MOLECULAR CHARACTERISATION OF TSETSE-ENDOSYMBIONT INTERACTIONS IN KENYAN POPULATIONS OF *Glossina austeni* AND *Glossina pallidipes* TSETSE FLIES

Florence Njeri Wamwiri
(SD14/0276/10)

A Thesis Submitted to the Graduate School in Fulfilment for the Requirements of the Doctor of Philosophy Degree in Biochemistry of Egerton University

EGERTON UNIVERSITY

March 2014

DECLARATION AND RECOMMENDATION

DECLARATION

I hereby declare that this thesis is my original work and has not been submitted for an award of a degree in any institution to the best of my knowledge.

Florence Njeri Wamwiri

Signed:.....Date: 17 MARCH 2014

RECOMMENDATION

We confirm that this thesis has our approval as supervisors to be presented for examination as per the Egerton University regulations.

Prof. Raphael Muchangi Ngure
Egerton University

Signed:.....Date: 17/3/2014

Dr Johnson Odera Ouma
Kenya Agricultural Research Institute-Trypanosomiasis Research Centre (KARI-TRC)

Signed:.....Date: 17 March 2014

Prof. Serap Aksoy
Yale University, New Haven, USA

Signed: Serap Aksoy Date: 17 March 2014

COPYRIGHT

DEDICATION

Dedicated to the memory of my loving parents, Wallace Wamwiri and MaryAnne Nyambura

ACKNOWLEDGEMENTS

I acknowledge with gratitude the guidance and advice from my supervisors: Dr Johnson Ouma for his keen mentorship and personal support towards developing my interest and skills in molecular entomology; Prof Raphael Ngure for continued guidance from the proposal stage to the completion of this work and Prof Serap Aksoy, who was always ready to share her enthusiasm, wealth of knowledge and experience on my thesis subject.

This work was possible through funding received directly or indirectly from various sources, whose support I hereby acknowledge. The tuition fees at Egerton University were covered by WHO/TDR Project A80132 grant to Dr J. Ouma. Dr Aksoy's NIH Training Grant D43TW007391 supported my 6-month sojourn at the Aksoy Laboratory, Yale University. The costs associated with field collections and laboratory analyses were covered by National Council for Science and Technology (NCST) PhD Grant and International Atomic Energy Agency Co-ordinated Research Project (IAEA-CRP) 14134. I acknowledge the Director, KARI and the Centre Director, TRC who granted me study leave, allowed me to conduct the research at KARI-TRC and provided the necessary facilitation and support.

I am grateful for the valuable technical assistance of the staff of TRC Entomology and Biochemistry Divisions in both field and laboratory work, especially Paul Thande, Samuel Guya and Kariuki Ndungu. A special appreciation goes Kenya Wildlife Service (KWS) for granting access into national parks for sample collection, and to Beatrice Elesani of ICIPE Muhaka Field Station for providing bench space in her laboratory while I worked in the field. I am thankful for the invaluable guidance and interactions with members of the Aksoy laboratory at Yale University. In particular, I am indebted to Dr Uzma Alam for her patience, mentorship and guidance. My fellow members of the TRC post-graduate study group and Dr Paul Mireji are highly appreciated for offering fruitful interactions and encouragement to reach the finish line.

Finally, I thank my family for their prayers, steadfast support and encouragement which has sustained me throughout the study period. I give honour and glory to the Almighty God for His abundant grace, through which I have been able to complete this work.

ABSTRACT

Tsetse flies (Diptera: Glossinidae), which are the major vectors of human and animal trypanosomes, harbour bacterial gut symbionts *Wigglesworthia glossinidia, Wolbachia pipientis* and *Sodalis glossinidius* in close association with trypanosomes. The objective of this study was to determine the prevalence, temporal variation and strain diversity of these endosymbionts in *G. austeni* and *G. pallidipes* from the Kenyan coast. The co-relation between symbiont and trypanosomes infection was also assessed, in order to provide insight into tsetse-symbiont-trypanosome interactions. Fly age was estimated using ovarian ageing and wing fray analysis. Symbiont infection was determined using symbiont-specific assays targeting *wsp*, 16s rRNA, *GPO1* genes. Trypanosome infection was also determined by dissection and generic primers. The diversity of infecting symbiont strains was evaluated by *wsp* typing, multi-locus strain typing (MLST) and variable number of tandem repeats (VNTRs) methods. Trypanosome-symbiont co-infection was assessed in both natural and experimental populations and in ampicillin-treated flies. The results showed that although *Wolbachia* infected all *G. austeni* individuals sampled, no *G. pallidipes* sampled was *Wolbachia*-infected. *Sodalis* infection was found in 3.7% of *G. austeni* and in 15.9% of *G. pallidipes* sampled. *Wolbachia* strains were found to be homogenous based on *wsp* typing, but slight variations were detected using MLST. In addition, multiple *Sodalis* strain variations were detected. Age grading revealed that female flies of both species had comparable longevity; but that male *G. pallidipes* analysis had longer lifespan than *G. austeni.* Trypanosome infection rates of 12.8% and 9.6% were recorded in *G. austeni* and *G. pallidipes* respectively, of which 45.6% were *T. congolense* infections. In natural populations, *Sodalis*-trypanosome co-infection was less than 4% in both species, predominantly with *T. congolense* or *T. simiae* trypanosomes. The level of significance of the association between trypanosome and *Sodalis* infection was found to differ depending on fly species and trypanosome species. This study concludes that there is marked heterogeneity in gut microfauna in terms of infection incidence, strain diversity and temporal infection patterns in natural tsetse populations. Additionally, infection with a particular symbiont does not appear to influence either the presence of the other symbionts or trypanosome infection. This study highlights inter-specific differences in tsetse-symbiont-trypanosomes tripartite interactions that proposed symbiont-mediated tsetse control interventions should take into account.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAT	African animal trypanosomosis
AW-IPM	Area-wide integrated pest management
CI	Cytoplasmic incompatibility
DNA	Deoxyribonucleic acid
ftsZ	Filamenting temperature-sensitive mutant Z
GPS	Geographic positioning system
HAT	Human African trypanosomosis
HVR	Hyper-variable regions
ITS	Internal transcribed spacer
KARI-TRC	Kenya Agricultural Research Institute-Trypanosomiasis Research Centre
MLST	Multi-locus sequence typing
mtDNA	Mitochondrial deoxyribonucleic acid
PATTEC	Pan African Tsetse and Trypanosomosis Eradication Campaign
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QT-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SAT	Sequential aerosol technique
SIT	Sterile insect technique
T&T	Tsetse and Trypanosomiasis
VNTR	Variable number of tandem repeats
WHO	World Health Organization
wsp	*Wolbachia* cell-surface specific protein

APPENDICES

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Trypanosomiasis, or trypanosomosis as it is now referred to, is one of the most studied zoonotic diseases, affecting man and his livestock. The tsetse fly, *Glossina* species, is the most important vector for the trypanosome, the parasite that causes trypanosomiasis. This disease has attracted immense scientific interest with some of the earliest research about the disease published in the late 1800's. That notwithstanding, trypanosomosis continues to be a constraint to livestock-based rural livelihoods and a potentially fatal human disease (Allsopp, 2001). Earlier research was based mainly on tsetse ecological studies and concentrated on ways to reduce the numbers of tsetse flies using bait-based methods such as use of traps, insecticide-treated targets and spraying, which have been seen to be effective over small operational areas (Willemse, 1991). As more advanced scientific techniques become more available, current research is directed more towards tsetse biology and in particular, methods to stop or reduce trypanosome acquisition, development and transmission. This line of research has led to the discovery of symbiotic microbes that live within the tsetse gut and which may influence tsetse and trypanosome interactions. Three of these symbiotic microbes (gut endosymbionts) have been identified: *Wigglesworthia glossinidia, Wolbachia pipientis* and *Sodalis glossinidius*. The endosymbionts inhabit the tsetse gut which is also the site of trypanosome maturation. The interactions between symbionts and trypanosomes in nature may provide an avenue for disease control. Not all tsetse individuals carry these symbionts and indeed some carry none except the primary symbiont *Wigglesworthia glossinidia*. This then raises the question as to whether the presence of one, or all or none of these symbionts affects the fly's vectorial capacity. The present study attempted to characterize tsetse-symbiont interactions in *Glossina pallidipes* and *G. austeni* from the Kenyan coast. Specifically, prevalence rates of *Wolbachia* and *Sodalis* infection were evaluated and the similarity or otherwise of the infecting strains determined. Co-infection with *Sodalis* and trypanosomes was investigated in both naturally and experimentally-infected *G. pallidipes.*

1.2 Statement of the Problem

Agriculture is considered to be the economic backbone of many sub-Saharan Africa (SSA) countries. The livestock sector is a key contributor to the economy, especially in the arid and semi-arid areas of the continent. However, many SSA countries are infested by tsetse fly, which is a major constraint to increased livestock productivity. In addition, Human African Trypanosomiasis (HAT) is re-emerging as a debilitating human disease in some of these countries. It is therefore imperative to achieve sustainable control of tsetse and trypanosomiasis (T&T) if our full economic potential is to be realized. However, traditional methods of tsetse control such as the use of baits and insecticides are neither sustainable nor environmentally acceptable. In order to effectively combat the problem of T&T, newer and more effective control interventions need to be identified. One such novel intervention is the manipulation of the bacterial gut endosymbionts that occur naturally in tsetse flies, such as *Wolbachia* and *Sodalis*, to develop applications to deliver targeted population reduction and/or replacement or the interruption of the parasite development and transmission cycle. This study seeks to understand tsetse-symbiont interaction in two fly species that are important for transmission of both animal and human disease, and to understand how these interactions can be harnessed for vector and disease control.

1.3 General Objective

The overall objective of the study was to characterize interactions between tsetse flies and their symbionts in terms of prevalence and effects of infection in relation to the feasibility of using the symbiont-based strategies for tsetse and trypanosomosis control.

1.4 Specific Objectives

1. To determine the prevalence, tissue distribution, infection types and temporal variations of *Sodalis* and *Wolbachia* in *Glossina austeni* and *G. pallidipes.*
2. To identify genetic differences in strains of *Wolbachia* and *Sodalis* symbionts from different geographical populations.
3. To determine the co-relation between trypanosome and symbiont infection in naturally and experimentally-infected flies *Glossina austeni* and *G. pallidipes.*

4. To determine the effect of ampicillin on *G. pallidipes* survival, symbiont clearance and trypanosome infection.

1.5 Hypotheses

The following hypotheses were tested in the study:

1. The prevalence, tissue distribution, infection types and temporal variations of *Wolbachia* and *Sodalis* do not differ among *Glossina austeni* and *G. pallidipes* populations.
2. Strains of *Wolbachia* and *Sodalis* from different geographical populations are homogenous.
3. There is no co-relation between trypanosome and symbiont infection in *G. austeni* and *G. pallidipes* populations.
4. Ampicillin has no effect on *G. pallidipes* survival, symbiont clearance and trypanosome infection.

1.6 Justification for the Study

Trypanosomiasis is a constraint to agricultural productivity, due to reduced output in both man and his livestock. Traditional methods to control or eliminate the tsetse fly have had limited sustainability. To overcome the problem of inefficient control interventions, current focus is now on sustainable control interventions which apply novel knowledge on fly and trypanosome biology. The concept of manipulating naturally occurring symbionts towards pest and disease control has been applied in various insect pests. This has not been applied in the control of T&T. Incorporation of symbiont-based control methods such as *Wolbachia*-mediated CI and paratransgenic applications into SIT may serve to increase the efficacy and reduce costs associated with this technique. However, this approach has not been well explored due to the lack of adequate information on tsetse-symbiont interactions, particularly in natural populations. Against this background, this study is justified to generate supportive data and information on tsetse-symbiont-trypanosome tripartite interactions, in order to identify interactions that can be harnessed to contribute to vector and disease control.

1.7 Scope and Limitations of the Study

This study assessed the prevalence and diversity of bacterial symbionts in various tsetse populations. Although three symbionts have been described in tsetse, namely *Wigglesworthia*, *Wolbachia* and *Sodalis*, this study focused on the latter two, given that *Wigglesworthia* is found in all tsetse individuals. The effect of symbiont infection on trypanosome maturation was also evaluated experimentally. However, due to colony maintenance considerations, female flies were unavailable for these experiments and the results reported are based on experiments with male flies only. The results obtained using male flies are considered to be representative of both sexes, as previous research has shown that the parameters of interest to this study are not sex-biased. Further, unavailability of high resolution gel systems such as polyacrylamide gel electrophoresis (PAGE) limited our analysis of strain diversity. However, this limitation was overcome by use of sequence data instead.

CHAPTER TWO

LITERATURE REVIEW

2.1 General Introduction

"Trypanosomiases" or trypanosomoses describe a group of closely related diseases which are caused by a single-celled organism, the trypanosome and which affect man and his livestock and. There are two types of trypanosomosis: South American trypanosomosis which is caused by the parasite *Trypanosoma cruzi* transmitted by triatomine bugs. This disease is limited to the South American countries, where it causes the human disease known as Chaga's disease (Beard *et al.*, 2002). The second type of trypanosomosis is the African trypanosomosis, transmitted by the tsetse fly. Further discussions in this study are related to the African trypanosomosis only.

2.2 Historical Perspective

The first case report of sleeping sickness or Human African Trypanosomosis (HAT) comes from the Arab historian Ibn Khaldun. In his historical work, he reported about the death of Sultan Mari Jata, Emperor of Mali, who died of an illness with a description similar to human trypanosomosis (Williams, 1996). In early modern times, the history of HAT was linked to traders and missionaries coming to Africa as explorers. In 1734, the English naval surgeon John Aktins published the first accurate medical report on African sleeping sickness (Cox, 2004), describing mainly the neurological symptoms of the late stage of sleeping sickness. In 1803, the English physician Thomas Winterbottom published a report referring to the characteristic sign of swollen lymph glands along the back of the neck in the early stage of the disease, as quoted in (Cox, 2004). He also mentioned that this symptom was known long ago by Arabian slave-traders who refrained from buying slaves with this symptom. Although throughout the 19^{th} century, reports on sleeping sickness increased and human African trypanosomosis became a well-recognized disease, however no one had any real idea about the nature of the illness. Winkle notes that the explorer David Livingstone first suggested that nagana is caused by the bite of tsetse flies, quoted by (Steverding, 2008). However, it took another 40–50 years for trypanosomes to be identified as the causative agents of nagana and sleeping sickness. In 1895, the Scottish pathologist and microbiologist David Bruce discovered *T. brucei* as the cause of cattle trypanosomosis (nagana) (Bruce, 1895). The first unequivocal observation of

trypanosomes in human blood was made by the British Colonial surgeon Robert Michael Forde in 1901 when he examined a steamboat captain in The Gambia (Forde, 1902). He first thought that the organisms he found were worms but the English physician Joseph Everett Dutton identified them as trypanosomes a few months later and proposed the species name *Trypanosoma gambiense,* now called *T. b. gambiense* (Dutton, 1902). In the same year, the Italian physician and pathologist Aldo Castellani found trypanosomes in the cerebrospinal fluid of sleeping sickness patients and suggested that they were the cause of sleeping sickness (Castellani, 1903).

2.3 The Vector – Tsetse Fly *Glossina* sp

Tsetse flies *Glossina* spp. (Diptera: Glossinidae) are the key vectors of African trypanosomosis although biting flies such as tabanids and stomoxys can also transmit the parasite (Desquesnes and Dia, 2004). The distribution of the tsetse fly is determined mainly by climate, but is influenced by altitude, vegetative cover and the presence of suitable host animals (Leak, 1999). Generally, tsetse flies occur in a belt across tropical Africa between 15°N and 29°S, covering about 10 million km^2 of sub-Saharan Africa and extending through 37 countries (WHO, 2006). Outside the known tsetse distribution limits, mechanical transmission of *T. vivax* and *T. evansi* by biting flies such as tabanids causes trypanosomosis in camels and other livestock, while *T. equiperdum* is transmitted among equines through coitus (Uilenberg, 1998). The problem of T&T is finely correlated with poverty in the continent. A total of 20 out of the 37 tsetse-infested countries rank among the world's poorest 25 countries (PAAT, 2008).

The life cycle of the tsetse fly is described as adenotrophic viviparity, whereby the larva develops inside the mother. The tsetse fly, a *k*-strategist, differs from many insects in that only one offspring is produced approximately every nine days (Leak, 1999). The egg produced from the ovary is maintained and nourished in the uterus until it reaches the 3rd instar larval stage. It is then deposited in sand or soft soil and within 1-2 hours, this larva becomes barrel-shaped and darkens as it changes into a pupa. The pupa incubates for 4-5 weeks after which the adult fly emerges to continue the life cycle (Figure 2.1). During development of the larva within the uterus, nourishment is provided via the milk gland tubules which carry nutrients from mother to offspring. It is during this period when transmission of the symbionts *Wigglesworthia glossinidia*

and *Sodalis glossinidius* takes place from the maternal milk gland structure to the intrauterine larvae (Aksoy *et al.*, 1997).

