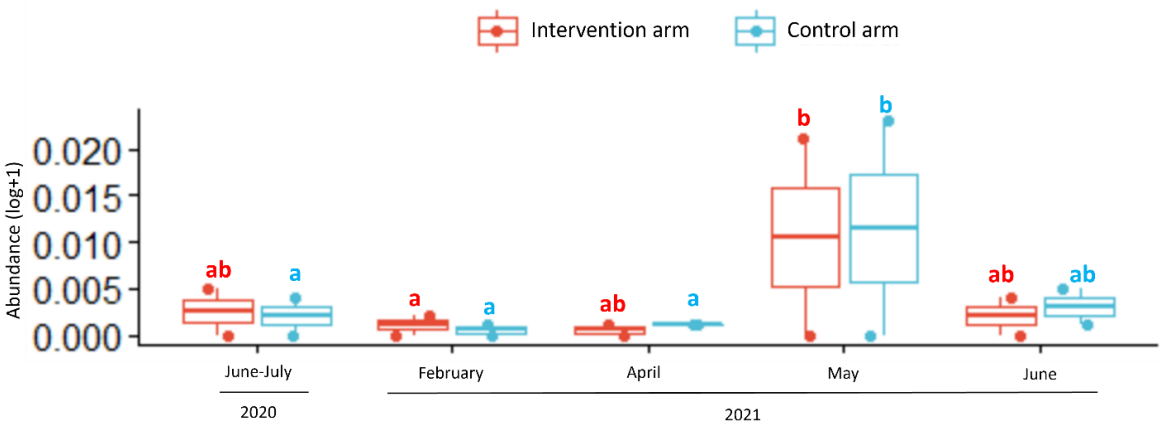


Figure 5. 6: Boxplots showing the abundance of horse flies during the cluster RCT

5.6 Discussion

Disease control interventions spatially targeted to epidemiological hotspots are important for effective and efficient reduction of pathogen risk (Bousema et al. 2012). However, it was not possible in the present cluster RCT to systematically evaluate the impact that ITFs targeted to epidemiological hotspots had on cattle infection risk. This could have been achieved if ITFs were deployed to trypanosome hotspots in one arm and outside the parasite hotspots in a second arm. Instead, ITFs were deployed in both arms in Shimba Hills to trypanosome hotspots (Chapter 2). In any case, the RCT was able to show that ITFs assigned to entomologically defined trypanosome hotspots (the entire area within one thousand metres from the boundary of the wildlife reserve) significantly reduced epidemiological risk of infection in cattle in Shimba Hills. This finding provides support for the use of ITFs for trypanosome vector control in Shimba Hills and corroborates previous reports on the effectiveness of the technologies for tsetse management in sub-Saharan Africa (Mahamat et al. 2017; Tirados et al. 2015, 2020).

The vegetation in Shimba Hills being predominantly savannah posed no hinderance to tsetse flies from being visually attracted to ITFs. ITFs in Shimba Hills were deployed at an average density of four ITFs per kilometer. This unequivocally increased the probability of tsetse contacts with the treated screens and helped to reduce the vector abundance and hence cattle infection risk (Vale et al. 1988; Hargrove 2003). The fact that tsetse control had plausibly driven reductions in trypanosome risk was observed between February and April 2021 when decline in tsetse abundance was matched with a decrease in trypanosome incidence.

However, trypanosome incidence increased after April 2021 at a time when more tsetse flies were collected in traps. Increased tsetse numbers in traps during the wet

season beginning from the time around April reflected higher levels of the vector infestations in sampling sites bordering the Shimba Hills National Reserve. Gashururu et al. (2021) in the Akagera National Reserve (NR) in Rwanda observed higher abundance of *G. pallidipes* in the wet than dry season. Tsetse flies were not collected inside the Shimba Hills National Reserve as was the case in the Gashururu et al. (2021) study in the Akagera NR. However, it is likely that increased tsetse fly abundance within 1000m of the Shimba Hills National Reserve boundary during the wet season was due to vector invasion of the interface area from the Shimba Hills reserve. An alternative or complementary hypothesis is that ITFs had declined in quality at this time when rainfall must have reduced the amount of deltamethrin insecticides on ITFs and sunlight have decolorized the devices, hence tsetse flies had reduced visual attraction to the ITF killing devices (Vale et al. 1988; Kuzoe & Schofield, 2005). However, ITFs destroyed by bush fire, wind, or animals were replaced.

The addition of tsetse repellents (WB collars) to ITFs helped to further reduce trypanosome incidences. However, this was far from the predetermined percentage reduction rate of 70.00%. Similar findings of vector repellent odours increasing but not significantly improving the efficacy of interventions have been reported in RCTs that used insecticide-treated nets for malaria control in Cambodia (Sluydts et al. 2016) and the Lao People's Democratic Republic (Chen-Hussey et al. 2013). However, RCTs in the Bolivian Amazon (Hill et al. 2007) and southern Ethiopia (Deressa et al. 2014) observed significant reductions in malaria infections in interventions that combined insecticide-treated nets with repellent odours for control of mosquito vectors. As in malaria interventions, field trials that add vector repellents to treated baits for trypanosome control might perform differently in different geographical locations

thus should be carried out under different environmental conditions outside of Shimba Hills.

Stomoxys were significantly more abundant in the control arm than the intervention arm. There are evidence that certain semio-chemicals affect the behaviours of multiple dipterans species across different genera (Leak et al. 2008; Kweka et al. 2011), hence the likelihood that *Stomoxys* lower abundance in the intervention arm may have been underpinned by effect of the tsetse-repellent WB collars. *Stomoxys* are *T. vivax* mechanical vectors. This and the insect's high abundance in the control arm perhaps explains why ITFs deployed for tsetse control were unable to drive significant reductions in *T. vivax* infections in the arm. That *T. vivax* incidence remained stable over time contributed to a minimised difference in treatment-effect on overall trypanosome infection between study arms.

In cattle farming communities in southwest Nigeria, Odeniran et al. (2019) reported trypanosome infection rate of 2.00% for *T. congolense* and 27.00% for *T. vivax* in *Stomoxys calcitrans* and *S. niger*. In the study, reports were also made of cattle bloodmeals in ~40.00% of *Stomoxys* samples. Similar studies to understand *Stomoxys* involvement in trypanosome epidemiology are currently lacking in Shimba Hills but will be important to effectively design intervention strategies aimed at reducing the parasite infection risk in the area. Such studies may also consider tabanids which also play roles in *T. vivax* transmission in certain locations and which in the Shimba Hills are highly diverse (Mugasa et al. 2018).

Insect repellent odours lacking active ingredients are recommended as placebo in RCT to assess efficacy of allomonal substances for disease vector control (WHO, 2017). However, this was difficult to implement in the present RCT because of constrained budget which made no provision for the procurement of additional

repellent collars lacking the synthetic 4-component waterbuck-mimicking odours (Saini et al. 2017). Even if this were possible, farmers in the control arm would still be able to tell by the lack of smell that their cattle were assigned placebo treatments. Another limitation was the delayed collection of entomological data. This was occasioned by the covid-19 pandemic which restricted movement and travels for field staff.

ITFs are an effective device for trypanosome control in Shimba Hills. However, efforts should be made to ensure regular maintenance of the devices during interventions. Saini et al. (2017) in a previous field trial showed significant effect of tsetse repellent odours on trypanosome incidence in cattle. However, combination of repellent odours with ITFs only marginally improved the intervention-effect outcome on trypanosome risk. A clear understanding of the epidemiological effect of adding tsetse repellent odours to ITFs was blurred by the likely involvement of mechanical vectors in *T. vivax* transmission in Shimba Hills. The present study provides no evidence that the addition of tsetse repellent odours to ITF-based interventions provides additional benefit for trypanosome control in Shimba Hills. However, replication of similar studies outside the area and elucidation of non-tsetse vector roles in trypanosome epidemiology in the wildlife interface are recommended.