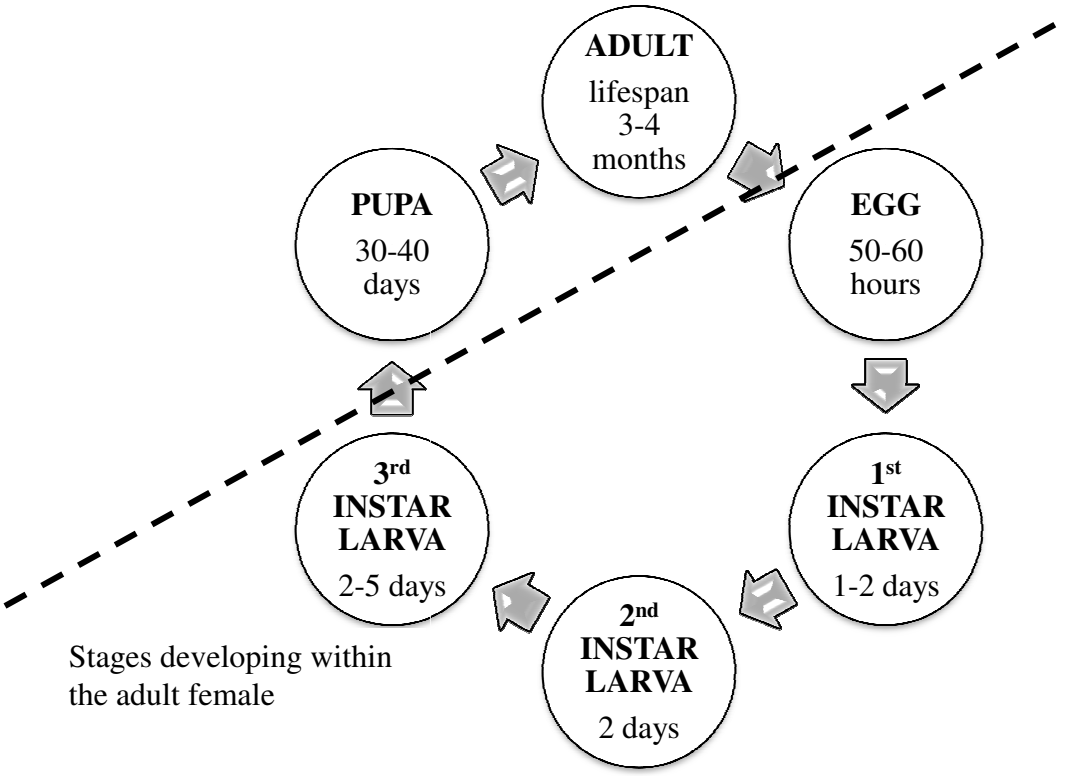

Figure 2.1: Life cycle of the tsetse fly *Glossina* species

2.4 The Disease - Trypanosomosis

African trypanosomosis is a zoonotic disease, affecting both man and his livestock. Wild animals act as reservoirs for the disease, capable of being infected but without any apparent adverse effects (Anderson *et al.*, 2011). Domestic animals such as cattle and pigs also act as reservoirs and these have been implicated in transmission of parasites to humans (von Wissmann *et al.*, 2011). The link between tsetse flies and HAT was first described by David Bruce (Bruce, 1895). There are two distinct forms of sleeping sickness; the chronic form caused by *Trypanosoma brucei gambiense* occurs in West and Central Africa while the acute form, Rhodesian sleeping sickness is caused by *T. brucei rhodesiense* and occurs in eastern and southern Africa (Welburn *et al.*, 2004). The WHO estimates that although 60 million people are at risk of contracting sleeping sickness, only about 10% of these people are under surveillance. In the late 90's, it was estimated that about 300,000 new cases occurred each year, but only about

30,000 - 40,000 of these were diagnosed and treated (WHO, 1998). Very few new cases are diagnosed per year (Gibson *et al.*, 2002). This under-surveillance is attributed to weaknesses in control programs, difficulty and/or inadequacies in disease diagnosis and often, inaccessibility of affected areas (Cattand *et al.*, 2009). HAT is a re-emerging zoonotic disease (Etchegorry *et al.*, 2001). From its first appearance around 1900 to the early 1960s, extensive control programs managed to control the disease throughout the continent. However, with the onset of independence in many African countries, T&T control was neglected, leading to recrudescence of the disease in the 1970's and 1980's (Hotez, 2008). Recent resurgence of HAT has been attributed to political and civil unrest in countries such as Sudan, Angola and the Democratic Republic of Congo (DRC), which has resulted in mass migration of populations into risk situations and the breakdown of traditional government support systems (Ford, 2007). The spread of HAT in south-eastern Uganda has been linked to movement of cattle for re-stocking purposes. This cattle movement caused the disease to spread into 8 new districts between 1998 and 2006 (Fevre *et al.*, 2003). Although HAT is a debilitating disease that is fatal unless treated, no new drugs have been developed since 1949 and those that have come into recent use are chance discoveries, initially intended to treat other diseases such as cancer and Chagas' disease (Truc, 2003). Currently, HAT drugs are provided free of charge by WHO in conjunction with some pharmaceutical companies. Continued availability and affordability of HAT drugs is not guaranteed, therefore control of the vector is essential (Etchegorry *et al.,* 2001). African animal trypanosomosis (AAT), also known as nagana, is a major constraint to animal productivity with about 46 million cattle in sub-Saharan Africa at risk of contracting AAT (Kristjanson *et al.*, 1999). Nagana causes general weakness and morbidity, leading to reduced meat and milk production and loss of traction power (Swallow, 2000). Other adverse effects include infertility, abortion, poor health, poor body condition and ultimately death of affected animals. Infected animals and wild animals act as reservoir for the human disease (Anderson *et al.*, 2011). Rising population levels in Africa have caused an increase in land-use pressure, which has resulted in more people settling in tsetse infested marginal areas thereby exposing themselves and their livestock to trypanosomosis risk (De La Rocque *et al.*, 2001).

2.5 Disease Transmission Cycle

The transmission of trypanosomes after they undergo a cycle of development, culminating in the production of infective metacyclic trypanosomes, is referred to as cyclical transmission. This mode of transmission is more common than mechanical transmission, which requires that blood containing infectious trypanosomes be transferred from one animal to another. The length of the cyclical transmission cycle for tsetse-vectored trypanosomes varies with trypanosome species and fly species. The *Trypanosoma brucei* transmission cycle begins when a tsetse fly picks up infective trypomastigotes while feeding on an infected mammalian host (Figure 2.2). In the fly midgut, the parasites multiply by binary fission over a period of 2-3 weeks and transform into procyclic trypomastigotes. These forms then leave the midgut, transform into 'stumpy form" epimastigotes and migrate into the salivary glands, where they transform further and mature into metacyclic trypomastigotes (Roberts and Janovy, 2000). The metacyclics are the only forms that are infective to mammalian hosts. When feeding on an uninfected host, the infected fly injects metacyclic trypomastigotes into the skin tissue. The parasites then enter the lymphatic system, pass into the bloodstream and multiply, and finally transform into bloodstream trypomastigotes (O'Neill *et al.*, 1992). The bloodstream forms can then be picked up by another uninfected fly and the cycle continues. A simplified developmental cycle is shown in figure 2.2 (Lee *et al.*, 2007).

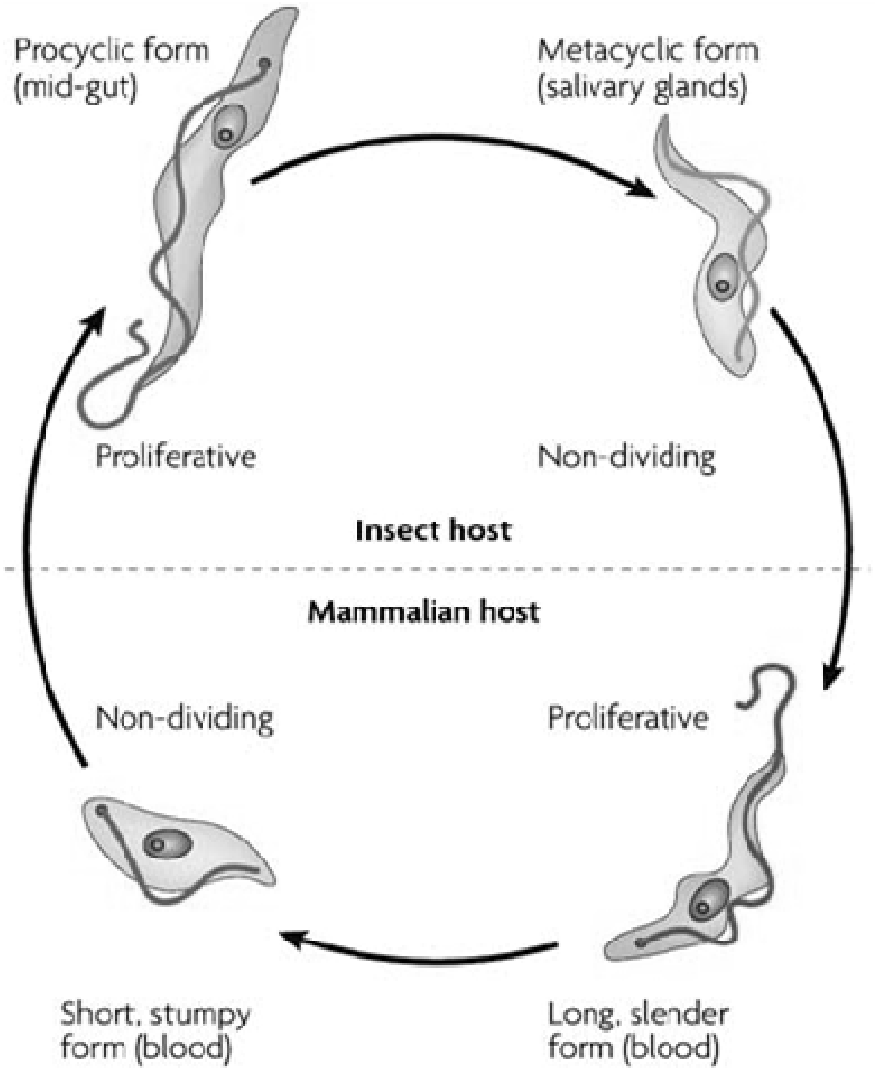

Figure 2.2: Life cycle of *Trypanozoon* species in both mammalian host and tsetse host

2.6 Current State of Tsetse and Trypanosomosis in Kenya

There are 8 species of tsetse found in Kenya, grouped into three sub-genuses (Table 2.1). Tsetse flies are widespread in the country, infesting about 138,000 km^2 and covering 38 out of the 48 counties (KETRI, 1997). The distribution is confined to protected areas such as national parks and reserves (Bourn *et al.*, 2001) although isolated peridomestic populations can still be found. Only one riverine species *G. f. fuscipes* is found and it is concentrated along the shores of Lake Victoria. Tsetse distribution in 1997 is shown in figure 2.3, but an alteration in this distribution is to be expected, mainly due to changes in land-use patterns.

Table 2.1: *Glossina* sub-species found in Kenya

Glossina sub-genus	Species found in Kenya
Morsitans (savannah) group	*Glossina pallidipes* Austen
	G. morsitans morsitans Westwood
	G. austeni Newstead
	G. swynnertoni Austen
Fusca (forest) group	*G. brevipalpis* Newstead
	G. fuscapleuris Austen
	G. longipennis Corti
Palpalis (riverine) group	*G. fuscipes fuscipes* Newstead

Glossina pallidipes is the most important tsetse species in Kenya, due to its widespread distribution and high vectorial capacity. This species is a key vector for both livestock and human-infective trypanosomes (Wellde *et al.*, 1989). HAT in Kenya has previously occurred in periodic outbreaks in Nyanza and Western provinces, resulting in 3,539 patients diagnosed at public hospitals in western Kenya and eastern Uganda between 1950 and 2007 (Rutto and Karuga, 2009). However, many other infected persons remain undiagnosed, often due to difficulty or inadequacies in disease diagnosis at primary health centers. In 2012, two European tourists were diagnosed with sleeping sickness after visiting the Maasai Mara game reserve (Clerinx *et al.*, 2012). On the other hand, AAT (nagana) is more widespread and prevalent than the human disease and a high prevalence of infection is obtained throughout the country. For instance, in western Kenya, prevalence rates of 41%, 29% and 12% have been reported in Suba, Teso and Busia districts, respectively (Thumbi *et al.*, 2010; von Wissmann *et al.*, 2011) while in Kwale district, a prevalence rate of 18% was reported (Ohaga *et al.*, 2007). Livestock farmers rely on on-farm disease control using veterinary drugs to prevent and treat nagana (Cattand *et al.*, 2009). Previous tsetse control interventions have included community trapping by the Maasai in Nguruman (Leak, 1999) and Teso districts (Barrett and Okali, 1998) and the use of impregnated targets in Lambwe Valley (Opiyo *et al.*, 1990) among other interventions. None of these interventions have been sustainable. The current T&T operations in Kenya strive to use a synergistic combination of methods to promote sustainable control as part of rural development.

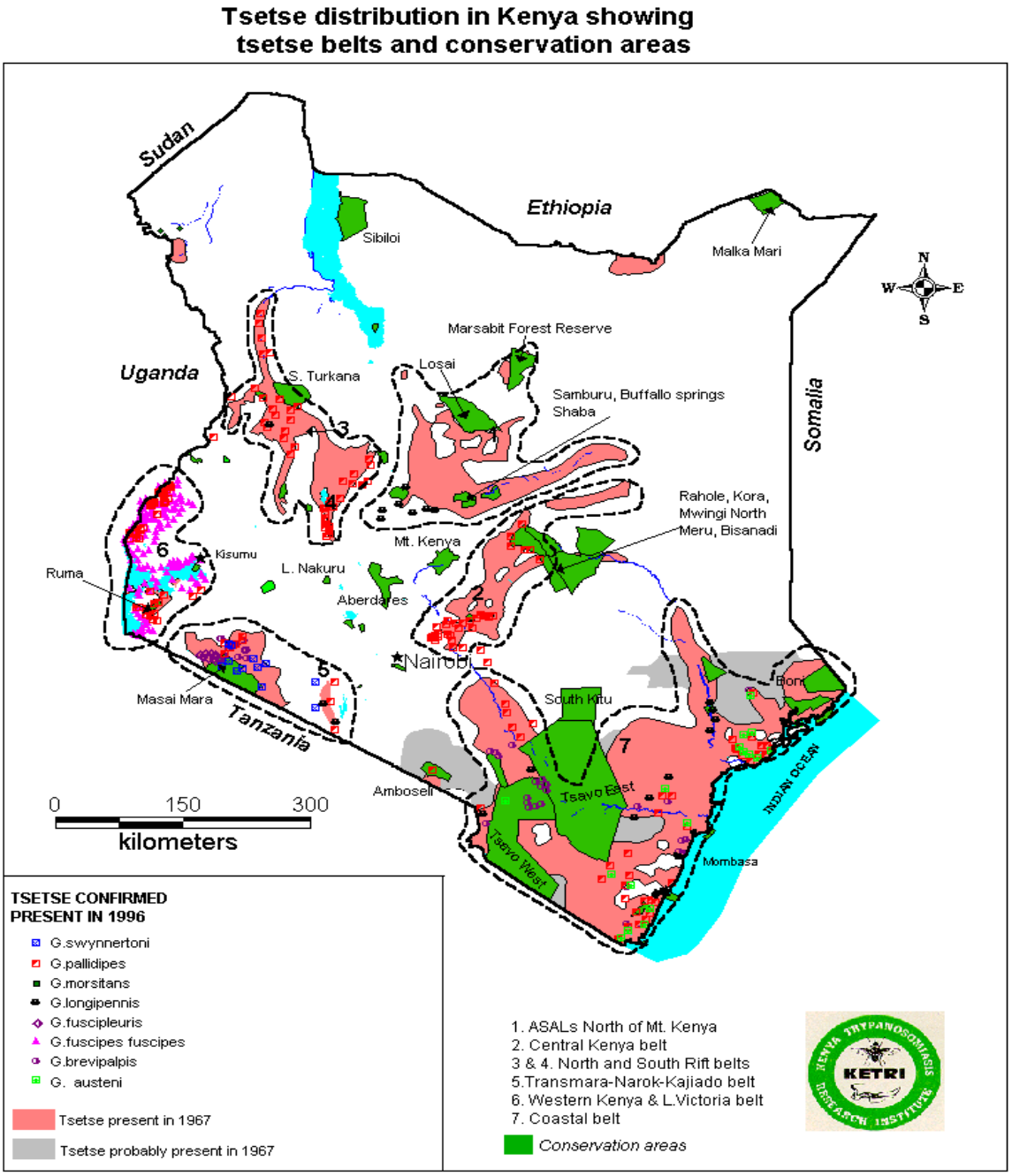

Figure 2.3: Tsetse fly distribution in Kenya (source: KETRI, 1997)

2.7 Trypanosomosis Control

Tsetse and trypanosomosis are unique to sub-Saharan Africa. The "tsetse belt" covers about 9 million km^2 of the humid and sub-humid zones, representing roughly 39% of the agricultural land in these countries. The control of animal trypanosomosis is achieved mainly through the use of preventive and curative trypanocidal drugs. Keeping of trypanotolerant cattle breeds such as N'dama in tsetse infested areas has also helped livestock keepers evade the effect of the disease (d'Ieteren *et al.*, 1998). Control of HAT continues to depend on early diagnosis and prompt treatment, and vector control to interrupt disease transmission (Hotez, 2008). However, management of HAT is affected by several factors; production of HAT drugs is challenging because pharmaceutical companies consider them to be unprofitable (Etchegorry *et al.*, 2001) in addition to the occurrence of treatment failures with the available drugs due to drug resistance (Legros *et al.*, 1999). To counter these challenges, WHO has created a network of stakeholders to monitor drug resistance, as well as to find and recommend solutions for the treatment of sleeping sickness (Etchegorry *et al.*, 2001). As detailed in figure 2.4, T & T control has cross-cutting inter-sectoral benefits covering livestock productivity, human health and ultimately enhancing rural development (Cattand *et al.*, 2009).