References

1. Wilson AL, Courtenay O, Kelly-Hope LA, Scott TW, Takken W, Torr SJ, Lindsay SW. The importance of vector control for the control and elimination of vector-borne diseases. PLoS Neglected Tropical Diseases. 2020 Jan 16;14(1):e0007831.

2. Pérez D, Robert E, Perez EJ, Vanlerberghe V, Lefèvre P, Ridde V. A Realist Synthesis of Community-Based Interventions in Vector-Borne Diseases. The American Journal of Tropical Medicine and Hygiene. 2021 Apr;104(4):1202.

3. Wilson AL, Dhiman RC, Kitron U, Scott TW, van den Berg H, Lindsay SW. Benefit of insecticide-treated nets, curtains and screening on vector borne diseases, excluding malaria: a systematic review and meta-analysis. PLoS Neglected Tropical Diseases. 2014 Oct 9;8(10):e3228.

4. Han CY, Issa H, Rychtář J, Taylor D, Umana N. A voluntary use of insecticide treated nets can stop the vector transmission of Chagas disease. PLoS Neglected Tropical Diseases. 2020 Nov 3;14(11):e0008833.

5. Brun R, Blum J, Chappuis F, Burri C. Human 226frican trypanosomiasis. The Lancet. 2010 Jan 9;375(9709):148-59.

6. Giordani F, Morrison LJ, Rowan TG, De Koning HP, Barrett MP. The animal trypanosomiases and their chemotherapy: a review. Parasitology. 2016 Dec;143(14):1862-89.

7. Mungube EO, Vitouley HS, Allegye-Cudjoe E, Diall O, Boucoum Z, Diarra B, Sanogo Y, Randolph T, Bauer B, Zessin KH, Clausen PH. Detection of multiple drug-resistant Trypanosoma congolense populations in village cattle of south-east Mali. Parasites & Vectors. 2012 Dec;5(1):1-9.

8. Sow A, Sidibé I, Bengaly Z, Marcotty T, Séré M, Diallo A, Vitouley HS, Nebié RL, Ouédraogo M, Akoda GK, Van den Bossche P. Field detection of resistance to isometamidium chloride and diminazene aceturate in *Trypanosoma vivax* from the region of the Boucle du Mouhoun in Burkina Faso. Veterinary parasitology. 2012 Jun 8;187(1-2):105-11.

9. Anderson NE, Mubanga J, Fevre EM, Picozzi K, Eisler MC, Thomas R, Welburn SC. Characterisation of the wildlife reservoir community for human and animal trypanosomiasis in the Luangwa Valley, Zambia. PLoS Neglected Tropical Diseases. 2011 Jun 21;5(6):e1211.

10. Auty H, Anderson NE, Picozzi K, Lembo T, Mubanga J, Hoare R, Fyumagwa RD, Mable B, Hamill L, Cleaveland S, Welburn SC. Trypanosome diversity in wildlife species from the Serengeti and Luangwa Valley ecosystems. PloS Negl Trop Dis. 2012 6(10): e1828.

11. Büscher P, Bart JM, Boelaert M, Bucheton B, Cecchi G, Chitnis N, Courtin D, Figueiredo LM, Franco JR, Grébaut P, Hasker E. Do cryptic reservoirs threaten gambiense-sleeping sickness elimination?. Trends in Parasitology. 2018 Mar 1;34(3):197-207.

12. Kasozi KI, Zirintunda G, Ssempijja F, Buyinza B, Alzahrani KJ, Matama K, Nakimbugwe HN, Alkazmi L, Onanyang D, Bogere P, Ochieng JJ. Epidemiology of Trypanosomiasis in Wildlife—Implications for Humans at the Wildlife Interface in Africa. Frontiers in Veterinary Science. 2021 Jun 14;8:565.

13. Torr SJ, Hargrove JW, Vale GA. Towards a rational policy for dealing with tsetse. Trends in Parasitology. 2005 Nov 1;21(11):537-41.

14. Meyer A, Holt HR, Selby R, Guitian J. Past and ongoing tsetse and animal trypanosomiasis control operations in five African countries: a systematic review. PLoS Neglected Tropical Diseases. 2016 Dec 27;10(12):e0005247.

15. Barrett K, Okali C. Community participation in the management of Tsetse: a comparative assessment of impact and sustainability. Final technical report. (1998) https://www.gov.uk/research-for-development-outputs/community-participation-in-the-management-of-tsetse-a-comparative-assessment-of-impact-and-sustainability-final-technical-report (Assessed 2 December 2021).

16. Kuzoe FA, Schofield C. Strategic review of traps and targets for tsetse and African trypanosomiasis control. (2005). **https://apps.who.int/iris/handle/10665/69013** (Assessed 2 December 2021).

17. Stone CM, Chitnis N. Implications of heterogeneous biting exposure and animal hosts on Trypanosomiasis brucei gambiense transmission and control. PloS computational biology. 2015 Oct 1;11(10):e1004514.

18. Auty H, Morrison LJ, Torr SJ, Lord J. Transmission dynamics of Rhodesian sleeping sickness at the interface of wildlife and livestock areas. Trends in Parasitology. 2016 Aug 1;32(8):608-21.

19. Bauer B, Holzgrefe B, Mahama CI, Baumann MP, Mehlitz D, Clausen PH. Managing tsetse transmitted trypanosomosis by insecticide treated nets-an affordable and sustainable method for resource poor pig farmers in Ghana. PLoS Neglected Tropical Diseases. 2011 Oct 11;5(10):e1343.

20. Saini RK, Orindi BO, Mbahin N, Andoke JA, Muasa PN, Mbuvi DM, Muya CM, Pickett JA, Borgemeister CW. Protecting cows in small holder farms in East Africa

from tsetse flies by mimicking the odor profile of a non-host bovid. PLoS Neglected Tropical Diseases. 2017 Oct 17;11(10):e0005977.

21. Bett B, Hargrove J, Randolph TF, Saini R, Hassanali A, Ndung'u JM, McDermott JJ. Modelling the impact of tsetse-repellent on the transmission of cattle trypanosomosis by *Glossina pallidipes* at Nguruman, Kenya. 2013. Proceedings of the 10th International Symposium on Veterinary Epidemiology and Economics.

22. Bett B, Randolph TF, Irungu P, Nyamwaro SO, Kitala P, Gathuma J, Grace D, Vale G, Hargrove J, McDermott J. Field trial of a synthetic tsetse-repellent technology developed for the control of bovine trypanosomosis in Kenya. Preventive Veterinary Medicine. 2010 Dec 1;97(3-4):220-7.

23. Byamungu M, Zacarie T, Makumyaviri M'Pondi A, Mansinsa Diabakana P, McMullin A, Kröber T, Mihok S, Guerin PM. Standardising visual control devices for Tsetse: East and Central African Savannah species Glossina swynnertoni, Glossina morsitans centralis and *Glossina pallidipes*. PLoS Neglected Tropical Diseases. 2018 Sep 25;12(9):e0006831.

24. Malonza PK, Mulwa DM, Nyamache JO, Jones G. Biogeography of the Shimba Hills ecosystem herpetofauna in Kenya. Zoological research. 2018 Mar 18;39(2):97.

25. Ochieng B, Okeyo B, Tamooh F. Evaluation of population status and foraging ecology of Sable antelope (Hippotragus niger roosevelti, Heller, 1910) in Shimba Hills National Reserve, Kenya. African Journal of Ecology. 2020 Sep;58(3):446-54.

26. Government of Kenya (GoK). Kwale County Integrated Development Plan (2018-2022) [Internet]. Kwale County, Kenya; 2018; [cited 2021 December 2]. Available from:

27. Muriithi BW, Gathogo NG, Diiro GM, Kidoido MM, Okal MN, Masiga DK. Farmer perceptions and willingness to pay for novel livestock pest control technologies: A case of tsetse repellent collar in Kwale County in Kenya. PLoS Neglected Tropical Diseases. 2021 Aug 17;15(8):e0009663.

28. Bett MK, Saini RK, Hassanali A. Repellency of tsetse-refractory waterbuck (Kobus defassa) body odour to Glossina pallidipes (Diptera: Glossinidae): Assessment of relative contribution of different classes and individual constituents. Acta Tropica. 2015 Jun 1;146:17-24.

29. Eldridge SM, Ashby D, Kerry S. Sample size for cluster randomized trials: effect of coefficient of variation of cluster size and analysis method. International journal of epidemiology. 2006 Oct 1;35(5):1292-300.

30. Moher D, Schulz KF, Altman D, Consort Group, CONSORT Group. The CONSORT statement: revised recommendations for improving the quality of reports of parallel-group randomized trials. Jama. 2001 Apr 18;285(15):1987-91.

31. Chakraborty H, Hossain A. R package to estimate intracluster correlation coefficient with confidence interval for binary data. Computer methods and programs in biomedicine. 2018 Mar 1;155:85-92.

32. Leak SG, Ejigu D, Vreysen MJ. Collection of entomological baseline data for tsetse area-wide integrated pest management programmes. Food and Agriculture Organization of the United Nations (FAO); 2008.

33. Team RC. R: A Language and Environment for Statistical Computing; Vienna, Austria. 2013. Available online: **https://www.R-project.org/** (accessed on 30 December 2021).

34. Ripley B, Venables B, Bates DM, Hornik K, Gebhardt A, Firth D, Ripley MB. Package 'mass'. Cran r. 2013 Jan 8;538:113-20.

35. Brooks, M.E.; Kristensen, K.; Van Benthem, K.J.; Magnusson, A.; Berg, C.W.; Nielsen, A.; Skaug, H.J.; Machler, M.; Bolker, B.M. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R J.* **2017**, *9*, 378–400.

36. Hothorn, T.; Bretz, F.; Westfall, P.; Heiberger, R.M.; Schuetzenmeister, A.; Scheibe, S.; Hothorn, M.T. Package 'multcomp'. In *Simultaneous Inference in General Parametric Models*; Project for Statistical Computing: Vienna, Austria, 2016.

37. Bousema T, Griffin JT, Sauerwein RW, Smith DL, Churcher TS, Takken W, Ghani A, Drakeley C, Gosling R. Hitting hotspots: spatial targeting of malaria for control and elimination. PLoS medicine. 2012 Jan 31;9(1):e1001165.

38. Mahamat MH, Peka M, Rayaisse JB, Rock KS, Toko MA, Darnas J, Brahim GM, Alkatib AB, Yoni W, Tirados I, Courtin F. Adding tsetse control to medical activities contributes to decreasing transmission of sleeping sickness in the Mandoul focus (Chad). PLoS Neglected Tropical Diseases. 2017 Jul 27;11(7):e0005792.

39. Tirados I, Esterhuizen J, Kovacic V, Mangwiro TC, Vale GA, Hastings I, Solano P, Lehane MJ, Torr SJ. Tsetse control and Gambian sleeping sickness; implications for control strategy. PLoS Neglected Tropical Diseases. 2015 Aug 12;9(8):e0003822.

40. Tirados I, Hope A, Selby R, Mpembele F, Miaka EM, Boelaert M, Lehane MJ, Torr SJ, Stanton MC. Impact of tiny targets on Glossina fuscipes quanzensis, the primary vector of human African trypanosomiasis in the Democratic Republic of the Congo. PLoS Neglected Tropical Diseases. 2020 Oct 16;14(10):e0008270.

41. Vale GA, Lovemore DF, Flint S, Cockbill GF. Odour-baited targets to control tsetse flies, Glossina spp.(Diptera: Glossinidae), in Zimbabwe. Bulletin of Entomological Research. 1988 Mar;78(1):31-49.

42. Hargrove JW. Optimized simulation of the control of tsetse flies *Glossina pallidipes* and G. m. morsitans (Diptera: Glossinidae) using odour-baited targets in Zimbabwe. Bulletin of Entomological Research. 2003 Jan;93(1):19-29.

43. Gashururu RS, Githigia SM, Gasana MN, Habimana R, Maingi N, Cecchi G, Paone M, Zhao W, Masiga DK, Gashumba J. An update on the distribution of Glossina (tsetse flies) at the wildlife-human-livestock interface of Akagera National Park, Rwanda. Parasites & Vectors. 2021 Dec;14(1):1-3.

44. Sluydts V, Durnez L, Heng S, Gryseels C, Canier L, Kim S, Van Roey K, Kerkhof K, Khim N, Mao S, Uk S. Efficacy of topical mosquito repellent (picaridin) plus long-lasting insecticidal nets versus long-lasting insecticidal nets alone for control of malaria: a cluster randomised controlled trial. The Lancet Infectious Diseases. 2016 Oct 1;16(10):1169-77.

45. Chen-Hussey V, Carneiro I, Keomanila H, Gray R, Bannavong S, Phanalasy S, Lindsay SW. Can topical insect repellents reduce malaria? A cluster-randomised controlled trial of the insect repellent N, N-diethyl-m-toluamide (DEET) in Lao PDR. PloS One. 2013 Aug 14;8(8):e70664.

46. Hill N, Lenglet A, Arnez AM, Carneiro I. Plant based insect repellent and insecticide treated bed nets to protect against malaria in areas of early evening biting vectors: double blind randomised placebo controlled clinical trial in the Bolivian Amazon. bmj. 2007 Nov 15;335(7628):1023.

47. Deressa W, Yihdego YY, Kebede Z, Batisso E, Tekalegne A, Dagne GA. Effect of combining mosquito repellent and insecticide treated net on malaria prevalence in Southern Ethiopia: a cluster-randomised trial. Parasites & Vectors. 2014 Dec;7(1):1-0.

48. Kweka EJ, Owino EA, Mwang'onde BJ, Mahande AM, Nyindo M, Mosha F. The role of cow urine in the oviposition site preference of culicine and Anopheles mosquitoes. Parasites & vectors. 2011 Dec;4(1):1-6.

49. Odeniran PO, Macleod ET, Ademola IO, Welburn SC. Molecular identification of bloodmeal sources and trypanosomes in Glossina spp., *Tabanus* spp. and *Stomoxys* spp. trapped on cattle farm settlements in southwest Nigeria. Medical and Veterinary Entomology. 2019 Jun;33(2):269-81.

50. Mugasa CM, Villinger J, Gitau J, Ndungu N, Ciosi M, Masiga D. Morphological re-description and molecular identification of tabanidae (Diptera) in East Africa. ZooKeys. 2018(769):117.

51. World Health Organization (WHO). How to design vector control efficacy trials. 2017.

CHAPTER 6

GENERAL DISCUSSION

6.0 Background

Trypanosomes infect humans and animals worldwide and have profound adverse effects on livestock health and production in sub-Saharan Africa where tsetse flies are the main vectors. Shimba Hills in southeast Kenya is among areas endemic to trypanosomes in sub-Saharan Africa (Saini et al. 2017) and a major focus of tsetse-borne trypanosome parasites of livestock in East Africa (Shaw et al. 2014). Data from epidemiological and sociological surveys in the area associate poor cattle health with trypanosome infections (Saini et al. 2017; Muriithi et al. 2021).