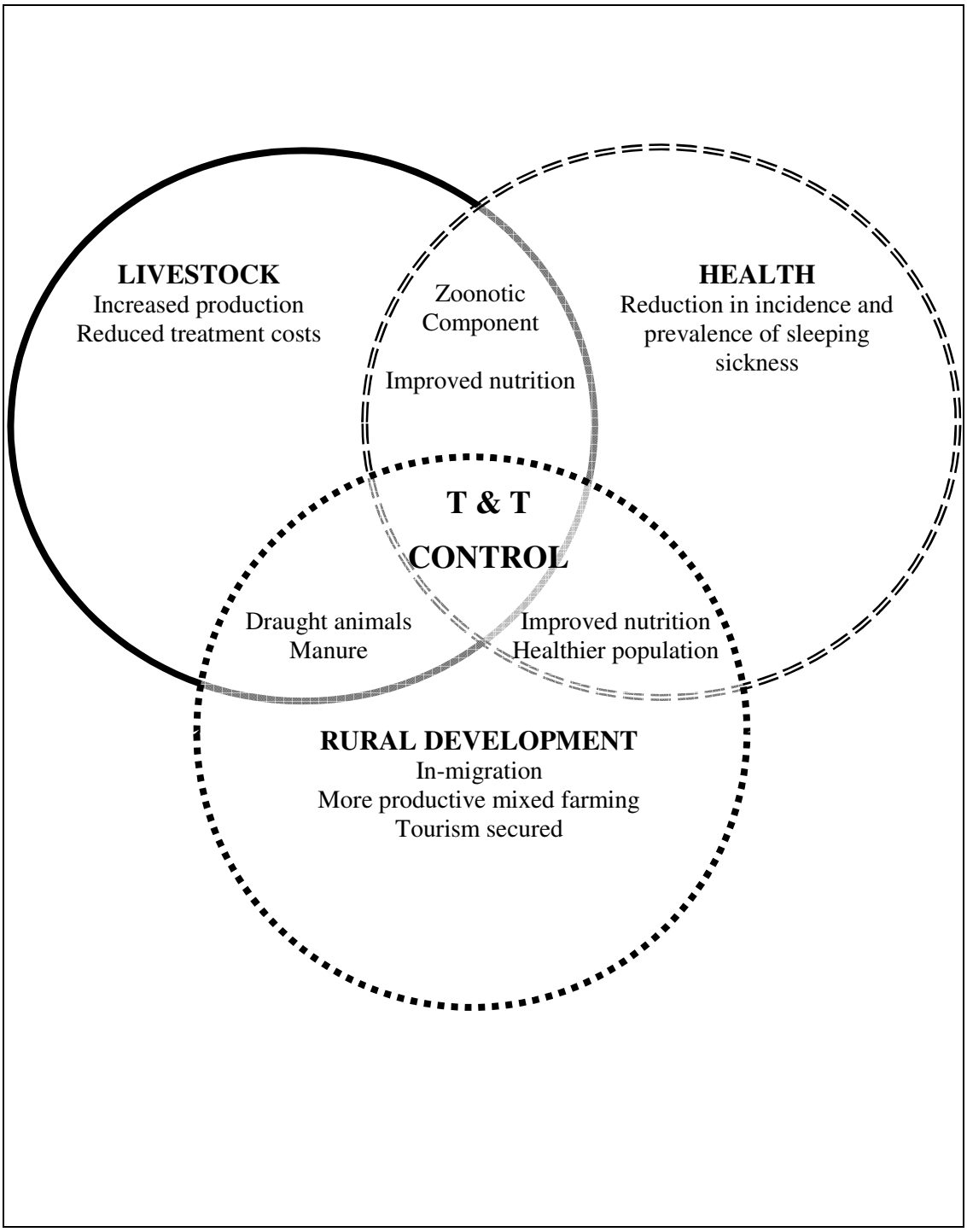

Figure 2.4: Inter-sectoral nature of benefits resulting from tsetse and trypanosomosis control

2.8 Vector-based Control Strategies

Tsetse fly control and eradication methods have generally exploited one or more behavioural characteristics of the tsetse fly. Thus, use of bait methods including traps and insecticide-treated targets, which attract flies based on visual and odour cues have been used to reduce tsetse populations over small areas (Willemse, 1991). Cattle which have had an application of pour-on insecticides via dip or spray pump formulations can act as bait animals to attract and knock-down tsetse (Okiria *et al.*, 2002). Spraying tsetse habitats by either aerial or ground-spraying has been used to control tsetse in many countries including Botswana, Cote d'Ivoire, Nigeria, Uganda, Tanzania, Somalia, Zambia and Zimbabwe (Hargrove, 2003). Between 1968 and 1991, approximately 143,500 km^2 in 9 African countries was treated using the Sequential Aerial Technique (SAT) to control tsetse flies (Allsopp *et al.*, 2010). The SAT efforts have been sustainable in areas such as the Okavango delta in Botswana where *Glossina morsitans centralis* has been eradicated (Kgori *et al.*, 2006).

Only a very limited number of tsetse control interventions throughout the African continent have been sustainable or permanently employed. One oft-quoted example is the eradication of *G. austeni* in Unguja Island, Zanzibar by use of the Sterile Insect Technique (SIT) (Vreysen *et al.,* 2000a). The SIT is based on the mating behaviour of insects on the premise that they mate once during their lifetimes (Knipling, 1955). The recent findings that multiple mating, referred to as polyandry, is a rather common event in tsetse flies (Bonomi *et al.*, 2011; Geiger *et al.*, 2013) do not, however, negate the basic principles of the technique (Barclay, 2005). SIT involves the sterilization of male flies through the use of gamma-irradiation, chemo-sterilization or physiological methods such as application of juvenile hormones (Hendrichs *et al.*, 2007). The sterilized males are then released into a native population and anywild female mating with a sterile male will be unable to produce an offspring thus decreasing the population to eradication (Knipling, 1979). The SIT approach has been used successfully for control and eradication of insect pests including the New World screwworm *Cochliomyia hominivorax* in parts of America and North Africa as well as for the Mediterranean fruit fly *Ceratitis capitata* in south America (Rendón *et al.*, 2004). SIT is very expensive, requiring high inputs of resources, especially for the production of large numbers of males in a breeding laboratory before release. Both the efficacy and cost-effectiveness of SIT programmes can be improved by various factors,

including: (a) improved and more efficient mass production of sterile males (b) incorporation of tsetse symbionts and pathogens to decrease the vectorial capacity of released males (c) near-total suppression of the target population before release of sterile male begins. Regarding the incorporation of symbionts, in theory *Wolbachia* may be used to improve SIT through expression of the cytoplasmic incompatibility (CI) effect, while tsetse-*Sodalis* interactions may be manipulated to influence parasite acquisition and/or maturation in tsetse flies. In 2000, African heads of state and government passed a resolution to create the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC), whose mandate is to assist in eliminating the scourge of tsetse-transmitted trypanosomosis from the African continent. This campaign is led by Burkina Faso, Ethiopia, Ghana, Kenya, Mali and Senegal. The programme seeks to create sustainable tsetse and trypanosomosis-free areas in sub-Saharan Africa by integrating suppression, control and eradication approaches while ensuring the reclaimed areas are sustainably, equitably and economically exploited. It is expected that PATTEC will employ a combination of different strategies to control both the vector and the disease and ultimately, lead to eradication of trypanosomosis in the continent.

2.9 Bacterial Gut Symbionts of the Tsetse Fly

Many species of arthropods are host to various bacterial microorganisms. These microorganisms have traditionally been classified as mutualists when beneficial, parasitic when harmful, or as commensal when neutral. Insects that feed on a single restricted diet throughout their lives such as plant sap, wood or animal blood often harbor mutualists which provide additional nutrients that may be unavailable through their normal diet, and which they are unable to synthesize (Aksoy *et al.*, 2001). Tsetse flies harbor at least three bacterial endosymbionts, some of which contribute to their nutrition by synthesis of vitamins such as folic acid and vitamin B complex metabolites (Nogge, 1981). The larger symbionts found in the mycetome, a giant-cell zone in the anterior midgut epithelium are classified under the γ-*Proteobacteria* and are called the primary or (P)-endosymbionts. *Wigglesworthia glossinidia,* the primary-endosymbiont of tsetse flies is transmitted through the maternal milk gland secretions and has obligate functions in host biology (Aksoy, 1995). *W. glossinidia* has a 700kb genome and is found intracellularly in the differentiated epithelial cells bacteriocytes of all tsetse individuals.

Wigglesworthia is involved in the synthesis of essential vitamins not found in tsetse blood diet and elimination of this symbiont results in loss of fecundity (Nogge, 1976; Nogge, 1981).

The secondary (S)-endosymbionts are not obligatory to host biology but may provide host benefits depending on environmental context, such as during periods of heat stress, parasitoid attack or towards the utilization of particular host plant substrates (Snyder *et al.*, 2011). In *Glossina*, the s-endosymbiont *Sodalis glossinidius* is α-*Proteobacteria* found in the midgut epithelium, ovaries and embryos (Aksoy *et al.*, 1997). It is both intra and extra-cellular and has a wider somatic distribution than *Wigglesworthia*. It is found principally in the midgut tissues, muscles, fat bodies, hemolymph, milk glands and in the salivary glands (Cheng and Aksoy, 1999). The sequenced genome of *Sodalis* is 4.2Mb and reveals features typically associated with closely related free-living bacteria (Toh *et al.*, 2006). The genome, with only 49% coding capacity and a high number of pseudogenes, is indicative of ongoing adaptation from a free-living to a symbiotic lifestyle. Similar to *Wigglesworthia*, *Sodalis* is acquired by the intra-uterine larvae through maternal milk secretions. The density of *Sodalis* infection varies with species and flies that harbor greater densities of *Sodalis* have been suggested to be more susceptible to trypanosome infection (Geiger *et al.*, 2007). Apart from the density-dependent effects, individual populations of tsetse may possess different genotypes of *Sodalis,* which in turn have different effects on the flies' ability to acquire and transmit trypanosomes (Geiger *et al.*, 2007).

The third endosymbiont present in some tsetse species is the parasitic *Wolbachia pipientis* (Cheng *et al.*, 2000). *Wolbachia pipientis* is a non-obligatory endosymbiont of arthropods infecting 75% of all arthropods species (Werren, 1997; Werren and O'Neill, 1997; Bourtzis and O'Neill, 1998; Jeyaprakash and Hoy, 2000). *Wolbachia* is usually maternally-transmitted through the mother's milk gland secretions into the developing larvae (Cheng and Aksoy, 1999) but horizontal transmission is thought to occur in rare instances (Rousset *et al.*, 1992). The functions of *Wolbachia* are not well known but its presence has been shown to cause a variety of reproductive abnormalities. The phenotypes expressed include cytoplasmic incompatibility (CI), male killing whereby only female eggs are fertilized, feminization of genetic males whereby infected genetic males convert to fertile females and thelytokous parthenogenesis whereby infected non-fertilized females produce daughters (Stouthamer *et al.*, 1999). The prevalence rates of *Wolbachia* infections in tsetse reared under colony conditions reach levels of 100%, while field prevalence rates is often lower (Cheng *et al.*, 2000). In *G. morsitans* and *G. brevipalpis,* this

bacterium has been shown to reside only in the reproductive tissues namely the ovary and testes while in *G. austeni* it can be detected in various somatic tissues (Cheng *et al.*, 2000). It is hypothesized that the particular phenotype of the bacterial strain harbored in *G. austeni* may be similar to that reported in the *Wolbachia* popcorn strain characterized from *Drosophila melanogaster* (Aksoy, 2000). This strain results in reduced fitness effects, forming heavy infections in nervous and muscle tissues and drastically reduces the life-span of adult flies (Min and Benzer, 1997). It would be of great value to investigate whether such infections in tsetse also cause reduced fly fitness.

2.10 Dynamics of Symbiont Infection in Tsetse Flies

There is scanty information available regarding *Sodalis* and *Wolbachia* infection dynamics or about the ecological factors that may impact upon their biology. Inside the fly itself, changes occur in the densities of the symbionts throughout the developmental cycle from early larval stages to adulthood. *Sodalis* shows increased proliferation during the late pupal and early adult development stages while *Wolbachia* density shows high variability between same-age individuals (Rio *et al.*, 2006). An evaluation of symbiont densities allows correlations to be drawn with characteristics such as expression of CI in case of *Wolbachia* and vectorial capacity in the case of *Sodalis*. The key parameters in *Wolbachia* infection dynamics include the strength of the incompatibility phenotype, fitness effects of the infection and the fidelity of the maternal inheritance (Hoffmann and Turelli, 1997). Examination of fitness effects associated with somatic infections addresses the potential adaptive significance of *Wolbachia,* because such somatic infections may improve the host's fecundity or the vertical transmission rates of *Wolbachia*. However, estimation of these three key parameters often suggests that the negative effects on host fitness are rare and limited (Poinsot *et al.*, 1998) and that CI level and transmission efficiency seem to be the main factors. Analysis of colony flies shows that the prevalence of infection is fixed at 100% and that the fidelity of maternal transmission of the bacterium to the progeny is complete, with each progeny carrying the infection (Alam *et al.*, 2011). These parameters determine the infection frequency after a population replacement event, an important consideration because the goal of population replacement is for the entire vector population to carry the desired genotype (Aksoy *et al.*, 2001).

Symbionts are basically bacteria and various antibiotics will eliminate them from the fly. The selective application of these antibiotics, either by continuous *per os* treatment or by intra-thoracic microinjection, has been used to help separate the specific effects of each symbiont. Ampicillin given by either route has no effect on fecundity or on symbiont viability. However, the resulting progeny lack *Wigglesworthia* but harbor *Sodalis* (Pais *et al.*, 2008). On the other hand, tetracycline feeding causes an almost complete clearance of *Sodalis, Wolbachia* and *Wigglesworthia* from treated flies, leading to total fly sterility. *Wigglesworthia*, which is essential for tsetse fecundity is destroyed by tetracycline supplementation causing loss of fertility (Nogge, 1976). Dietary supplementation with yeast extract however rescues fecundity in *G. m. morsitans* in the absence of *Wigglesworthia* (Alam *et al.*, 2011). Progeny produced from tetracycline and yeast fed flies are aposymbiotic but can be propagated through provision of yeast supplemented blood to produce an aposymbiotic line of tsetse flies.

2.11 Harnessing Insect-Symbiont Interactions for Disease Control

New knowledge in the fields of insect cell biology, immunology and developmental biology provides an opportunity to strengthen existing control methods, as well as to develop new approaches in vector control.