To date, chemotherapy remains the mainstay for trypanosome management in Shimba Hills (Muriithi et al. 2021), but recent reports of chemotherapeutic interventions against trypanosomes confirm trypanoresistance constraints among other challenges (Giordani et al. 2016; Assefa & Shibeshi 2018). Although cases of trypanoresistance are barely documented in Shimba Hills (Kulohoma et al. 2020), high trypanosome positive cases in treated livestock in the area indicate that domestic stock in smallholder communities have high risk of exposure to parasite reinfection from wildlife reservoirs (Saini et al. 2017).

However, locations in Shimba Hills where cattle are exposed to high trypanosome risk and infection from wildlife are not well known because epidemiological data that describe the parasite transmission patterns over space and among animal hosts are sparse. This has made it difficult to formulate rational tsetse control strategies for the area and contributes to jettison efforts aimed at complementing chemotherapeutic interventions and addressing constraints associated with trypanosome drug control. A dearth in data describing trypanosome molecular diversity in Shimba Hills also contributes to difficulties in identifying and targeting control of tsetse flies responsible for the transmission of the parasite genotypes that are virulent for cattle.

Preceding chapters in this thesis described tsetse-borne trypanosome epidemiology in Shimba Hills with a focus on the parasite risk, wildlife sources, and genotype diversity. Risks of cattle trypanosome infections posed by tsetse flies in Shimba Hills were assessed based on the entomological inoculation rate index and findings were compared to the parasite infections in cattle screened in contemporaneous parasitological surveys. Wildlife sources of trypanosomes were determined in xenomonitoring surveillance that used *Cytochrome* b, vertebrate *16S rRNA*, and *Cytochrome Oxidase* 1 gene-markers to characterise animal bloodmeal hosts of tsetse flies with and without trypanosome infections. Molecular phylogenetic analysis of *ITS*-1 and *18S* DNA amplicon sequences were used to unravel *Trypanosoma vivax* diversity with the aim of characterising the parasite genotypes in Shimba Hills. Findings on trypanosome risk assessment, xenomonitoring surveillance of trypanosome wildlife sources, and *T. vivax* genotype diversity were explored to rationalise a plan for community-led interventions against tsetse flies in Shimba Hills. Efficacy of the rationalised plan on trypanosome infections in cattle was assessed in a Cluster Randomised-Controlled Trial that aimed to control epidemiologically important tsetse flies in interventions targeted to transmission hotspots. These interventions utilised insecticide-treated fabrics with and without a synthetic blend of tsetse-repellent waterbuck-mimicking synthetic odours applied on cattle. Effort is made in the current chapter to collate and synthesize major findings from both epidemiological surveys and interventions with the aim of making recommendations for animal trypanosomiasis vector management in Shimba Hills and to point out grey areas requiring further research.

6.1.0 Summary of major findings

6.1.1 Chapter two

Trypanosome entomological risk assessment in Shimba Hills (Kenya) reveals high risk of cattle infections in grazing fields close to the wildlife reserve and identifies epidemiologically important tsetse-vectors for targeted control in interventions to manage animal trypanosomiasis

As irrational deployment of vector control tools continues to constrain infectious effective disease management worldwide, priority should be given to production of epidemiological data that adequately describe pathogen transmission hotspots where interventions can be targeted. However, field surveillances to understand pathogen hotspots have received insufficient attention globally and especially in sub-Saharan Africa (Diall et al. 2017). As a first step to elucidating the epidemiology of animal trypanosomiasis in the Shimba Hills human-wildlife-livestock interface in southeast Kenya, a study was carried out to assess the transmission patterns of tsetse-borne trypanosomes in smallholder agrarian communities at the edge of the Shimba Hills National Reserve. The objective was to identify the parasite hotspots where cattle are exposed to high risk of infections so that tsetse control to reduce trypanosome incidences can be effectively tailored to such hotspots while maximising use of scarce resources and accelerating intervention-effects.

High entomological inoculation rate, extensive spatial range, high abundance, and relatively longer lifespan observed for *G. pallidipes* compared with *G. brevipalpis* and *G. austeni* in Shimba Hills incriminate this fly species as responsible for exposing cattle to high risk of trypanosome infections in Shimba Hills. Tsetse abundance and entomological inoculation rates were highest close to the wildlife reserve. Tsetse-trypanosome-cattle interactions were uncovered for the first time in Shimba Hills.

Importantly, average entomological inoculation rate showed that cattle in Shimba Hills are potentially exposed to feeding attack from one trypanosome-positive tsetse fly every twenty-six days. This frequency of tsetse-cattle interactions in Shimba Hills was higher than the 50 days reported in the Ghibe Valley in Ethiopia (Lemecha et al. 2006). Far higher still is the attack rate in Kinangodongo close to the wildlife reserve in Shimba Hills where I estimated three days as the average duration for tsetse-cattle contacts to occur. However, trypanosome risk in Kinangodongo did not differ significantly from other study-blocks in Shimba Hills. Additionally, trypanosome risk was homogeneous between vegetation landscapes experiencing disparate anthropogenic disturbances.

Contrary to findings in previous field data in Zambia (Mweempwa et al. 2015; Chilongo et al. 2021), anthropogenic disturbances in Shimba Hills had no effect on tsetse age and phenotypic sizes —two demographic traits reported in prior entomological studies to influence pathogen infection risk in arthropod-vectors (Guo et. 2019; Channumsin et al. 2018). The likelihood that a tsetse would be positive for trypanosome was significantly influenced by the fly species while probability of detecting cattle bloodmeals in the vector depended on the fly sex, with the proportion of cattle bloodmeals being significantly higher in female than male tsetse flies. Although *G. austeni* presented with the highest infection rate among tsetse flies, this is very unlikely to translate into the fly species posing high risk of infection to cattle given that it rarely feeds on livestock (Weitz 1963) and has a constrained distribution and low abundance in Shimba Hills (Mbahin et al. 2013; Chapter 2).

The study identified *proximity-to-wildlife* as an important driver of trypanosome risk in Shimba Hills. Importantly, it provided epidemiological data to show that cattle are increasingly exposed to trypanosome infections closer to the wildlife protectorate. Programmes aiming to reduce the incidence of animal trypanosomiasis should target

deployment of control tools to these hotspots and tailor interventions to reduce the abundance of *G. pallidipes*, the species responsible for most trypanosome infections in cattle. Following confirmation of trypanosome parasites associated with wildlife in tsetse flies, the need for future field studies to assess the role and involvement of wildlife in trypanosome epidemiology in Shimba Hills was identified (Chapter 3).

Contemporaneous parasitological surveys on cattle in 14 study-blocks provided evidence for the observed clinical effects of trypanosomes on livestock in Shimba Hills. Contrary to prior reports (Fall et al. 1999; Bett et al. 2008), I uncovered a weak relationship between cattle infection rates and tsetse entomological inoculation rates. The Fall et al. (1999) and Bett et al. (2008) studies collected monthly data over longer durations than in the present work in Shimba Hills. The results from both studies also provide proof to the ability of limited spatio-temporal scale of data collection to blur detection of significant associations between cattle trypanosome rates and tsetse entomological inoculation rates. Monthly data collection over similar periods was constrained by logistical challenges in Shimba Hills. However, further studies conducted over extended periods will be important for providing a clearer understanding of the relationship between both variables in the area.

6.1.2 Chapter three

Molecular xenomonitoring surveillance characterises a wide diversity of animal bloodmeal hosts in tsetse flies and implicates the common warthog Phacochoerus africanus as an important trypanosome reservoir exposing cattle to the parasite infections in Shimba Hills

Wildlife reservoirs are currently contributing to maintenance of trypanosome endemicity in many sylvatic ecologies in the sub-Sahara region and frustrating efforts to successfully interrupt transmission of the Human and Animal African

Trypanosomiasis, particularly in East Africa (Büscher et al. 2018). Wildlife, by continuously exposing livestock to infections, are also jeopardising farmer-led chemotherapeutic interventions against *nagana* cattle disease in smallholder communities around National Reserves in tsetse-endemic countries in the continent, including the Shimba Hills National Reserve in Kenya. The presence of wildlife-associated trypanosome species in Shimba Hills was confirmed in tsetse flies sampled from the area (Chapter 2). A xenomonitoring study was therefore carried out to profile a broader range of animal bloodmeal hosts of tsetse flies (with and without infections), so as to untangle trypanosome transmission patterns among wild fauna and livestock and finally, identify major wildlife species maintaining endemicity of trypanosomes and exposing cattle to trypanosome infections. Bloodmeal analyses confirmed that tsetse had fed on thirteen mammalian species corroborating previous reports of extensive animal host range in areas with similar ecologies (Muturi 2011; Auty et al. 2016a; Gaithuma et al. 2020). High host species diversity was attributed to high faunal biodiversity in Shimba Hills (Government of Kenya 2018) and application of multiple gene-markers for vertebrate DNA detection and characterisation in high-throughput analyses (Ouso et al. 2020). A prior report of lower tsetse fly host diversity in Shimba Hills (Channumsin 2021) may have been due to the limited spatio-temporal scale of that study.

Tsetse preference for warthogs in Shimba Hills including locations where individuals of this wildlife species are sparse seems to deviate from the notion of animal selection being a function of host abundance. It further adds to evidence of the vector penchant for warthog bloodmeals (Weitz 1963) while clearly supporting the view that other factors aside from host numbers are fundamentally driving tsetse host selection behaviours in Shimba Hills. Among reasons explaining tsetse preference for warthogs in Shimba Hills are the high nutritional content of the animal blood (De

Beer et al. 2012), synchrony in daily activity patterns between tsetse flies and warthogs (Okiwelu 1977), and warthog emission of large amounts of tsetse-attractant semio-chemical volatiles (Späth 1997). Tsetse flies also showed high preference for cattle bloodmeals in Shimba Hills understandably on account of strong odour attraction from the livestock (Leak et al. 2008). These distinct host preferences were reflected in the detection of both warthog and cattle bloodmeals in individual tsetse flies in 0.57% of the flies screened. Importantly, tsetse flies positive for warthog bloodmeals also harboured a wide variety of trypanosomes, including cattle parasites reported in epidemiological surveys in Shimba Hills. This agreed with and gave empirical evidence to anecdotes claiming that warthogs are drivers of cattle trypanosome infections in Shimba Hills, an assertion sufficiently supported by findings of a significantly higher likelihood of *T. vivax*, *T. congolense* and *T. brucei sl* infections in tsetse flies that took bloodmeals from warthogs.

The present study notes that nutritional content of bloodmeals, semio-chemical odour profile of animal species, and anti-feeding behaviours of animal hosts, in addition to host abundance, are important factors underpinning tsetse host selection behaviours in Shimba Hills. It also highlights the need for extensive sampling both in space and time to gain a clearer understanding of bloodmeal hosts of tsetse flies in the vector endemic locations. Confirmation that tsetse flies feed on a wide range of animal species indicates complex transmission patterns for trypanosomes in Shimba Hills. However, the vectors preferred to feed on warthogs perhaps due to kairomones thus providing a basis for chemical ecological studies directed at unravelling chemical interactions between tsetse flies and warthogs. This avenue of research could potentially furnish *nagana* vector control programs with further potent attractants for luring tsetse flies to killing devices such as traps and insecticide-treated tiny targets used in community-led interventions. Meanwhile, it is clear that

warthogs contribute to the high risk of trypanosome infections close to the wildlife reserve in Shimba Hills. Control activities targeted to locations infested by tsetse flies and where warthogs are abundant and co-exist with cattle could deliver better intervention-effects in reducing incidences of trypanosome infections in cattle in Shimba Hills.

6.1.3 Chapter four

Phylogenetic studies to assess Trypanosoma vivax diversity in tsetse flies provide unequivocal evidence of Tvv4 endemicity in Kenya and incriminate this genotype as an important etiological agent of clinical cases of cattle trypanosomiasis in Shimba Hills

High rates of *T. vivax* are reported in livestock and tsetse flies in sub-Saharan Africa (Fetene et al. 2021) and infections are associated with anaemia in cattle in Shimba Hills in Kenya (Mbahin et al. 2013; Saini et al. 2017). However, a clear understanding of *T. vivax* diversity is lacking in Shimba Hills. There is, however, evidence that different *T. vivax* genotypes give rise to varying degrees of clinical conditions in infected livestock in wildlife environments (Rodrigues et al. 2008, 2017). Characterisation of parasite diversity in sylvatic ecologies such as Shimba Hills is therefore key to understanding the variable clinical presentation and for guiding targeted interventions to control virulent genotypes. This thesis investigated *T. vivax* diversity in Shimba Hills and confirmed the presence of multiple genotypes of the parasites in the area. The *T. vivax Tvv4* genotype which is reportedly highly virulent in infected livestock and which was first documented in a wild-caught nyala antelope in Mozambique and subsequently in tsetse flies and livestock in sylvatic ecologies in the country (Rodrigues et al. 2008, 2017), was found in tsetse flies in Shimba Hills. The discovery of *Tvv4* in Shimba Hills is the first unequivocal evidence of the parasite

endemicity in Kenya and provides evidence for a broader distributional range, inclusive of East Africa, and of circulation in wildlife ecosystems in the sub-region. The recent detection of *Tvv4* in cattle in Southern Africa specifically the Kafue National Park in Zambia (Nakamura et al. 2021) further buttresses the fact about the *T. vivax* genotype widespread and association with wildlife in the continent. *Tvv4*, which occurs in sympatry with the *T. vivax TvL1*-G genotype in Shimba Hills, was the dominant genotype of *T. vivax* trypanosome species in Shimba Hills. The *Tvv4* parasite was detected in *G. pallidipes*, known to infest grazing fields and recognised in the area as a prolific vector of animal trypanosome parasites and preferential feeder on cattle blood, consequently underscoring the high risk of cattle infection with the genotype in Shimba Hills. The *T. vivax Tvv1* genotype has been reported in tsetse-free areas in Ethiopia (Fkru et al. 2016) but was absent in tsetse flies in Shimba Hills. However, phylogenetic analysis of DNA sequences of mechanically transmitted *T. vivax* isolates from camels and camel flies revealed *Tvv1* presence in the tsetse-free camel-keeping northern Kenya. These findings suggest that *Tvv1* is adapted to different transmission patterns in East Africa and, together with *Tvv4* and *TvL1*-G detected in Shimba Hills, confirmed endemicity of at least three *T. vivax* genotypes in Kenya.

Similar studies to characterise *T. vivax* diversity should be extended to other sylvatic environments within and outside Kenya. This will assist to better understand the parasite diversity on the continent and allow for clearer assessment of *Tvv4* spatial distribution. *Trypanosoma vivax* characterisation in Shimba Hills was carried out on tsetse flies collected at the wildlife-livestock interface but should in subsequent studies be extended to tsetse flies within the Shimba Hills National Reserve to provide further insights into the parasite diversity in the area and possibly assist to identify genotypes not reported in the present study and perhaps other genotypes

yet undescribed in epidemiological surveys in sub-Saharan Africa. Findings of warthogs and cattle bloodmeals in tsetse flies positive for *Tvv4* pave the way for shuttling of the parasite between wildlife and domestic stock in Shimba Hills. However, subsequent studies to unravel *T. vivax* diversity in Shimba Hills should consider screening wildlife accommodated within the National Reserve and cattle and other livestock in farming communities bordering the wildlife reserve. This will be important to further strengthen the argument regarding *Tvv4* transmission between sylvatic and domestic cycles in the area.