2.11.1 Paratransgenesis for trypanosome transmission blocking in *Glossina*

Transformation systems based on deoxyribonucleic acid (DNA) have been developed with the goal of vector competence modulation (Beerntsen *et al.*, 2000). In transgenic approaches, the vector itself is modified to manifest certain desirable characteristics. In contrast, using the paratransgenic approach the symbiont in the vector is modified and not the vector itself. Paratransgenesis has been applied in control of Chagas' disease where the bacteria *Rhodococcus* has been modified to express foreign genes in the triatomine bug vector with the net effect of reducing the capacity of the vector to transmit the *T. cruzi* parasite (Beard *et al.*, 2002). Viability of the paratransgenic approach has also been demonstrated in sandflies for the control of *Leishmania* (Hurwitz *et al.*, 2011). Availability of *in vitro* cultures of *Sodalis* has enabled development of genetic transformation systems that introduce and express foreign products in *Sodalis* and subsequently into host insects (Beard *et al.*, 1993). In tsetse flies, modified *Sodalis*, termed recombinant *Sodalis* (rec*Sodalis*) can be injected into the parent female, and are passed

onto multiple subsequent generations, faithfully expressing the marker gene product (Cheng and Aksoy, 1999). In this way, if the rec*Sodalis* have been modified to express anti-trypanosomal products, they can potentially block parasite development, considering that *Sodalis* resides also in the midgut, where trypanosome development takes place. Such engineered trypanosome-refractory tsetse flies can be produced on an industrial scale and used for SIT releases.Several compounds have been identified as ideal candidates for transgenic expression in *S. glossinidius* as a strategy for inhibiting trypanosome survival, development, and maturation in tsetse and therefore interference with transmission of African sleeping sickness (Haines *et al.*, 2003).

2.11.2 *Wolbachia*-mediated cytoplasmic incompatibility

Wolbachia infection displays several phenotypes in the host, some of which can be harnessed for disease control. The most studied phenotype is cytoplasmic incompatibility (CI). This occurs when uninfected females inseminated by an infected male produce eggs that do not undergo embryogenesis. In such an incompatible cross, the sperm enters the egg but does not contribute its genetic material to the potential zygote, thus disrupting early fertilization events and resulting in embryonic death (Hofmann and Turelli, 1997). While the molecular basis of CI is still unknown, it has been suggested that two bacterial functions are involved. These include *modification* (*mod*) which modifies the sperm during spermatogenesis and *rescue* (*resc*) which acts in the female germline and/or in early embryos, neutralizing the modification (Venetia *et al.*, 2003). CI may be unidirectional between infected and uninfected individuals or bidirectional between individuals infected with two different *Wolbachia* strains (Werren *et al.*, 2008). On the other hand, infected females are fertile and fecund and can produce progeny with both the imprinted and normal sperm. This asymmetry in fertility causes the proportion of *Wolbachia*-infected insects to increase and spread into a population, given adequate fidelity of vertical transmission and strength of the CI (Rasgon and Scott, 2003). By taking advantage of this phenomenon, a novel *Wolbachia*-infected strain carrying a maternally inherited factor such as specific antiparasitic genes could be driven into a natural population, diminishing its vector potential (Aksoy *et al.*, 2008).

The CI phenomenon has led to proposals for use of the Incompatible Insect Technique (IIT) for vector control. In IIT, female sterility is artificially sustained by multiple releases of cytoplasmically incompatible males. Since *Wolbachia* is not paternally transmitted, the infection

type present in the release strain does not become established in the field. Due to incompatible matings, the field population decreases and the proportion of males with the release strain increases. Similar to conventional SIT, the increasing ratio of incompatible matings over time can lead to population suppression and possibly population elimination. As early as 1938, scientists showed that crosses between certain populations of mosquito *Culex pipiens* from England and France failed to produce progeny (Laven, 1967). They later proved that this phenomenon was cytoplasmic in nature, caused by a rickettsia-like bacterial symbiont and they went on to use CI to suppress *Culex fatigans.* Later experiments have shown that *Wolbachia*–mediated CI can be used to suppress populations of the medfly *Ceratitis capitata*, a major agricultural pest (Zabalou *et al.*, 2004) and *Aedes aegypti* (Brelsfoard and Dobson, 2011).

For practical applications in vector control, CI may be achieved through cross-mating of infected and non-infected individuals. Such mixed populations are already present in nature (Cheng *et al.*, 2000). Alternatively, flies may be bred in laboratories and *Wolbachia* eliminated through administration of antibiotics to produce a symbiont-free line, which could be used in SIT interventions. Antibiotic treatment clears off all bacterial symbionts and results in fly sterility. Supplementing the fly diet with yeast serves to rescue fertility (Weiss *et al.*, 2006). However, production of laboratory colonies of the preferred symbiont status would require identification and colonization of suitable wild populations, availability of an insectary with controlled environmental conditions and specialized technical skills for the maintenance of the colonies.

Other strains of *Wolbachia* display a life-shortening phenotype, depressing the longevity of infected hosts (Min and Benzer, 1997). The particular strain isolated from the fruitfly *D. melanogaster*, is referred to as *w*MelPop and displays an over-replicating phenotype, high rates of maternal inheritance, complete CI and a reduction of lifespan by half compared to uninfected controls. The *w*MelPop strain originally from *Drosophila*, was successfully introduced into the dengue vector *Aedes aegypti* (McMeniman *et al.*, 2009) and continued to express the same characteristics. This phenotype can therefore be applied for vector control to reduce the time required for development and/or replication namely the extrinsic incubation period of the vector. Therefore, life-shortening *Wolbachia* infections that impact the population age structure of the host insect, shifting it toward younger individuals, would reduce the percentage of adults that survive long enough to transmit the pathogen (Brownstein *et al.*, 2003).

Disease control can be achieved by decreasing the susceptibility of the vector to acquire the parasite, or by interfering with parasite development and maturation. It has been shown that infection with *Wolbachia* may limit co-infection with other pathogens, especially viruses. Indeed, infection with the *w*MelPop-CLA *Wolbachia* strain reduces the ability of the dengue and Chikungunya viruses, as well as *Plasmodium* to establish productive infections in the mosquito *Aedes aegypti* (Moreira *et al.*, 2009). However, the possibility of *Wolbachia* infection in *Glossina* causing a reduced susceptibility to trypanosome infection has not been investigated.

2.12 Bacterial Strain Typing for *Wolbachia* and *Sodalis*

For accurate characterization of a host-bacterial symbiotic association, determination and identification of the bacterial strain is crucial. In the case of CI-inducing *Wolbachia*, the strain of the infecting bacteria determines whether uni-directional or bi-directional cytoplasmic incompatibility will be induced (Blagrove *et al.*, 2011). Classical bacterial strain determination methods involve culturing and classification based on phenotypic characteristics. However, these phenotypic methods are unsuitable for strain determination in the intracellular *Wolbachia*, therefore molecular systematic methods based on DNA sequencing and phylogenetic analyses are used. *Wolbachia* form a monophyletic group composed of eight divergent clades or super-groups A-H based on the sequence of bacterial 16S subunit ribosomal ribonucleic acid (16S rRNA), the *Wolbachia* cell surface protein gene (*wsp)*, and the filamenting temperature-sensitive mutant Z (*ftsZ*) genes (Lo *et al.*, 2007). A single host group may contain several closely related strains. However, a single-locus approach to strain characterization may be misleading (Casiraghi *et al.*, 2005) and therefore, a multigene typing approach which provides greater discrimination has been developed to type *Wolbachia* strains. This Multilocus Sequence Typing (MLST) scheme differentiates strains based on direct nucleotide sequencing of five conserved housekeeping genes (Baldo *et al.*, 2006). The MLST scheme is based on the *Drosophila melanogaster* strain of *Wolbachia pipientis* (*w*Mel), which has a circular DNA genome of about 1,267,782 bp (Wu *et al.*, 2004). The position of the MLST loci on the *Wolbachia* genome is shown in figure 2.5.

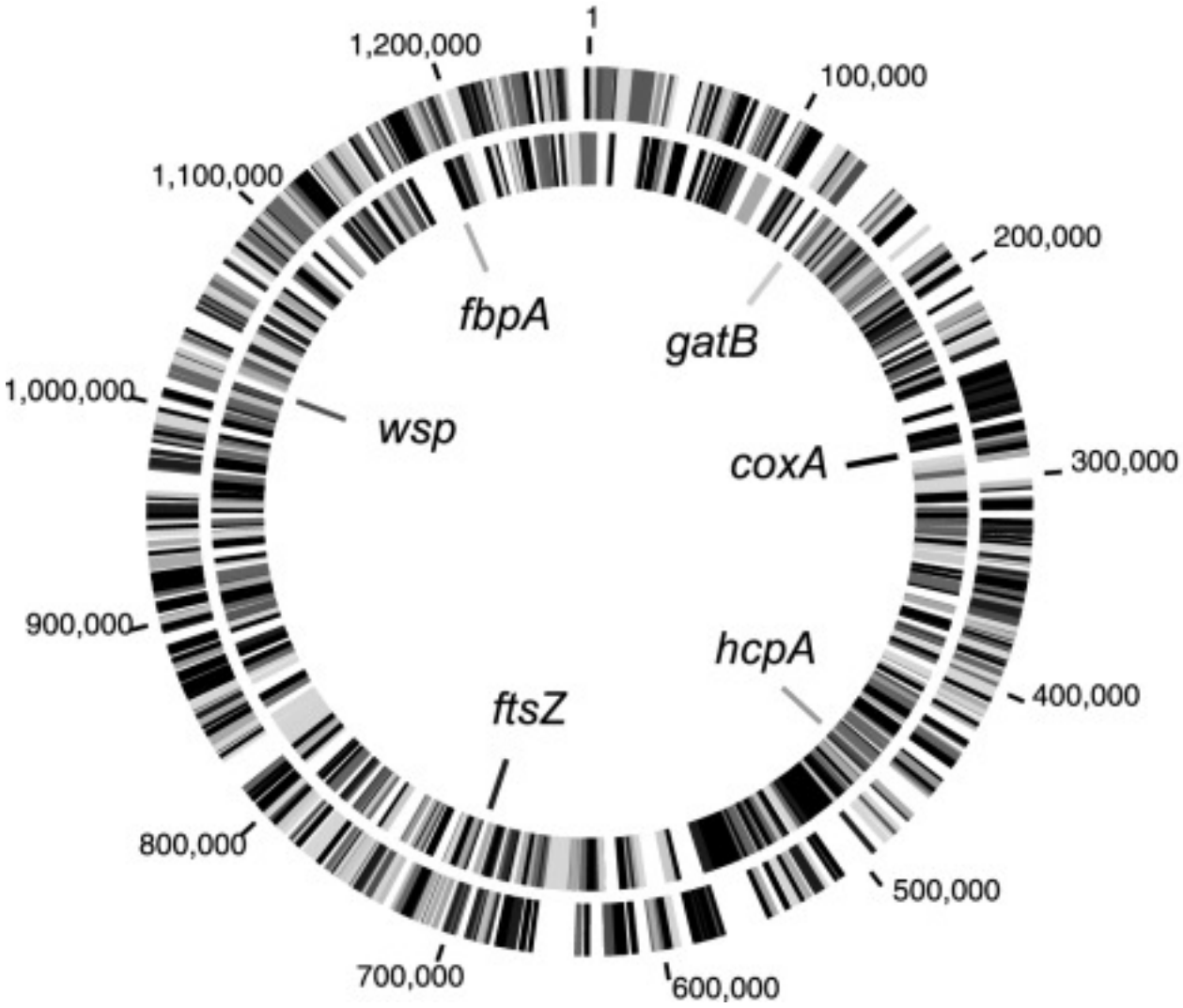

Figure 2.5: Location of MLST and *wsp* loci on the *w*Mel chromosome

The MLST and *wsp* loci are shown on the inner circle. The outer and middle circles show the open reading frames on the plus and minus chromosomal strands respectively (Baldo *et al*., 2006)

The MLST approach defines a strain as a sequence type (ST) identical to a haplotype on the basis of a combination of alleles referred to as allelic profile of a number of housekeeping genes. *Wolbachia* MLST uses the following genes: aspartyl/glutamyl-tRNA (Gln) amidotransferase subunit B (*gatB*): cytochrome C oxidase subunit I *(coxA)*: - conserved hypothetical protein (*hcpA)*: cell division protein (*ftsZ)*and the fructose-bisphosphate aldolase gene (*fbpA)*. These

genes have been chosen based on their conformity to the standard loci requirements for an MLST system (Baldo *et al.*, 2006). Several other genes have also been proposed as possible MLST scheme candidates (Paraskevopoulos *et al.*, 2006). The MLST system provides a universal and unambiguous tool for strain typing, population genetics and molecular evolutionary studies (Baldo *et al.,* 2006). Researchers can access a web-based interface (http://pubmlst.org/wolbachia/) which is the central database for storing and organizing *Wolbachia* bacterial and host information.

Wolbachia strain diversity can also be characterized based on the amino acid motifs of the four hypervariable regions (HVRs) of its *wsp* sequence (Baldo *et al.*, 2006). However, the *wsp* gene is subject to extensive recombination and strong diversifying selection, making it unsuitable for reliable strain characterization if used alone (Baldo *et al.*, 2006). The position of the *wsp* loci on the *Wolbachia* genome is shown in Figure 2.5. A relatively conserved set of amino acid motifs are present at each of the four HVRs, with shuffling of HVR motifs among strains. Each *wsp* amino acid sequence (amino acid range with respect to *w*Mel sequence is 52-222) is partitioned into four consecutive sections whose breakpoints fall within conserved regions between the hypervariable regions: HVR1 (amino acid range 52-84), HVR2 (85-134), HVR3 (135-185), HVR4 (186-222). Each section encompasses one of the four HVR motifs and a portion of the two conserved flanking regions, referred to as an HVR. The majority of amino acid changes among *wsp* sequences occur in the four HVRs, therefore these motifs can be used as signatures for discrimination of the different *wsp* protein types. The location of the four HVRs on the *w*Mel *wsp* sequence is shown in figure 2.6.

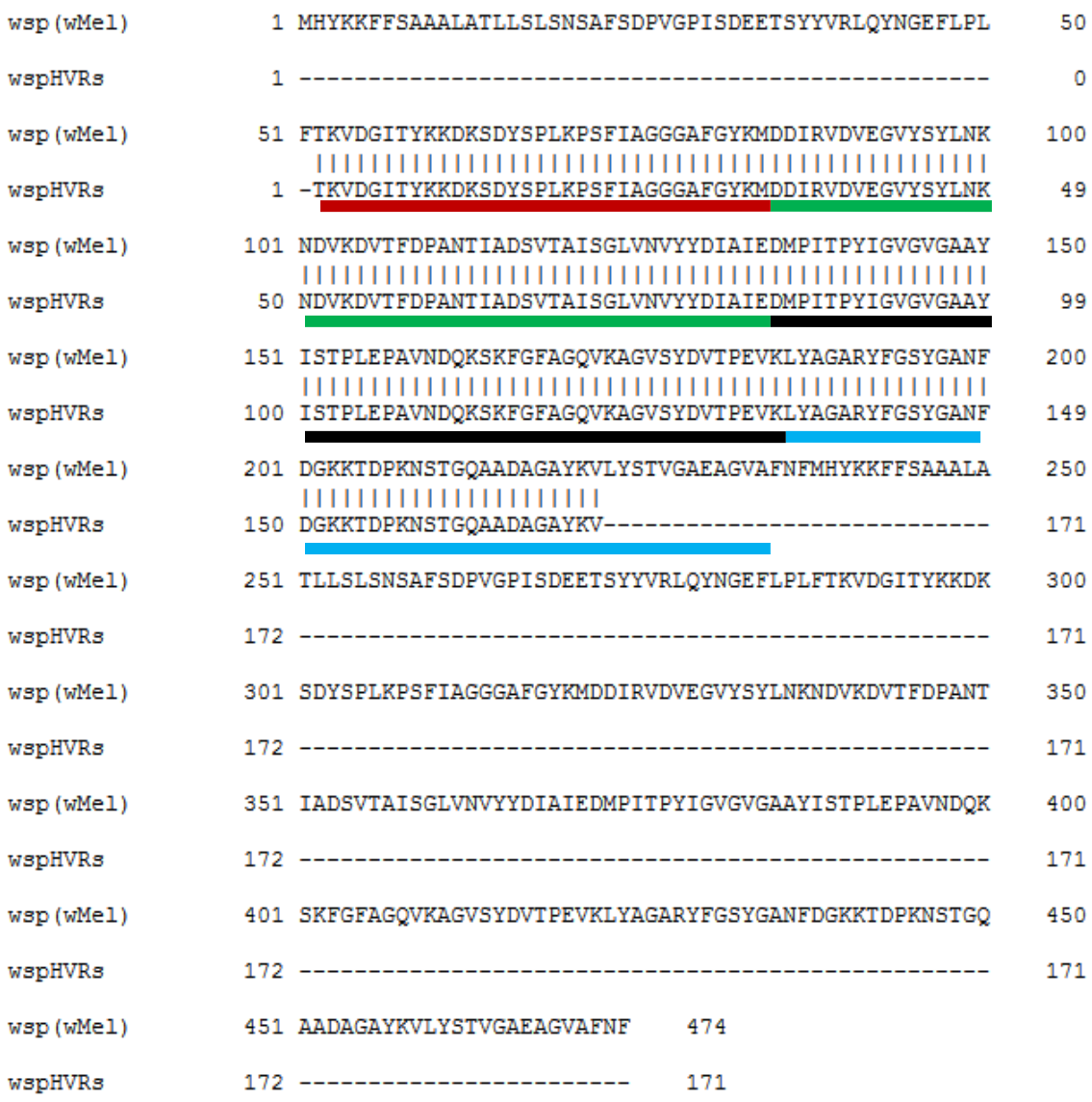

Figure 2.6: Location of the four HVRs on the *w*Mel *wsp* gene

wsp (*w*Mel)-sequence of surface antigen *wsp* (*Wolbachia* endosymbiont of *Drosophila melanogaster*); *wsp* HVRs: HVR1, HVR2, HVR3, HVR4