In conclusion, *T. vivax* is likely contributing to cattle pathologies in livestock farming communities in Shimba Hills, considering the parasite high entomological infection rate (2.44%) and the dominance of its population by the *Tvv4* genotype known to cause severe clinical conditions in infected livestock (Rodrigues et al. 2008). Drug experiment studies on *Tvv4* are currently lacking but needed to identify potent trypanocides for parasite management in Shimba Hills and other locations where the *Tvv4* genotype is endemic and constraining livestock health and production. However, control programs targeting *Tvv4* control in Shimba Hills can in the meantime continue to strategise interventions to target the *G. pallidipes* tsetse fly vectors of the parasite.

6.1.4 Chapter five

Insecticide-treated fabrics targeted to entomologically defined trypanosome hotpots with or without synthetic tsetse-repellent odours significantly reduced cattle infection risk in the Shimba Hills, Kenya

Push-Pull disease-vector management is an emerging strategy for pathogen control worldwide and involves the integrated application of repellent odours to interrupt vector-host contacts and visual cues or kairomones to attract vectors to insecticide-

treated killing points (Takken 2010). This strategy has been explored for malaria vector control and, in most cases, was shown to improve intervention-effects compared with programmes that use only repellent odours or insecticide-treated materials (Hill et al. 2007; Deressa et al. 2014). Additionally, the *push-pull* strategy has the advantage of being low-cost, environmentally safe, and locally adaptable and thus presents enormous potential for control of infectious diseases of poverty such as tsetse-borne trypanosomiasis endemic in resource-limited rural communities in different parts of sub-Saharan Africa, including East Africa. A cluster randomised controlled trial was therefore carried out in smallholder communities in the Shimba Hills human-wildlife-livestock interface in Kenya with an objective to assess the epidemiological effect of insecticide-treated fabrics (ITFs) with and without a synthetic blend of waterbuck-mimicking tsetse-repellent odours on cattle.

ITF strategic allocation to trypanosome high risk sites where tsetse flies are highly abundant and deployment at an optimal density of four ITFs per km square as well as low vegetation cover in the intervention sites allowed tsetse flies to easily encounter ITFs so that the vector infestations were quickly reduced within a limited timeframe. The reduction in tsetse infestations were reflected in significant reductions in cattle infection risk in March-April 2021, five months after the intervention was rolled-out. However, there was a surge in trypanosome risk in the period after April 2021 following an increase in tsetse fly abundance. Possible reasons for this include, one, the vector high invasion pressure from within the Shimba Hills National Reserve and, two, compromised quality of ITFs due to sunlight discolouration of the tsetse-attractive blue fabric and the action of rainfall on deltamethrin insecticides applied on the black fabric, on which tsetse flies alight after they have been attracted by the blue fabric (Vale 1988). A high abundance of *Stomoxys* was observed in the control arm of the cluster RCT where ITFs were used without

tsetse-repellent odours on cattle. It is possible that this influenced the risk of *T. vivax* in those locations since *Stomoxys* are capable of mechanically transmitting parasite infections (Odeniran et al. 2019). The addition of tsetse-repellent odours to ITFs improved intervention-effects, but the high *T. vivax* rates in the control arm limited the difference between treatment arms with intervention-effect being far below the 70.00% level specified *a priori*.

ITFs targeted to trypanosome hotpots are effective for reducing the parasite risk in cattle populations in Shimba Hills. However, efforts must be made to ensure maintenance of ITFs which should ideally be deployed during tsetse peak periods in wet seasons. Tsetse-repellent odours, since they are cheap and easy-to-use, could be applied on cattle year-round including times when tsetse infestations are low. However, adding the odours to ITFs may be unnecessary as it is unlikely to significantly improve intervention-effects. Following disparities between geographical areas in the outcome of *push-pull* malaria vector control (Hill et al. 2007; Chen-Hussey et al. 2013; Deressa et al. 2014; Sluydts et al. 2016), there is a need to reassess the potential of ITFs plus tsetse-repellent odours for trypanosome control in locations outside Shimba Hills. Meanwhile, subsequent studies in Shimba Hills should evaluate mechanical vector involvement in trypanosome transmission as this could assist to better strategise interventions to reduce trypanosome incidences and alleviate the parasite burden on cattle.

6.2 Conclusion

This thesis has described the molecular epidemiology of tsetse-borne trypanosome parasites of livestock in the Shimba Hills human-wildlife-livestock interface in Kenya. The findings were useful for identification of trypanosome high-risk locations where tsetse flies are more likely to carry out infectious biting attacks on cattle and

where wildlife reservoirs of trypanosomes expose cattle to infections including virulent genotypes of trypanosomes believed to be responsible for major livestock pathologies in Shimba Hills. Using data from the epidemiological surveys, tsetse interventions were targeted to trypanosome hotpots (areas within 1000m of the wildlife reserve) with results of significant reductions in the parasite incidence in cattle populations. The present study is the most extensive on trypanosome epidemiology in Shimba Hills and contributes to current understanding of the parasite infections in human-wildlife-livestock interfaces in East Africa. The study has generated important baseline data for future geospatial analyses of trypanosome transmission dynamics in Shimba Hills and has also identified important areas of research in subsequent studies.

References

1. Saini RK, Orindi BO, Mbahin N, Andoke JA, Muasa PN, Mbuvi DM, Muya CM, Pickett JA, Borgemeister CW. Protecting cows in small holder farms in East Africa from tsetse flies by mimicking the odor profile of a non-host bovid. PLoS Neglected Tropical Diseases. 2017 Oct 17;11(10):e0005977.
2. Shaw AP, Cecchi G, Wint GR, Mattioli RC, Robinson TP. Mapping the economic benefits to livestock keepers from intervening against bovine trypanosomosis in Eastern Africa. Preventive Veterinary Medicine. 2014 Feb 1;113(2):197-210.
3. Muriithi BW, Gathogo NG, Diiro GM, Kidoido MM, Okal MN, Masiga DK. Farmer perceptions and willingness to pay for novel livestock pest control technologies: A case of tsetse repellent collar in Kwale County in Kenya. PLoS Neglected Tropical Diseases. 2021 Aug 17;15(8):e0009663.
4. Giordani F, Morrison LJ, Rowan TG, De Koning HP, Barrett MP. The animal trypanosomiases and their chemotherapy: a review. Parasitology. 2016 Dec;143(14):1862-89.
5. Assefa S, Shibeshi W. Drug resistance in African animal trypanosomes: A review. African Journal of Microbiology Research. 2018 May 7;12(17):380-6.
6. Kulohoma BW, Wamwenje SA, Wangwe II, Masila N, Mirieri CK, Wambua L. Prevalence of trypanosomes associated with drug resistance in Shimba Hills, Kwale County, Kenya. BMC research notes. 2020 Dec;13(1):1-6.
7. Diall O, Cecchi G, Wanda G, Argilés-Herrero R, Vreysen MJ, Cattoli G, Viljoen GJ, Mattioli R, Bouyer J. Developing a progressive control pathway for African animal trypanosomosis. Trends in Parasitology. 2017 Jul 1;33(7):499-509.
8. Lemecha H, Mulatu W, Hussein I, Rege E, Tekle T, Abdicho S, Ayalew W. Response of four indigenous cattle breeds to natural tsetse and trypanosomosis

challenge in the Ghibe valley of Ethiopia. Veterinary parasitology. 2006 Oct 10;141(1-2):165-76.