Additional methods are available to produce higher resolution of strain phylogenies and to distinguish closely related *Wolbachia* strains. The *wsp* Blot PCR method involves blotting and hybridization with an internal *wsp* probe and greatly enhances *Wolbachia* detection limits (Arthofer *et al.*, 2009a; Arthofer *et al.*, 2009b). Additional methods for strain differentiation include the use of multi-copy mobile insertion sequences (IS elements) primer sets (Miller *et al.*, 2010) and the variable number of tandem repeats (VNTR) loci to fingerprint *Wolbachia* types (Riegler *et al.*, 2005). VNTRs consist of short lengths of DNA that are repeated multiple times within a genome. The number of repeats is variable and different individuals within a population may have different number of repeats. The number of repeats can be a source of variation because they are subject to very high mutation rates (Fondon and Garner, 2004). These loci can be used for a PCR-based typing system in an approach that analyses multiple loci simultaneously termed Multi-Locus VNTR Analysis (MLVA). MLVA uses highly polymorphic markers that allow fine-scale typing of very closely related isolates (Pourcel *et al.*, 2004).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

Glossina austeni and *G. pallidipes* flies were trapped in the Shimba Hills National Reserve (SHNR) in Kwale County and the Arabuko-Sokoke National Reserve (ASNR) in Malindi County (Figure 3.1).These two areas are national reserves which the government has declared as protected in order to preserve their natural flora and fauna. The Shimba Hills Reserve lies between latitude 4°20′S and longitude 39°31′E, at an altitude of 120-450m above sea level in the coastal lowlands agro-ecological zones 2-4 and covers an area of about 250km^2. Temperatures range between 24-36^{o}C. Rainfall averages 900-1200mm per annum, with the short rains falling in the months of October-December and the long rains during March-June. A wide range of wild animals can be found in SHNR including elephants, buffalo, antelope and waterbuck. The Arabuko-Sokoke National Reserve is located at latitude 3°16′S and longitude 39°49′E. The park itself covers an area of about 6km² and is part of the larger Arabuko-Sokoke forest, which is the largest remaining fragment of coastal forest in East Africa. The forest contains three forest types namely mixed forest, *Brachystegia* and *Cynometra*, each of which protects different communities of plants and animals. Animals found here include a variety of birdlife, mongoose, duikers, forest elephants, African civets, baboons and vervet monkeys. There are three species of tsetse found in the study area namely *G. pallidipes*, *G. austeni* and *G. brevipalpis*. Although *G. austeni* is classified among the savannah (*morsitans*) group, it is considered a unique member retaining some primitive characters. *G. austeni* is found in secondary shrub, thickets and islands of forests along the East African coast including Somalia, Kenya, Tanzania, Mozambique and South Africa. The spread of *G. austeni* is limited to not more than 250km inland and under 900m above sea level. For this study, a comparative sample of *G. austeni* from South Africa was kindly provided by Dr Otto Koekomoer, Onderstepoort Veterinary Institute, Pretoria, South Africa. For purposes of analysis, the South African samples were divided into two regions: those from the south of Lake St. Lucia, South Africa (denoted hereafter as "LSLSA") and those from the northern part of the lake, specifically Mbazwana, Muzi, Phinda and Tembe regions (hereafter "northern"). Laboratory samples were obtained from the KARI-TRC *G. pallidipes* and the Tsetse and Trypanosomiasis Research Institute, Tanzania (TTRI) *G. austeni* colonies.

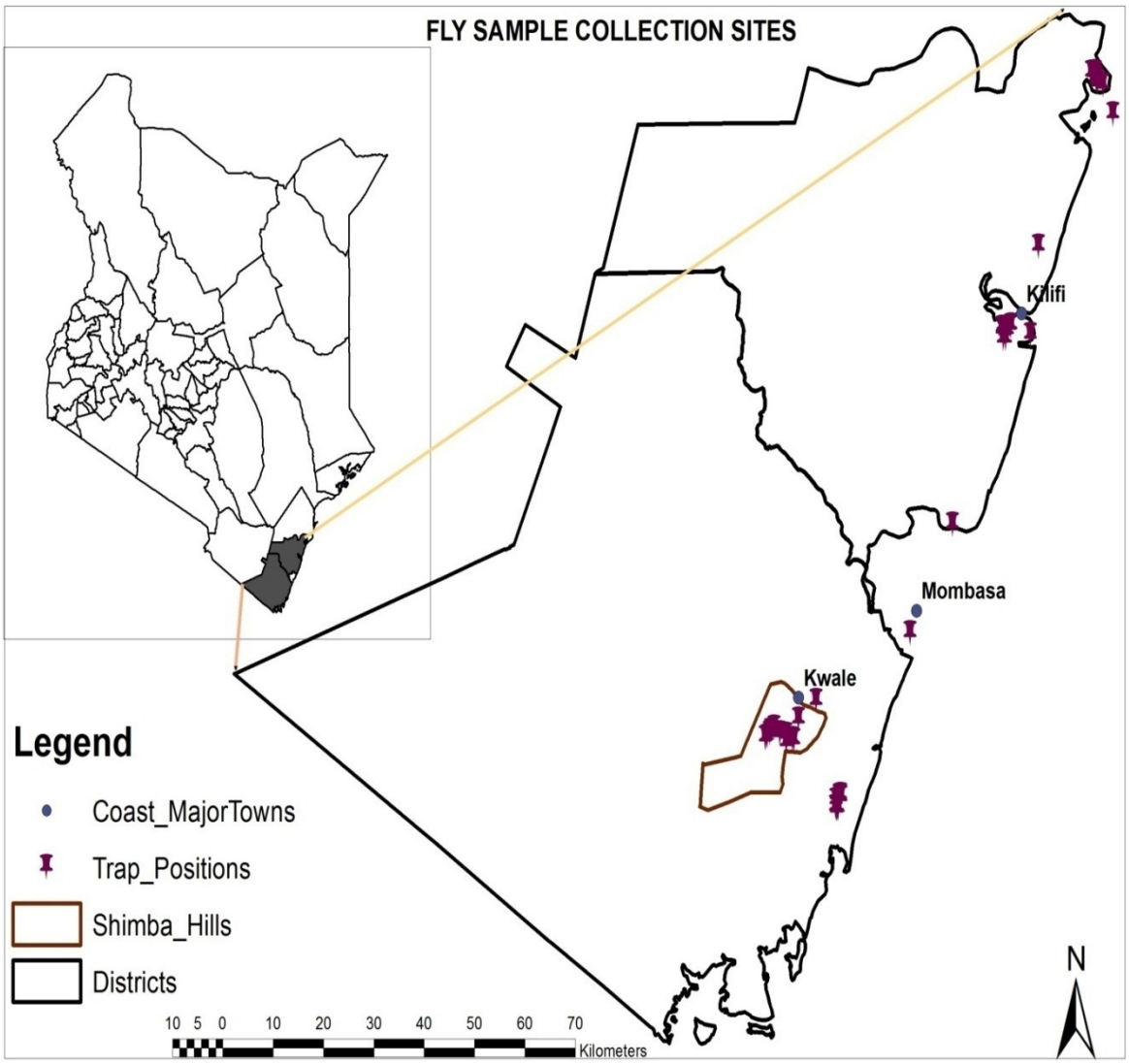

Figure 3.1: Fly sample collection sites in Kwale and Kilifi

3.2 Fly Sample Collection

Flies were trapped using either standard biconical trap, theNGU2G trap (Brightwell *et al.*, 1987) or the sticky mono-panel trap (Vreysen *et al*., 2000b) whose lower half was coated with a thin layer of Temoocid® adhesive (Kollant SPA, Italy) pictured in Appendix 1. The traps were baited with a plastic sachet containing 1ml each of 4-methylphenol, 3-*n*-propyl phenol and 1-octen-3-ol. In addition, a soda bottle containing acetone was placed next to the trap. All the odours were dispensed by placing them on the windward side (Torr *et al.*, 1992). The traps were left in place for between 6 - 24 hours depending on the tsetse density in the particular area. All trap sites were geo-referenced using Garmin GPSmap76 (Garmin Corporation, Olathe, KS, USA). During the study period, all fly collections were performed in the months of October-November, coinciding with the short rainy season. On collection, catches from each trap were sorted by species and a proportion of them preserved in 50ml Falcon tubes containing absolute ethanol. The remaining proportion of flies was reserved for dissection. Records of total catches, sex, date and area of collection were maintained for cross-referencing purposes.

3.3 DNA Extraction

Upon removal from ethanol storage, individual flies were blotted dry on a paper towel and air-dried overnight at room temperature. Total genomic DNA was prepared from individual whole fly samples (minus legs and wings) using either the DNeasy® Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions or by using the salting out protocol (Sunnucks and Hales, 1996). For the latter procedure, flies were crushed in 300µl TNES buffer (50mM Tris pH 7.5, 400mM NaCl, 20mM EDTA, 0.5% SDS) and incubated at 56°C for 3 hours in the presence of 20µl proteinase K (20mg/ml). A volume of 85µl of 5M NaCl solution was then added to the solution. The tubes inverted several times and then centrifuged at 14,000 revolutions per minute (rpm) for 5 minutes to precipitate the proteins. The supernatant was transferred to a new Eppendorf tube and one volume of cold 100% ethanol was added to it. The solution was mixed by inverting and then centrifuged at 14,000 rpm for 5 minutes. The resulting supernatant was disposed of using a pipette and 500µl of 70% ethanol added to the DNA deposited at the bottom of the tube. The tube was spun again at the same speed for 5 minutes and the ethanol removed as above. An additional spin was done to remove any remaining ethanol. The tubes were then air-dried and 50µl TE buffer (10mM Tris-HCl pH

7.5, 1mM EDTA) added. The tubes were kept at room temperature for one hour to resuspend the DNA. The final product was then stored at -20°C until further processing.

3.4 Determination of the Prevalence of *Wolbachia* and *Sodalis*

General screening for the presence of *Wolbachia* was determined by PCR amplification of the *wsp* gene using the primers 81F/691R (Zhou *et al.*, 1998) and the heat shock protein *groEL* gene (Casiraghi *et al.*, 2005). These primers amplify products of 600bp and 795bp, respectively. The presence of *Sodalis* was determined using the primers *GPO13*/*GPO15* (Dale and Maudlin, 1999) which amplifies a 1.2kb product of the extra-chromosomal plasmid. Cycling conditions were as detailed in table 3.1. To determine the prevalence of the symbionts, screening was carried out in 20µl reactions using 1µl of template DNA in a buffer containing 5X PCR reaction buffer, 2.5mM $MgCl_2$, 0.5mM dNTPs, 500nM of each primer and 0.3µl of GoTaq® Flexi DNA polymerase 5 units/µl (Promega, Madison, WI, USA). For each PCR run, a negative control using molecular grade water (HyClone Laboratories Inc., UT, USA) and a positive control from sequenced *Wolbachia* and *Sodalis* PCR product were included. The quality of template DNA was verified to be adequate by amplification of insect-specific 12S rRNA, with a product size ~400bp (Simon *et al.*, 1994). After completion of the PCR run, 10µl of the amplification products were analyzed by electrophoresis in TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.0) on a 1-2% agarose gel together with a 100bp DNA ladder size standard (Invitrogen, Carlsbad, CA, USA) and visualized using ethidium bromide (EtBr) staining.

Table 3.1: Primers and conditions used for symbiont detection

Symbiont	Gene	Primer Names	PCR cycling conditions	Expected product size	Reference
Wolbachia	*wsp* (*Wolbachia* surface protein*)*	81F/691R	94°C for 3min, [94°C for 30sec, 47°C for 30 sec, 72°C for 30sec]x35, 72°C for 5min	600bp	(Braig *et al.*, 1998; Zhou *et al.*, 1998)
	groEL (heat shock protein)	WgroF1/WgroRev1	[94°C for 45 sec, 60°C for 45 sec, 72°C for 80 sec]x4, [94°C for 45 sec, 55°C for 45 sec, 72°C for 80sec]x33, 72°C for 7 min	795bp	(Casiraghi *et al.*, 2005)
Sodalis	Extrachromosomal plasmid	*GPO15*/*GPO13*	95°C for 5min, [95°C for 60sec, 52°C for 60sec, 72°C for 60sec]x30, 72°C for 5min	1.2kb	(O'Neill *et al.*, 1993 ; Dale and Maudlin, 1999)

3.5 Tissue Distribution of Symbionts in Tsetse Flies

Laboratory colony flies were used to determine tissue distribution of the symbionts. *G. austeni* flies emerging from pupae obtained from the Tsetse and Trypanosomiasis Research Institute (TTRI) Tanga, Tanzania and *G. pallidipes* from the TRC colony were used. A sample of five10-12 day old flies each of either sex were dissected in phosphate buffer saline (PBS) and the reproductive tract, gut, salivary gland and carcass separated out. To prevent cross-contamination, the dissecting instruments were sterilized in between organs by dipping in 10% bleach which is available commercially as Jik® household bleach followed by rinsing in distilled water. The separate organs were placed in 1.5µl microfuge tubes containing 100µl lysis buffer. DNA extraction and subsequent analysis was carried out as described in sections 3.3 and 3.4 respectively. As described in section 3.4, to ensure that extraction of DNA from the organs was successful, insect-specific 12S rRNA mitochondrial markers were used. Samples that did not amplify this gene were excluded from further analysis.

3.6 Age Structure of the Sympatric *G. austeni* and *G. pallidipes*

The age structure of the sympatric populations was estimated using the wingfray method for males (Jackson, 1946) and ovarian ageing for females (Saunders, 1962). The wings from randomly selected male flies of both species were snipped off from the body using a pair of fine scissors and mounted on a sheet of paper using cellotape. The wings were then examined under a dissecting microscope. The degree of fraying as indicated by the tearing in the trailing edge of the wing was scored on a scale of 1-6. The smooth-edged wings were assigned to category 1, while very tattered wings were assigned to category 6. The mean wing fray value (MWFV) was then calculated for each sample. Ageing of females was performed using the ovarian ageing method, which is based on the changes that occur in the ovaries during successive gonotrophic cycles (Saunders, 1962). In a normal tsetse fly, each ovary consists of two polytrophic ovarioles with a single developing egg follicle. Only one egg is produced at a time, and a sequence of egg maturation and ovulation occurs such that the eggs are produced alternately by the right and left ovary (Saunders, 1960). For ovarian ageing, a fly was placed dorsal side up on a microscope slide and two cuts made on the lower abdominal segments of the fly using fine scissors. The reproductive organs were gently pulled out using forceps. The ovarioles were examined under a dissecting microscope for evidence of ovulation either in the form of an expanded sac which

appears immediately after ovulation, or a small follicular relic seen as a pale yellow pigmented area at the end of the follicular tube. The number and position of these structures and the relative sizes of the four follicles developing in the ovarioles were noted. When pregnant the stage of the developing egg or larva in the uterus was noted. Individual females were then assigned ovarian age categories as described by Saunders (1962).

3.7 Strain Variation in *G. austeni Wolbachia* Based on *wsp* Typing

Sequence analysis of *Wolbachia* strains infecting *G. austeni* at the Kenyan coast was carried out using both *wsp* and MLST typing protocols. *G. austeni* from South Africa was included in the analysis as an outgroup. Genomic DNA was extracted from four individual flies from each trapping area as described in section 3.3 using the Qiagen extraction kit. The PCR reaction was run as described in section 3.4 but making up a final reaction volume of 50µl. The PCR product was cleaned up using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer´s instructions. The purified product was then ligated overnight at 4°C using the commercial pGEM®-T Easy Vector kit (Promega, Madison, USA). The ligation reactions were transformed using *Escherichia coli* DH5α competent cells and then plated onto LB/ampicillin/IPTG/X-Gal plates which were prepared by addition of ampicillin to Luria Broth (LB) medium (100µl/ml) and then supplementing with 0.5mM isopropyl thiogalactoside (IPTG) and 80µg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). The plates were incubated overnight and blue-white selection system was used to identify positive transformants. At least three positive white appearing colonies were added to 1.5ml microtubes of starter culture (1µl ampicillin per ml of LB). A *Wolbachia* PCR was conducted as above to confirm that the selected colonies contained the inserts. After confirmation, 200µl of the starter culture was added to 4ml LB/ampicillin and cultured overnight at 37°C with shaking at 150rpm. Plasmid extraction was carried out using QIAprep Miniprep kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer´s instructions. A sample of 500ng of purified plasmid was sequenced using T7 primer at the Science Hill Sequencing Facility, Yale University, USA. The sequences returned were downloaded from the sequencing facility website using EditSeq and trimmed of the vector sequence. These sequences were compared with those existing in the databases using a Basic Local Alignment Search Tool (BLAST), reverse complementing where necessary. Multiple alignment of the sequences was performed using DNAStar MegAlign and ClustalW (Thompson

et al., 1994). The *wsp* sequences were queried into the *wsp* database to determine strain divergence based on the amino acid sequences of the four hypervariable regions (HVR) of the *wsp* gene.

3.8 Strain Variation in *G. austeni Wolbachia* Based on Multi-Locus Strain Typing

For *Wolbachia* MLST, five housekeeping genes namely *hcpA, gatB, ftsZ, coxA* and *fbpA* as described by Baldo *et al.,* (2006) were used. The reactions were carried out in a buffer containing 1.8µM $MgCl_2$, 0.1mMdeoxyribonucleotide triphosphates (dNTPS) and 500nM of each primer in 20µl reactions. Optimization of annealing temperature for each gene analyzed was carried out before selection of the appropriate temperatures. Thus the annealing temperature for *hcpA, gatB, ftsZ, coxA* was 50°C, while for amplification of *fbpA,* an annealing temperature of 58°C was used. Details of the primers used are presented in table 3.2. For cloning purposes, 50µl reactions with equivalent proportions of these reagents were used. After PCR, the amplification products were analyzed by electrophoresis in TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.0) on a 1% agarose gel stained with ethidium bromide. The band containing the gene of interest was excised from the agarose gel using a sharp scalpel and purified using Qiagen gel purification kit (Qiagen GmbH, Hilden, Germany). The purified product was then cloned as described in section 3.7 and submitted for sequencing. The MLST sequences obtained were inputted into the *Wolbachia* MLST database (http://pubmlst.org/wolbachia). In concordance with MLST convention, alleles that were identical at specific loci to sequences existing in the database were assigned the same number, whereas new allele sequences were submitted to the curator for allele number assignment. Characterization of each strain was based on the combination of the five allelic numbers to give a specific sequence type.

Table 3.2: PCR primers used for *Wolbachia* MLST typing

Locus Code (*w*Mel)[a]	**Gene**	**Product**	**Primer name and sequence (5'-3')[b]**	**Gene length (bp)[c]**	**Fragment size (bp)**
WD_0146	*gatB*	glutamyl-tRNA amidotransferase, subunitB	gatB_F1: GAKTTAAAYCGYGCAGGBGTT gatB_R1: TGGYAAYTCRGGYAAAGATGA	1,425	471
WD_0301	*coxA*	cytochrome *c* oxidase, subunit1	coxA_F1: TTGGRGCRATYAACTTTATAG coxA_R1: CTAAAGACTTTKACRCCAGT	1,551	487
WD_0484	*hcpA*	conserved hypothetical protein	hcpA_F1: GAAATARCAGTTGCTGCAAA hcpA_R1: GAAAGTYRAGCAAGYTCTG	741	515
WD_0723	*ftsZ*	cell division protein	ftsZ_F1: ATYATGGARCATATAAARGATAG ftsZ_R1: TCRAGYAATGGATTRGATAT	1,197	524
WD_1238	*fbpA*	fructose-biphosphate aldolase	fbpA_F1: GCTGCTCCRCTTGGYWTGAT fbpA_R1: CCRCCAGARAAAAYYACTATT C	900	509

[a] *w*Mel –*Wolbachia* strain in *Drosophila melanogaster*

[b] Reference for all primers Baldo *et al.,* 2006

[c] with reference to the *w*Mel genome

3.9 *Sodalis* Strain Diversity Based on *GPO1* and VNTRs

Strain diversity of *Sodalis* in *G. pallidipes* was analyzed using two methods. Firstly, diversity was assessed through direct sequencing of the *GPO1* PCR product. Secondly, microsatellite genotyping was used to test for diversity. The *Sodalis* specific *GPO1* amplification was conducted as described in section 3.4. The reaction volumes were adjusted to total reaction volume of 50µl. A volume of 10µl sample of the product was run on a 2% agarose gel and visualized in ultra violet (UV) light to ensure that only one band was amplified. The PCR product was then cleaned up using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer´s instructions. The samples were then sequenced and the sequences returned were aligned using ClustalW. A further attempt was made to differentiate *Sodalis* strains in *G. pallidipes* based on the different allele sizes of the microsatellite markers ADNg 15/16 and ADNg 21/22 (Farikou *et al.,* 2011a). These loci display allelic polymorphism producing 2–4 alleles (Table 3.3). One microliter of DNA from selected *Sodalis* positive individuals was used for genotyping. A total reaction volume of 20µl comprising 2µl 10x reaction buffer, 2.5mM $MgCl_2$, 0.5mM dNTPs and 500nM of each primer was used. Samples were first denatured at 94°C for 3 min and then processed through 35 cycles consisting in a denaturation step at 94°Cfor 30 seconds, an annealing step at 55 - 58°C for 30 seconds and an extension step at 72°C for 1 min. The final elongation step was performed for 10 minutes at 72°C. After PCR, the amplification products were analyzed by electrophoresis in TAE buffer on a 1% agarose gel stained with ethidium bromide. The number of separated bands was noted. Single bands were then excised from the agarose gel using a sharp scalpel and purified using Qiagen gel purification kit. The purified product was then submitted for sequencing. The Tandem Repeat Finder (TRF) program was used to determine the number and location of the repeat sequence (Benson, 1999).

Table 3.3: Details of the *Sodalis* microsatellite markers used

Loci	Repeat sequence	Primer sequences (5'- 3') [1]	No. of alleles	Allele size (bp)
ADNg 15/16	(AGG)	ATACGGCGAAGCAATGAGAC CAGCCTCTAAGCGCTCAACTC	4	163/166/172/178
ADNg 21/22	(GCC)	GAGCAAATCTCCCAGCACAT TTCTTGTCCCTCAACCCATC	2	106/112

[1] Reference for all primers (Farikou *et al.*, 2011)

3.10 *Sodalis* - Trypanosome Co-infection in Wild *G. pallidipes*

The presence of trypanosomes in flies was investigated using one or both of two methods. In the field, where possible a small proportion of captured flies were dissected and the organs preserved individually in 1.5ml Eppendorf tubes. The remainder of the flies caught were preserved in ethanol as whole flies. Fly dissection was carried out following the basic protocol (Lloyd and Johnson, 1924) whereby prior to dissection, the wings and legs were removed from the fly. Using sharp-pointed forceps, the thecal bulb was removed onto a drop of PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4,1.47mM KH_2PO_4, pH 7.4)on a microscope slide. The mouthparts namely proboscis, hypopharnyx and the labrum were then separated from each other, covered with a coverslip under a drop of saline and examined at x40 magnification under a compound microscope. Sequentially, the same was done for the gut and the salivary glands. Identification of the infecting trypanosome was done based on the site of infection, which is related to the development cycles of the different *Trypanosoma* species in tsetse flies that is mouthparts only for *T. vivax*; mouthparts and midgut for mature *T. congolense*, midgut only for immature *T. congolense* infections; midgut and salivary gland for *T. brucei*. In situations whereby dissection was not performed in the field, flies were preserved in absolute ethanol. In the laboratory, the presence of trypanosomes in these samples was established through PCR. PCR was also used to confirm the identity of trypanosomes detected by dissection. The internal transcribed spacer region (ITS) is a preferred target for universal test because of its highly conserved flanking regions and size variability among trypanosome species and sub-groups. This locus has 100-200 copies and each transcribed unit is composed of 18S, 5.8S and 28S rRNA

genes separated by two ITS regions (Hernández *et al.*, 1993; Desquenes *et al.*, 2001). The ITS1 primers used in this study are designed to amplify the ITS1 region (Njiru *et al.*, 2005). A schematic diagram of rDNA showing the annealing positions of this primer set is shown in figure 3.2. DNA was extracted from the whole flies as described in section 3.3. The PCR reaction was performed in 20µl reactions using 2-4µl test sample and the forward (ITS1 CF) and the reverse (ITS1 BR) primers at a concentration of 500nM each. The reaction contained 2µl of 10x PCR reaction buffer, 2.5mM $MgCl_2$, 0.5mM dNTPs, 500nM of each primer and 0.3µl of GoTaq® Flexi DNA polymerase 5units/µl (Promega, Madison, WI, USA). The initial denaturing step performed at 94°C for 5 minutes was followed by 35 amplification cycles of denaturation at 94°C for 40 seconds, annealing at 58°C for 40 seconds and extension at 72 °C for 90 seconds. A final extension step at 72°C for 5 minutes followed. For each PCR run, a negative control (PCR water) and a number of positive controls of samples obtained from certified trypanosome isolates were included. Electrophoresis and visualization was carried out as described previously in section 3.4. Identification of the infecting trypanosome(s) was based on the product size, details of which are presented in table 3.4. The assay previously described in section 3.4 was used to detect *Sodalis*-trypanosome co-infection in individual flies.

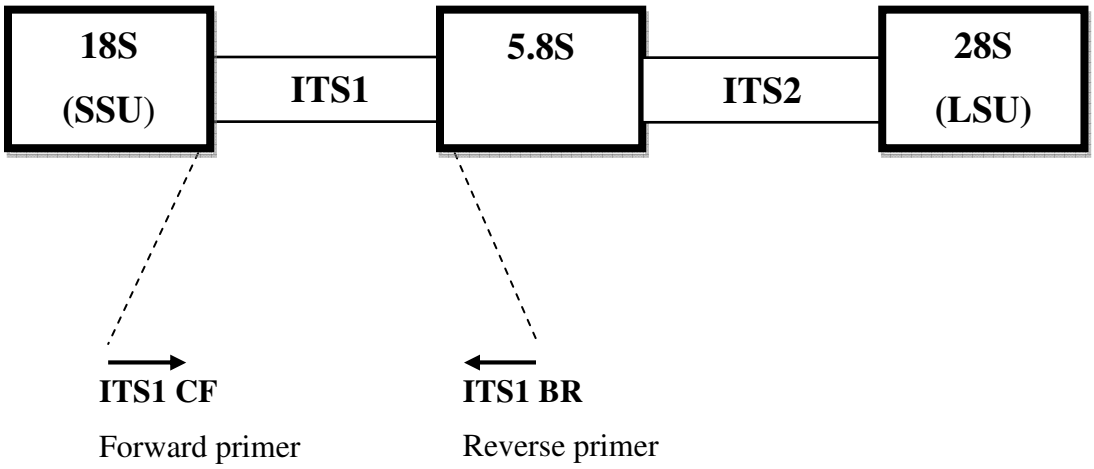

Figure 3.2: Schematic representation of rDNA showing ITS1 CF/BR primers annealing positions The small subunit (SSU) and the large subunit (LSU) are separated by two internal transcribed spacers (ITS). The ITS 1 separates the coding region of the 18S subunit and the 5.8 S rDNA, and the ITS2 separates 5.8 S rDNA sequences from the 28S rDNA.

Table 3.4: Expected ITS1 band sizes of different trypanosome species

Trypanosome species	Approximate PCR product band size
T. vivax	250bp
T. simiae	370-400bp
Trypanozoon (*T. brucei sub-group, T. evansi*)	480bp
T. congolense Kilifi (TCK)	620bp
T. congolense savannah (TCS	700bp
T. congolense forest (TCF)	710bp

bp – size in base pairs

3.11 *Sodalis* - Trypanosome Co-infection in Experimentally Challenged *G. pallidipes*

The rate of co-infection with both symbiont and trypanosomes was evaluated in experimentally challenged *G. pallidipes*.

3.11.1 *In vivo* infection of tsetse flies

Male teneral *Glossina pallidipes* aged 0-2 days old from the TRC colony were used. Details of the three trypanosome isolates used for the fly infection are presented in table 3.5. Before the experiment was started, the identity of each stabilate was confirmed using specific PCR assays. The stabilates were then expanded in two donor Swiss White mice which had been immuno-suppressed with cyclophosphamide at a dose of 300mg/kg body weight. Disease progression in the mice was monitored by collection and microscopic examination of blood obtained through tail snips on alternate days. At the peak of parasitaemia, the mice were euthanized using concentrated CO_2. Blood from the heart was then collected by cardiac puncture into a tube containing ethylene di-amine tetra acetic acid (EDTA). The level of parasiteamia was estimated using the matching method (Herbert and Lumsden, 1976) and subsequently, an inoculum dose of 1×10^6 trypanosomes/ml was prepared in phosphate saline glucose (PSG) pH 8.0. Two milliliters of this inoculum used to infect 12 recipient mice. At peak parasitaemia, teneral flies assigned to three treatment groups designated TBR, TBB and TC (Table 3.5) were allowed to feed on the belly of infected mice. Feeding success was confirmed by visual observation of engorged fly abdomens. Flies that did not feed were excluded from the experiment. After 10-15 minutes, feeding was interrupted and the engorged flies transferred to the insectary which is maintained at

a temperature of 24±1^{0}C and 70±5% humidity. These flies were fed on defibrinated bovine blood on alternate days using the *in vitro* feeding system (Feldmann, 1994).

Table 3.5: Details of trypanosome stabilates and experimental groups

Experimental Group	TBR	TBB	TC
Parasite	*T. b. rhodesiense*	*T. b. brucei*	*T. congolense*
Isolate code	KETRI 2537	EATRO 1784	EATRO993
Host	Human	*G. pallidipes*	*G. pallidipes*
Origin, year of isolation	Busoga, 1972	Lambwe, 1970	S. Nyanza, 1962
n	118 (25, 22, 26, 27, 18)[a]	77	98
Dissection (dpi)	7, 14, 21, 28,35[b]	40	30

dpi – days post-infection; [a] number of flies in TBR group dissected the different time points; [b]Temporal dissection for TBR group at 7, 14, 21, 28 and 35 dpi

3.11.2 Dissections and DNA extraction

In the TBR group, sequential dissections were carried out after 7, 14, 21, 28 and 35 days post-infection (dpi). Dissections for flies in the TBB and TC groups were all performed at parasite maturity stage, which is at 40 and 30 dpi respectively. Dissections were performed on a microscope slide using PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, pH 7.4). Following the classical method (Lloyd and Johnson, 1924), the mouthparts, gut and the salivary glands (in the *brucei* infection groups only) were isolated and examined microscopically. Subsequently, individual midguts from the dissected tsetse flies were placed in a 1.5ml microfuge tube. Total genomic DNA was isolated from the midgut samples using the DNeasy® Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), with a minor modification to the manufacturer's instructions being that the final elution step was performed with 50µl instead of 100 µl of elution buffer.

3.11.3 Determination of *Sodalis* and trypanosome co-infection

The presence of *Sodalis* in the gut tissues was determined using the primers *GPO1*F/R which amplify a 1.2kb product (Dale and Maudlin, 1999). The 20µl final PCR reaction contained 2µl of

10x PCR reaction buffer, 2.5mM $MgCl_2$, 0.5mM dNTPs, 500nM of each primer and 0.3µl of GoTaq® Flexi DNA polymerase 5 units/µl (Promega, Madison, WI, USA). The amplification cycle was performed as previously detailed in section 3.4. Midgut trypanosome infection in the *T. congolense* treatment group was identified using *T. congolense* savannah sub-type (TCS) specific primers TCS1 and TCS2 (Masiga *et al.*, 1992) while infections in the *T. brucei* infection groups were determined using TBR1 and TBR2 primers (Moser *et al.*, 1989). In both cases, the initial denaturing step was performed at 94°C for 3 minutes, followed by 30 amplification cycles consisting of a denaturation step at 94°C for 30 seconds, an annealing step and an extension step at 72 °C for 2 minutes. The final extension step was carried out at 72°C for 10 minutes. The annealing temperatures were 55°C and 60°C for TCS and TBR respectively. For each PCR run, a negative control (water) and the respective positive controls were included. After completion of the PCR run, 10µl of the amplification products was analyzed by electrophoresis in TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.0) on a 1.5% agarose gel together with a 100bp DNA ladder size standard (Invitrogen, Carlsbad, CA, USA) and visualized using ethidium bromide (EtBr) staining.