9. Mweempwa C, Marcotty T, De Pus C, Penzhorn BL, Dicko AH, Bouyer J, De Deken R. Impact of habitat fragmentation on tsetse populations and trypanosomosis risk in Eastern Zambia. Parasites & Vectors. 2015 Dec;8(1):1-0.
10. Chilongo K, Manyangadze T, Mukaratirwa S. Effects of human settlements and spatial distribution of wing vein length, wing fray categories and hunger stages in Glossina morsitans morsitans (Diptera: Glossinidae) and *Glossina pallidipes* (Diptera: Glossinidae) in areas devoid of cattle in North-Eastern Zambia. Journal of medical entomology. 2021 Mar;58(2):891-9.
11. Guo F, Bonebrake TC, Gibson L. Land-use change alters host and vector communities and may elevate disease risk. Ecohealth. 2019 Dec;16(4):647-58.
12. Channumsin M, Ciosi M, Masiga D, Turner CM, Mable BK. Sodalis glossinidius presence in wild tsetse is only associated with presence of trypanosomes in complex interactions with other tsetse-specific factors. BMC microbiology. 2018 Nov;18(1):69-82.
13. Weitz, B. The feeding habits of *Glossina*. *Bull. World Health Organ.* 1963, *28*, 711.
14. Mbahin N, Affognon H, Andoke J, Tiberius M, Mbuvi D, Otieno J, Muasa P, Saini RK. Parasitological prevalence of bovine trypanosomosis in Kubo division of Kwale county of coastal: baseline survey. Am J Anim Vet Sci. 2013 Jan 1;8(1):28-36.
15. Fall A, Diack A, Diaite A, Seye M, d'Ieteren GD. Tsetse challenge, trypanosome and helminth infection in relation to productivity of village Ndama cattle in Senegal. Veterinary parasitology. 1999 Mar 1;81(3):235-47.
16. Bett B, Irungu P, Nyamwaro SO, Murilla G, Kitala P, Gathuma J, Randolph TF, McDermott J. Estimation of tsetse challenge and its relationship with

trypanosomosis incidence in cattle kept under pastoral production systems in Kenya. Veterinary Parasitology. 2008 Aug 17;155(3-4):287-98.

17. Büscher P, Bart JM, Boelaert M, Bucheton B, Cecchi G, Chitnis N, Courtin D, Figueiredo LM, Franco JR, Grébaut P, Hasker E. Do cryptic reservoirs threaten gambiense-sleeping sickness elimination?. Trends in Parasitology. 2018 Mar 1;34(3):197-207.
18. Muturi, C.N.; Ouma, J.O.; Malele, I.I.; Ngure, R.M.; Rutto, J.J.; Mithöfer, K.M.; Enyaru, J.; Masiga, D.K. Tracking the feeding patterns of tsetse flies (*Glossina* genus) by analysis of bloodmeals using mitochondrial cytochromes genes. *PloS One* 2011, *6*, e17284.
19. Auty H, Cleaveland S, Malele I, Masoy J, Lembo T, Bessell P, Torr S, Picozzi K, Welburn SC. Quantifying heterogeneity in host-vector contact: tsetse (Glossina swynnertoni and G. pallidipes) host choice in Serengeti National Park, Tanzania. PloS One. 2016a Oct 5;11(10):e0161291.
20. Gaithuma, A.; Yamagishi, J.; Hayashida, K.; Kawai, N.; Namangala, B.; Sugimoto, C. Blood meal sources and bacterial microbiome diversity in wild-caught tsetse flies. *Sci. Rep.* 2020, *10*, 1–10.
21. Government of Kenya (GoK). Kwale County Integrated Development Plan (2018-2022) [Internet]. Kwale County, Kenya; 2018; [cited 2021 December 2].
22. Ouso, D.O.; Otiende, M.Y.; Jeneby, M.M.; Oundo, J.W.; Bargul, J.L.; Miller, S.E.; Wambua, L.; Villinger, J. Three-gene PCR and high-resolution melting analysis for differentiating vertebrate species mitochondrial DNA for biodiversity research and complementing forensic surveillance. *Sci. Rep.* 2020, *10*, 1–13.
23. Channumsin M, Ciosi M, Masiga D, Auty H, Turner CM, Kilbride E, Mable BK. Blood meal analysis of tsetse flies (*Glossina pallidipes*: Glossinidae) reveals higher

host fidelity on wild compared with domestic hosts. Wellcome Open Research. 2021;6.

24. De Beer, C.J.; Venter, G.J.; Potgieter, F.T. *Developing Quality Control Procedures to Sustain a Supply of High Quality Blood for Mass Rearing Tsetse Flies*; IAEA: Vienna, Austria, 2012.
25. Okiwelu SN. Host preference and trypanosome infection rates of Glossina morsitans morsitans Westwood in the Republic of Zambia. Annals of Tropical Medicine & Parasitology. 1977 Mar 1;71(1):101-7. *In:* Munang'andu HM, Siamudaala V, Munyeme M, Nalubamba KS. A review of ecological factors associated with the epidemiology of wildlife trypanosomiasis in the Luangwa and Zambezi valley ecosystems of Zambia. Interdisciplinary Perspectives on Infectious Diseases. 2012 May 27;2012.
26. Späth, J. Natural host odours as possible attractants for *Glossina tachinoides* and *G. longipalpis* (Diptera: Glossinidae). *Acta Trop.* 1997, *68*, 149–158.
27. Leak SG, Ejigu D, Vreysen MJ. Collection of entomological baseline data for tsetse area-wide integrated pest management programmes. Food and Agriculture Organization of the United Nations (FAO); 2008.
28. Fetene E, Leta S, Regassa F, Büscher P. Global distribution, host range and prevalence of *Trypanosoma vivax*: a systematic review and meta-analysis. Parasites & Vectors. 2021 Dec;14(1):1-20.
29. Rodrigues AC, Neves L, Garcia HA, Viola LB, Marcili A, Da Silva FM, Sigauque I, Batista JS, Paiva F, Teixeira MM. Phylogenetic analysis of *Trypanosoma vivax* supports the separation of South American/West African from East African isolates and a new *T. vivax*-like genotype infecting a nyala antelope from Mozambique. Parasitology. 2008 Sep;135(11):1317-28.

30. Rodrigues CM, Garcia HA, Rodrigues AC, Costa-Martins AG, Pereira CL, Pereira DL, Bengaly Z, Neves L, Camargo EP, Hamilton PB, Teixeira MM. New insights from Gorongosa National Park and Niassa National Reserve of Mozambique increasing the genetic diversity of *Trypanosoma vivax* and *Trypanosoma vivax*-like in tsetse flies, wild ungulates and livestock from East Africa. Parasites & Vectors. 2017 Dec;10(1):1-6.
31. Nakamura Y, Hayashida K, Delesalle V, Qiu Y, Omori R, Simuunza M, Sugimoto C, Namangala B, Yamagishi J. Genetic Diversity of African Trypanosomes in Tsetse Flies and Cattle From the Kafue Ecosystem. Frontiers in veterinary science. 2021 Jan 27;8:5.
32. R. Fikru, I. Matetovici, S. Rogé, B. Merga, B.M. Goddeeris, P. Büscher, N. Van Reet. Ribosomal DNA analysis of tsetse and non tsetse transmitted Ethiopian *Trypanosoma vivax* strains in view of improved molecular diagnosis. Vet. Parasitol., 220 (2016 Apr 15), pp. 15-22.
33. Takken W. Push-pull strategies for vector control. Malaria Journal. 2010 Dec;9(2):1-1.
34. Hill N, Lenglet A, Arnez AM, Carneiro I. Plant based insect repellent and insecticide treated bed nets to protect against malaria in areas of early evening biting vectors: double blind randomised placebo controlled clinical trial in the Bolivian Amazon. bmj. 2007 Nov 15;335(7628):1023.
35. Deressa W, Yihdego YY, Kebede Z, Batisso E, Tekalegne A, Dagne GA. Effect of combining mosquito repellent and insecticide treated net on malaria prevalence in Southern Ethiopia: a cluster-randomised trial. Parasites & Vectors. 2014 Dec;7(1):1-0.