3.12 Effect of Ampicillin Feeding on *G. pallidipes* Survival and Symbiont Clearance

Two groups of 100 male *G. pallidipes* were used to establish the effect of *per os* administration of ampicillin on the tsetse fly survival. The antibiotic diet was prepared by spiking clean defibrinated bovine blood with ampicillin (American Bioanalytical, MA, USA) at a dose of 30µg/ml of blood. Newly-emerged flies were fed *in vitro* on the ampicillin diet on alternate days (Feldmann, 1994). Fly survival was thereafter assessed for a period of three weeks. A control group comprising of 200 male flies fed on a normal diet was included for comparison. To determine the period required for tsetse to clear the symbionts from the gut, flies were exposed to a number of ampicillin feeds after which they were dissected and the midgut isolated for symbiont assay. Over a period of 6 weeks, 5 individual flies were dissected after 3, 6, 9, 12, 15 and 18 ampicillin feeds, corresponding to 1-5 weeks old, respectively. Upon dissection, the midgut was isolated and the DNA extracted as described in section 3.3. PCR assay for *Sodalis* was performed as described previously (section 3.4) whereas presence of *Wigglesworthia* was determined by amplification of a 365bp fragment of the thiamine biosynthesis factor (*thiC*) gene (Pais *et al.*, 2008). The *Wigglesworthia* amplification program was 94°C for 2 min, with 30

amplification cycles at 94°C for 30 sec, 54°C for 40 sec and 72°C for 60 sec. A final step of 72°C for 7 minutes was done. Previous assays using *G. pallidipes* did not detect *Wolbachia* and therefore no further assays for this symbiont were performed.

3.13 Trypanosome Establishment in Ampicillin-Treated *G. pallidipes*

The stabilates *T. b. brucei* EATRO1784 and *T. congolense* EATRO993 were used to infect ampicillin-treated flies. Expansion of the stabilates and subsequent infection of recipient mice was performed as previously described (section 3.11.1). The presence of motile trypanosomes was verified microscopically before the infected blood was used to feed experimental flies. The blood was also examined at the end of the feeding process to confirm presence of trypanosomes.

Newly-emerged teneral flies were first offered ampicillin-spiked bloodmeals for a period of one week totaling to three ampicillin feeds. At age 8-10 days old, the flies were presented with an infective feed, containing either *T. b. brucei* or *T. congolense* parasites. The flies were allowed to feed *in vitro* on a silicone membrane and visually inspected to confirm feeding success by observing them for engorged abdomens. The flies were thereafter maintained in the insectary and fed on clean blood. Surviving flies from the *T. b. brucei* group were dissected at 40 dpi, while those in the *T. congolense* group were dissected at 30 dpi. Dissection and DNA extraction were thereafter performed (section 3.11.2). Trypanosome positive flies were re-analyzed to confirm presence or absence of *Sodalis* infection as described in section 3.4. For each stabilate, a control group comprising seventy 10-day old males that had fed on normal blood was similarly infected, maintained and subsequently dissected.

3.14 Statistical Analysis

Average tsetse catches among species were compared by expressing total catches as flies/trap/day. Comparative analysis for symbiont and parasite infection prevalence between species and localities, temporal prevalence variations and age frequency was performed using chi-square and Fisher's exact tests.

CHAPTER FOUR

RESULTS

4.1 Tsetse Fly Density

More than 7,900 tsetse flies, comprising three species of tsetse, namely *G. pallidipes, G. austeni* and *G. brevipalpis* were captured in a total of 226 trap-days during the study period 2009-2011. The total catch comprised *G. pallidipes* (80.6%), *G. austeni* (13.0%) and *G. brevipalpis* (6.4%). Of these 6,384 *G. pallidipes,* 360 *G. brevipalpis* and 447 *G. austeni* were from SHNR while 144 *G. brevipalpis,* 583 *G. austeni* were captured in ASNR (Figure 4.1). No *G. pallidipes* flies were captured in the latter site. Average tsetse density as expressed by fly/trap/day (FTD) over the 3-yr period was 24.0, 4.1 and 1.9 for *G. pallidipes, G. austeni* and *G. brevipalpis* respectively. Both study sites recorded lower FTDs in the year 2010 as compared to 2009 and 2011. The highest FTDs were recorded for *G. pallidipes* in SHNR with densities in excess of 60 flies per trap per day. The overall *G. austeni* FTD in ASNR (11.2 FTD) was comparatively higher than that in SHNR which was 2.6 flies/trap/day. Details of annual FTDs are shown in figure 4.2.

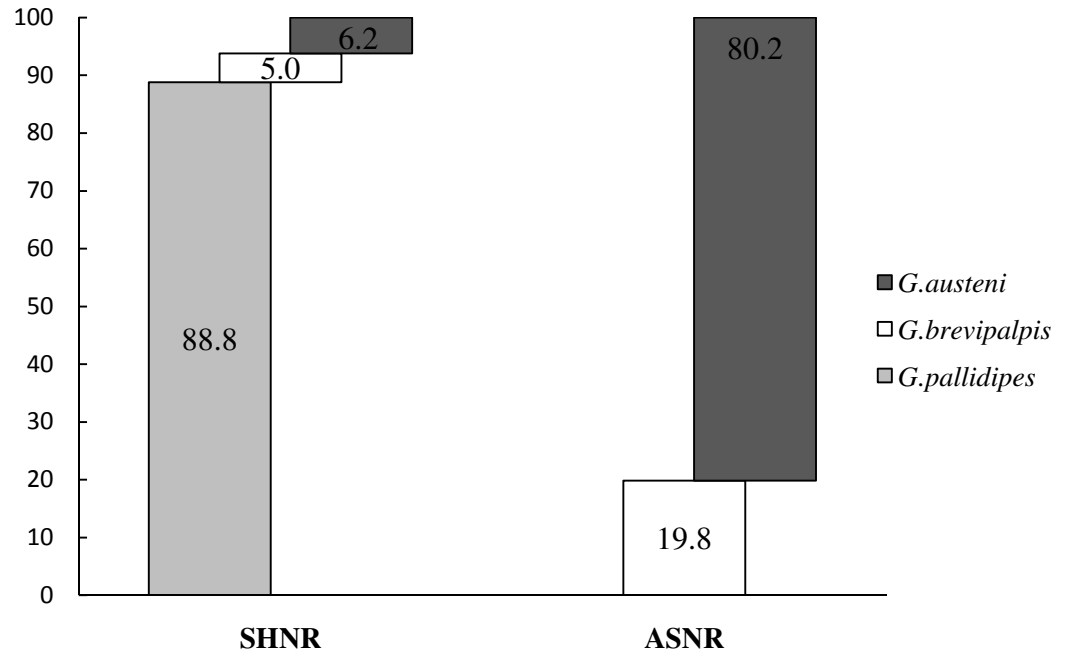

Figure 4.1: Total relative catches (%) of different tsetse species captured at the two coastal sites

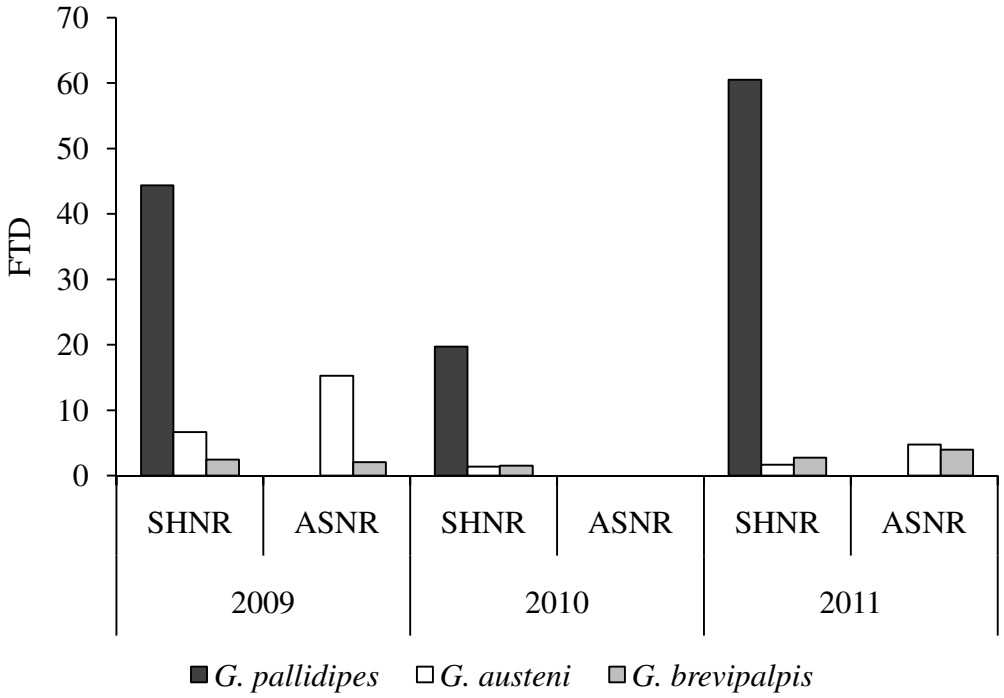

Figure 4.2: 2009-2011 FTD values in SHNR and ASNR for the three tsetse species

4.2 Symbiont Infection in *G. austeni*

All *G. austeni* field samples from Kenya (n=296) comprising 147 females and 149 males analyzed were positive *Wolbachia* infection. Total infection was also recorded in all the South African samples (n=74). A proportion of 83% of the TTRI laboratory colony flies were infected. *Wolbachia* infection was significantly higher in the wild population than the colonized population ($p<0.0001$). The incidence of *Sodalis* infections in *G. austeni* was 2% (n=100) and 4.6% (n=196) in ASF and SHNR, respectively (Table 4.1). The difference in the incidence of infection between the two areas was not significant ($p=0.3443$) and therefore the data were pooled. Total *Sodalis* incidence was not significantly different at 3.7% and 5% for field and laboratory colony respectively (Table 4.1). In the South African samples, *Sodalis* infection in the LSLSA population was 70.8% (n=24), while only one out of 50 flies analyzed from the "northern" population was *Sodalis* positive. The incidence of *Sodalis* in *G. austeni* was significantly higher in South Africa than in Kenya ($p<0.0001$). In addition, significantly more *G. austeni* females than males were infected ($p<0.0001$).

Table 4.1: *Sodalis* prevalence in *G. austeni* from different locations

Source	*n*	number infected		Prevalence (CI 95%)
		F	M	
ASF, Kenya	100	2	0	2% (2.43 - 7.04)
SHNR, Kenya	196	6	3	4.6% (2.12 – 8.54)
Total Kenyan field infection	**296**	**8**	**3**	**3.7% (1.87-6.55%)[a]**
St. Lucia, S. Africa	24	17	0	70.8% (48.91 – 87.39)
northern, S. Africa	50	1	0	2% (0.05 – 10.65)
Total S. African field infection	**74**	**18**	**0**	**24.3% (15.11 – 35.69)[b]**
Laboratory flies	**60**	**0**	**3**	**5% (1.04-1.39%)[a]**

n= sample size, prevalence given with 95% confidence intervals in brackets. [a,b] Prevalence values followed by different letters are significantly different ($p < 0.01$). "northern" refers to sites north of Lake St. Lucia. F- female, M- male; prevalence % given with 95% confidence intervals in brackets.

4.3 Symbiont Infection in *G. pallidipes*

A total of 100 wild and 100 colonized *G. pallidipes* were analyzed for *Wolbachia* prevalence. None of the samples tested from both field and laboratory populations were positive for *Wolbachia*. In addition, 302 wild fly samples (161 female, 141 male) and 63 laboratory colony flies (23 female, 40 male) were analyzed for *Sodalis* using *GPO1* primers. The amplification produced the expected amplicon of 1.2kb, which subsequent sequence analysis confirmed to be of *Sodalis* origin (Figure 4.3).

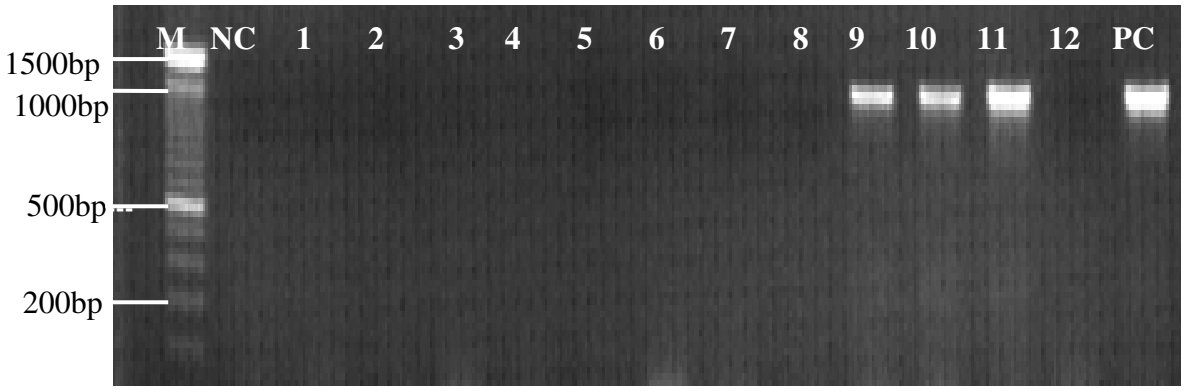

Figure 4.3: Amplification of *Sodalis GPO1* gene in *Glossina*

M-DNA size marker (bp), NC – negative control, Lane 1-8, 12 – *Sodalis* negative, Lane 9-11-*Sodalis* positive, Lane 13-positive control.

Despite having no apparent *Wolbachia* infection, *G. pallidipes* was found to be infected with *Sodalis* at a prevalence of 15.9% and 55.6% in field and laboratory flies, respectively (Table 4.2). In contrast to the findings with *G. austeni*, *Sodalis* infection prevalence in *G. pallidipes* was significantly higher in the field population than in the field population ($p< 0.0001$) Fisher's exact test. In this species as well, significantly more females than males were infected with *Sodalis* ($p=0.0249$). Comparison of infection prevalence between field and laboratory populations using Fisher's exact test showed that the difference was highly significant in *Wolbachia* infection ($p<0.0001$) but not significantly different in relation to *Sodalis* infection.

Table 4.2: *Sodalis* prevalence in wild and laboratory populations of *G. pallidipes*

Source	***n***	**number infected**		***Sodalis* Prevalence**
		F	**M**	
SHNR, Kenya	302	32	16	15.9% (11.96 – 20.52)
Laboratory flies	63	14	21	55.6% (42.49 – 68.08)

n= sample size, prevalence given with 95% confidence intervals in brackets. F-female, M-male

4.4 *Wolbachia* Infection Prevalence Based on 16S rRNA Analysis

All *G. austeni* samples were *Wolbachia* positive using the 16S rRNA, whereas all *G. pallidipes* samples were negative, thus in agreement with *wsp* analysis. However, when compared with *G. m. morsitans*, 16S rRNA analysis of *G. austeni Wolbachia* displayed a different banding pattern as shown in figure 4.4. While only one band of the expected size of 438bp was amplified in *G. austeni*, two bands were amplified in *G. m. morsitans,* with an additional 296bp band. The product bands were consistently more intense in the Kenya samples, indicating the possibility of a higher *Wolbachia* density in these samples. All the 16S rRNA products were sequenced i.e. the single bands produced by Kenyan and South African samples (lanes 3-7) and the two bands produced using *G. m. morsitans* (lane 2). Sequence similarity searches performed using the BLAST algorithm on the National Center for Biotechnology Information (NCBI) platform confirmed at that all bands were of *Wolbachia* origin. The sequences of all the 438bp bands were identical while the smaller *G. m. morsitans* fragment had 142bp deletion in the sequence (Figure 4.5).