36. Vale GA, Lovemore DF, Flint S, Cockbill GF. Odour-baited targets to control tsetse flies, Glossina spp.(Diptera: Glossinidae), in Zimbabwe. Bulletin of Entomological Research. 1988 Mar;78(1):31-49.
37. Odeniran PO, Macleod ET, Ademola IO, Welburn SC. Molecular identification of bloodmeal sources and trypanosomes in Glossina spp., *Tabanus* spp. and *Stomoxys* spp. trapped on cattle farm settlements in southwest Nigeria. Medical and Veterinary Entomology. 2019 Jun;33(2):269-81.
38. Sluydts V, Durnez L, Heng S, Gryseels C, Canier L, Kim S, Van Roey K, Kerkhof K, Khim N, Mao S, Uk S. Efficacy of topical mosquito repellent (picaridin) plus long-lasting insecticidal nets versus long-lasting insecticidal nets alone for control of malaria: a cluster randomised controlled trial. The Lancet Infectious Diseases. 2016 Oct 1;16(10):1169-77.
39. Chen-Hussey V, Carneiro I, Keomanila H, Gray R, Bannavong S, Phanalasy S, Lindsay SW. Can topical insect repellents reduce malaria? A cluster-randomised controlled trial of the insect repellent N, N-diethyl-m-toluamide (DEET) in Lao PDR. PloS One. 2013 Aug 14;8(8):e70664.

APPENDIX

pathogens

MDPI

Article

Tsetse Bloodmeal Analyses Incriminate the Common Warthog *Phacochoerus africanus* as an Important Cryptic Host of Animal Trypanosomes in Smallholder Cattle Farming Communities in Shimba Hills, Kenya

Faith I. Ebhodaghe [1,2,*], Michael N. Okal [1], Shewit Kalayou [1], Armanda D. S. Bastos [2] and Daniel K. Masiga [1,*]

[1] International Centre of Insect Physiology and Ecology, Nairobi P.O. Box 30772-00100, Kenya; mnyanganga@icipe.org (M.N.O.); skalayou@icipe.org (S.K.)
[2] Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Pretoria Hatfield 0083, South Africa; adbastos@zoology.up.ac.za
* Correspondence: febhodaghe@icipe.org (F.I.E.); dmasiga@icipe.org (D.K.M.)

Abstract: Trypanosomes are endemic and retard cattle health in Shimba Hills, Kenya. Wildlife in the area act as reservoirs of the parasites. However, wild animal species that harbor and expose cattle to tsetse-borne trypanosomes are not well known in Shimba Hills. Using xeno-monitoring surveillance to investigate wild animal reservoirs and sources of trypanosomes in Shimba Hills, we screened 696 trypanosome-infected and uninfected tsetse flies for vertebrate DNA using multiple-gene PCR-High Resolution Melting analysis and amplicon sequencing. Results revealed that tsetse flies fed on 13 mammalian species, preferentially *Phacochoerus africanus* (warthogs) (17.39%, 95% CI: 14.56–20.21) and *Bos taurus* (cattle) (11.35%, 95% CI: 8.99–13.71). Some tsetse flies showed positive cases of bloodmeals from multiple hosts (3.45%, 95% CI: 2.09–4.81), including warthog and cattle (0.57%, 95% CI: 0.01–1.14). Importantly, tsetse flies that took bloodmeals from warthog had significant risk of infections with *Trypanosoma vivax* (5.79%, 95% CI: 1.57–10.00), *T. congolense* (7.44%, 95% CI: 2.70–12.18), and *T. brucei sl* (2.48%, 95% CI: −0.33–5.29). These findings implicate warthogs as important reservoirs of tsetse-borne trypanosomes affecting cattle in Shimba Hills and provide valuable epidemiological insights to underpin the parasites targeted management in *Nagana* vector control programs in the area.

Keywords: Trypanosomiasis; nagana; epidemiology; pathogen; spill-over; reservoir; asymptomatic host; wildlife-livestock interface; Shimba Hills; Kenya

Citation: Ebhodaghe, F.I.; Okal, M.N.; Kalayou, S.; Bastos, A.D.S.; Masiga, D.K. Tsetse Bloodmeal Analyses Incriminate the Common Warthog *Phacochoerus africanus* as an Important Cryptic Host of Animal Trypanosomes in Smallholder Cattle Farming Communities in Shimba Hills, Kenya. *Pathogens* **2021**, *10*, 1501. https://doi.org/10.3390/pathogens10111501

Academic Editor: Lawrence S. Young

Received: 2 October 2021
Accepted: 2 November 2021
Published: 18 November 2021

Infection, Genetics and Evolution 93 (2021) 104953

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Molecular characterization of *Trypanosoma vivax* in tsetse flies confirms the presence of the virulent *Tvv4* genotype in Kenya: Potential implications for the control of trypanosomiasis in Shimba Hills

Faith I. Ebhodaghe [a,b,*], Armanda D.S. Bastos [b], Kevin O. Kidambasi [a], Shewit Kalayou [a], Daniel K. Masiga [a], Michael N. Okal [a,*]

[a] International Centre of Insect Physiology and Ecology, P. O. Box 30772-00100, Nairobi, Kenya
[b] Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0083, Pretoria, South Africa

ARTICLE INFO

Keywords:
Trypanosoma vivax
Tvv4
Kenya
Tsetse
Wildlife-livestock interface

ABSTRACT

Trypanosoma vivax is a vector-borne protozoan parasite of livestock endemic to Africa and South America. To date, fifteen genotypes of the parasite have been described in vertebrate and insect hosts in East Africa. However, information regarding *T. vivax* diversity remains limited in many endemic countries in the sub-region, including Kenya. Such information could deepen insight into the local epidemiology of animal trypanosomiasis in Shimba Hills, a wildlife area in southeast Kenya where *T. vivax* is endemic and infects livestock. We employed two-gene conventional-PCR-sequencing and phylogenetic analysis to characterize *T. vivax* genotypes in tsetse flies collected between November 2018 and September 2019 in the wildlife-livestock interface of the Shimba Hills National Reserve. Phylogenetic analysis of Internal Transcribed Spacer-1 (ITS-1) sequences of *T. vivax* isolates confirmed the presence of two *T. vivax* genotypes in Shimba Hills of which >80% of *T. vivax* isolates from tsetse flies clustered within the virulent *Tvv4*-genotype clade. Tsetse infections with the *Tvv4* genotype were also confirmed based on 18S *r*RNA gene sequencing. Expanded gene characterization identified three closely related haplotypes within the *Tvv4*-clade. The *Tvv4*-isolates were detected in male and female *Glossina pallidipes* tsetse flies, most of which were collected from grasslands and within two kilometres of the Shimba Hills National Reserve boundary. Considering that *T. vivax* is the most common trypanosome in the Shimba Hills area and causes severe clinical conditions in livestock, the *Tvv4* genotype reported here for the first time in Kenya contributes to our understanding of these pathologies. The effectiveness of trypanocidal drugs in the management of *Tvv4* is presently not clearly understood. Therefore, the parasite management in Shimba Hills should focus on vector control to reduce the density of *G. pallidipes*, especially in grasslands near the wildlife protectorate.