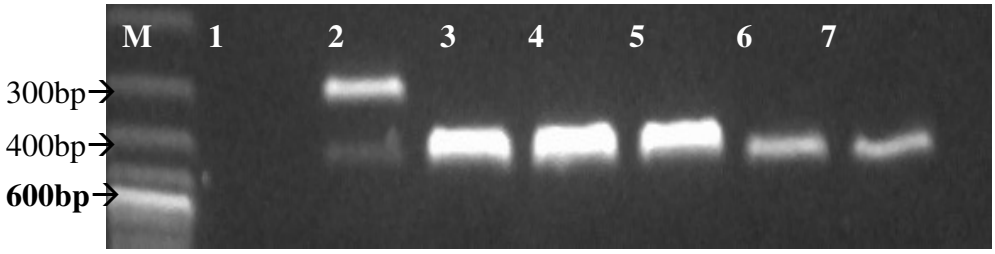

Figure 4.4: Size variation of the *Wolbachia* 16S rRNA gene in *G. austeni* and *G.m.morsitans*
M – DNA size marker, Lane 1-NC, Lane 2-*G.morsitans*, Lane 3,4,5-*G. austeni* Kenya, Lane 6,7-*G. austeni* South Africa

```
CLUSTAL 2.1 multiple sequence alignment


Gmm2_438bp_           CATACCTATTCGAAGGGATAGGGTCGGTTCGGCCGGGTTTCACACAGGTGTTGCATGGCT 60
G.austeni_438bp_      TATACCTATTCGAAGGGATAGGGTCGGTTCGGCCGGATTTCACACAGGTGTTGCATGGCT 60
Gmm1_296bp_           CATACCTATTCGAAGGGATAGGGTCGGTTCGACCGGGTTTCACACAGGTGTTGCATGGCT 60
                       ****************************** **** ********************


Gmm2_438bp_           GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCATCC 120
G.austeni_438bp_      GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCATCC 120
Gmm1_296bp_           GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGCGCGCAACCCTCATCC 120
                      ******************************************** ***************


Gmm2_438bp_           TTAGTTACCATCAGGTAATGCTGGGGACTTTAAGGAAACTGCCAGTGATAAACTGGAGGA 180
G.austeni_438bp_      TTAGTTACCATCAGGTAATGCTGGGGACTTTAAGGAAACTGCCAGTGATAAACTGGAGGA 180
Gmm1_296bp_           TTAGTTGCCATCAGGTAATGCTGGGGACTTTAAGGAAACTGCCAGTGATAAACTGGAGGA 180
                      ****** ************************************************


Gmm2_438bp_           AGGTGGGGATGATGTCAAGTCATCATGGCCCTTATGGAGTGGGCTACACACGTGCTACAA 240
G.austeni_438bp_      AGGTGGGGATGATGTCAAGTCATCATGGCCCTTATGGAGTGGGCTACACACGTGCTACAA 240
Gmm1_296bp_           AGGTGGGGATGATGTCAAGTCATCATGGCCCTTATGGAGTGGGCTACACACGTACTACG- 239
                      ************************************************** ****


Gmm2_438bp_           TGGTGGCTACAATGGGCTGCAAAGTCGCGAGGCTAAGCTAATCCCTTAAAAGCCATCTCA 300
G.austeni_438bp_      TGGTGGCTACAATGGGCTGCAAAGTCGCGAGGCTAAGCTAATCCCTTAAAAGCCATCTCA 300
Gmm1_296bp_           ------------------------------------------------------------


Gmm2_438bp_           GTTCGGATTGTACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGTGGATC 360
G.austeni_438bp_      GTTCGGATTGTACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGTGGATC 360
Gmm1_296bp_           ------------------------------------------------------------


Gmm2_438bp_           AGCACGCCACGGTGAATACGTTTTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAA 420
G.austeni_438bp_      AGCACGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAA 420
Gmm1_296bp_           ----------------------TTTCGTGTCTTGTACACACTGCCCGTCACGCCATGGGAA 278
                                            * *** ********************************


Gmm2_438bp_           TTGGTTTCACTCGAAGCT 438
G.austeni_438bp_      TTGGTTTCACTCGAAGCT 438
Gmm1_296bp_           TTGGTTTCACTCGAAGCT 296
                      ******************
```

Figure 4.5: Alignment of *Wolbachia* 16S rRNA sequences of *G. austeni* and *G. m. morsitans*

Gmm1-smaller product in *G. m. morsitans* (236bp), Gmm2-larger 438bp product in *G. m. morsitans.* A deletion of about 142bp is noted in the sequence for the shorter 16S rRNA product (third row).

4.5 Annual Variation of *Sodalis* Infection

The incidence of *Sodalis* infection in the two tsetse species was compared for three consecutive years. Over this period, the mean % incidence for *Sodalis* infection was significantly higher in *G. pallidipes* than in *G. austeni* (Table 4.3). The prevalence of *Sodalis* infection was not significantly different between the two species in the year 2009 (p=0.8014). However, in 2010 and 2011 the prevalence was significantly higher in *G. pallidipes* (p<0.0001, Fisher's exact test).

Table 4.3: Annual variation of % *Sodalis* infection prevalence

Tsetse Species	**Year**			**Mean ± SD**
	2009	**2010**	**2011**	
G. austeni	8.6% (116)	1.5% (66)	0 % (114)	3.36 ± 3.75%
G. pallidipes	7 % (100)	27 % (100)	13.7 % (102)	15.9 ±8.31%
p-value	p = 0.8014 (NS)	p< 0.0001	p< 0.0001	p< 0.0001

Numbers in brackets represent *n*. The difference in total infection prevalence between the two species was highly significant.

4.6 Distribution of *Wolbachia* and *Sodalis* Symbionts in Fly Organs

The presence of symbionts was assessed in the body tissues of both field and laboratory populations. *Wolbachia* was present in the following *G. austeni* tissues: head, thorax, legs, abdomen and reproductive tract (Figure 4.6). The apparent bacterial density was greater in females than in males, with the former showing stronger bands on the gel. On the other hand, *Sodalis* was detected in the reproductive tract, midgut and carcass of both *G. austeni* and *G. pallidipes*. *Sodalis* was however not detected in the salivary gland.

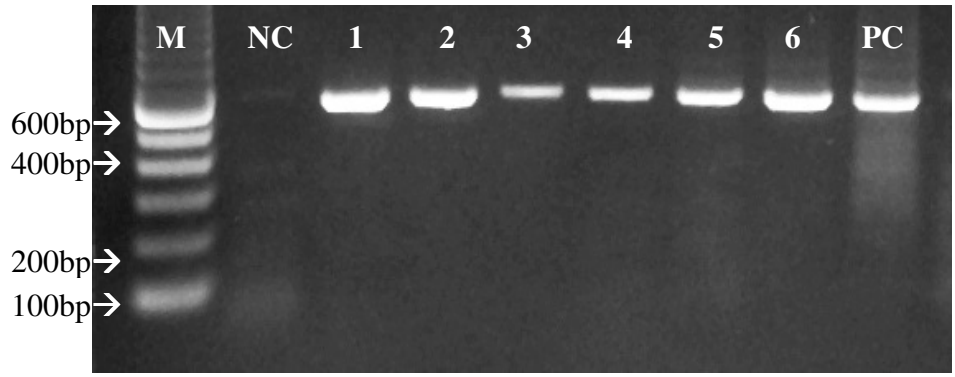

Figure 4.6: Amplification of *Wolbachia wsp* in different *G. austeni* body organs

The results of two representative flies are shown: M- DNA size marker, NC-Negative control, Lane 1-head, Lane 2-thorax, Lane 3-abdomen (male), Lane 4- reproductive tract (female), Lane 5-head, Lane 6- thorax, PC – positive control (*G. morsitans*)

4.7 Age Structure of the Sympatric *G. austeni* and *G. pallidipes*

Fly age was estimated for fly samples from SHNR where both *G. austeni* and *G. pallidipes* were present. Representative samples of *G. austeni* (n=78) and *G. pallidipes* (n=117) collected over the three year study period were used. Wing fray analysis of males indicated a relatively younger *G. austeni* population than that of *G. pallidipes*, with mean wing fray values of 2.4 and 3.8, respectively. The mean age of male *G. austeni* was estimated at 18 days while that of *G. pallidipes* was estimated at 30 days (Table 4.4).

Table 4.4: Mean wing fray value (MWFV) for *G. austeni* and *G. pallidipes*

Species	n	MWFV	Mean Age (days)
G. austeni	78	2.4	18
G. pallidipes	117	3.8	30

In contrast, ovarian ageing indicated that females of both species had comparable longevity and there was no significant difference between the frequency distribution of females among the eight ovarian categories (Table 4.5). In both species, majority of females were between 20-50 days old.

Table 4.5: Frequency distribution (%) of the ovarian categories in *G. austeni* and *G. pallidipes*

Ovarian category	Estimated age (days)	Frequency (%)		*p*- value
		G. pallidipes (n=103)	*G. austeni* (n=67)	
0	0-8	1.6	4.5	0.6812
1	8-19	7.8	14.9	0.2011
2	20-30	30.1	25.4	0.6016
3	30-40	22.3	22.4	1.0000
4	40-50	13.6	16.4	0.6607
5	50-60	5.8	3.0	0.4820
6	60-70	12.6	10.4	0.8090
7	70-80	4.9	3.0	0.7052

4.8 Strain Variation in *G. austeni Wolbachia* Based on *wsp* Typing

The sequences obtained from several *wsp* clones from *G. austeni* flies were identical, indicating that only one bacterial strain was present. This was confirmed by the appearance of single distinct peaks in the chromatograms (Appendix 2). The *wsp* sequences from both Kenyan and South African tsetse fly samples were identical to each other and to the GenBank entry for *Glossina austeni Wolbachia* (AF020077). However, these were different from the *Wolbachia* strain found in *G. m. morsitans*. The HVR profiles of the *G. austeni Wolbachia* strain from both countries (populations) were identical. The sequences of all four *G. austeni Wolbachia* HVRs were different from those of the *Wolbachia* strain found *G. m. morsitans* (Figure 4.7). The *wsp*-based phylogeny of *G. austeni Wolbachia* strains in relation to strains found in other *Glossina* and arthropod species is detailed in figure 4.8.

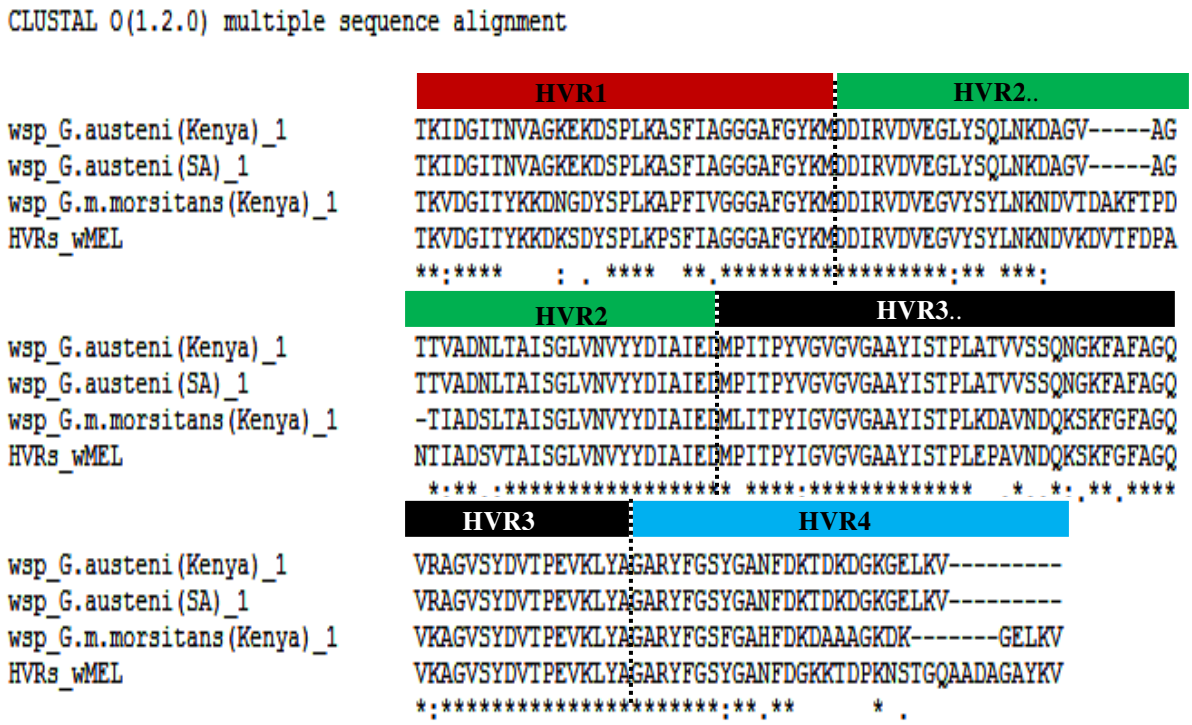

Figure 4.7: Alignment of partial tsetse fly *Wolbachia wsp* amino acid sequences in the HVR. Sequences of *G. m. morsitans* and *D. melanogaster* are included for comparison: SA-South Africa: *w*Mel-*Wolbachia* endosymbiont of *Drosophila melanogaster*

With reference to the *Wolbachia* database, the *wsp* HVR peptides/motifs were identical to others already deposited in the database and therefore were not novel. The HVRs 1-4 were assigned numbers 180, 40, 210 and 18, respectively and cumulatively corresponding to sequence type (ST) 507 (Table 4.6).

Table 4.6: *wsp* HVR profiles for *Wolbachia strains* from *G. austeni* and *G. m. morsitans*

Species (Origin)	**Sequence Type (ST)**	HVR typing			
	wsp	HVR1	HVR2	HVR3	HVR4
G. austeni (Kenya)	**507**	180	40	210	18
G. austeni (S. Africa)	**507**	180	40	210	18
G. m. morsitans (Kenya)	**549**	142	9	223	9

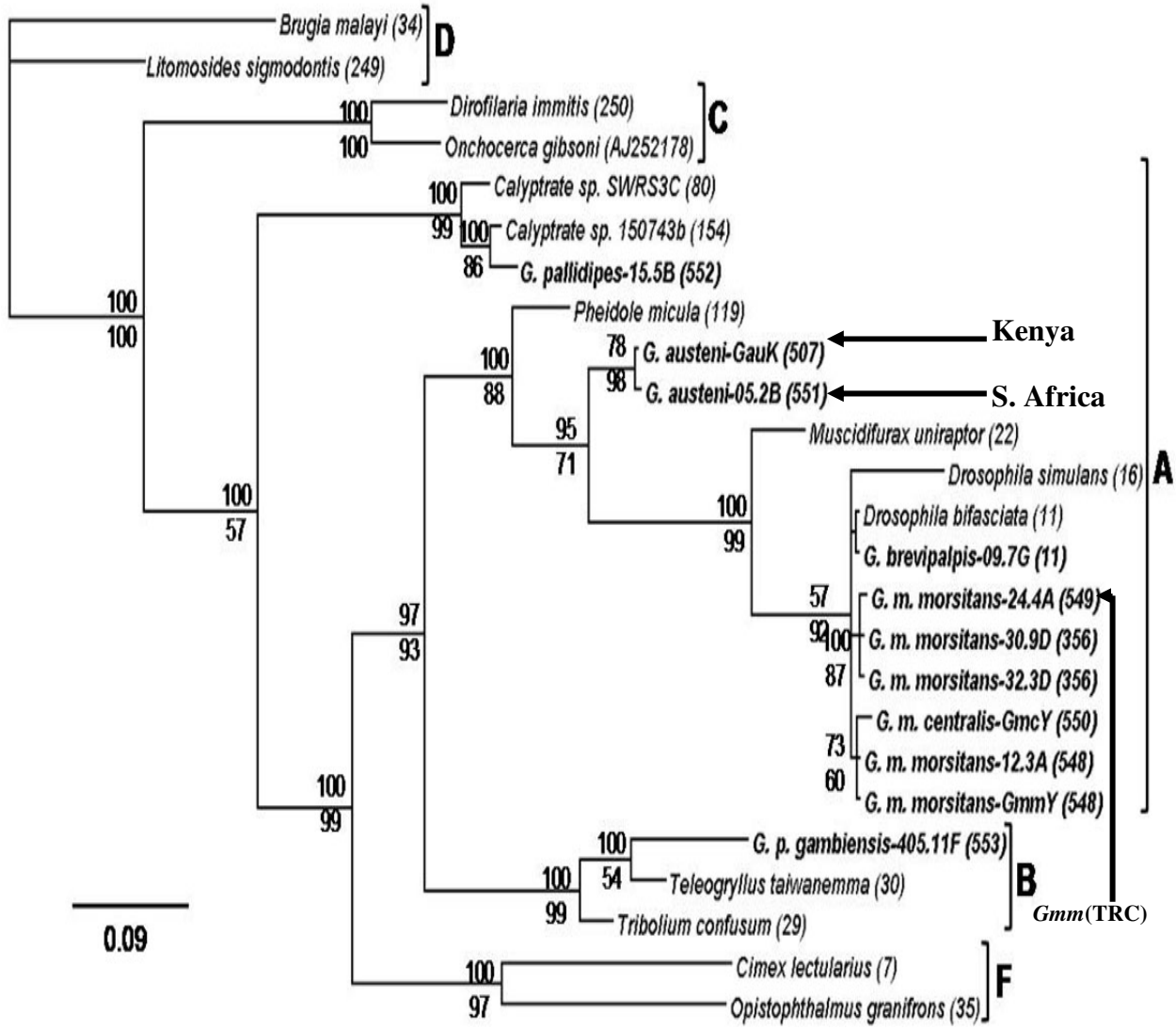

Figure 4.8: Bayesian inference phylogeny based on the *wsp* sequence

Eleven *Wolbachia* strains present in *Glossina* are indicated in bold letters. The *G. austeni* and *G. m. morsitans* isolates used in this study are indicated by the arrows. Strains are characterized by the names of their host species and their *wsp* allele number from the MLST database (except *O. gibsoni* for which the GenBank accession number is given). *Wolbachia* supergroups are shown to the right of the host species names. Bayesian posterior probabilities (top numbers) and ML bootstrap values based on 1000 replicates (bottom numbers) are given (only values >50% are indicated). Adapted from Doudoumis *et al*. (2012).

4.9 Strain Variation in *G. austeni Wolbachia* Based on Multi-Locus Strain Typing

All the five *Wolbachia* genes targeted in the MLST assay were readily amplified in *G. austeni*(Figure 4.9) and the nucleotide sequences obtained deposited in Genbank under the accession numbers JF906102 – JF906106 (Appendix 3).

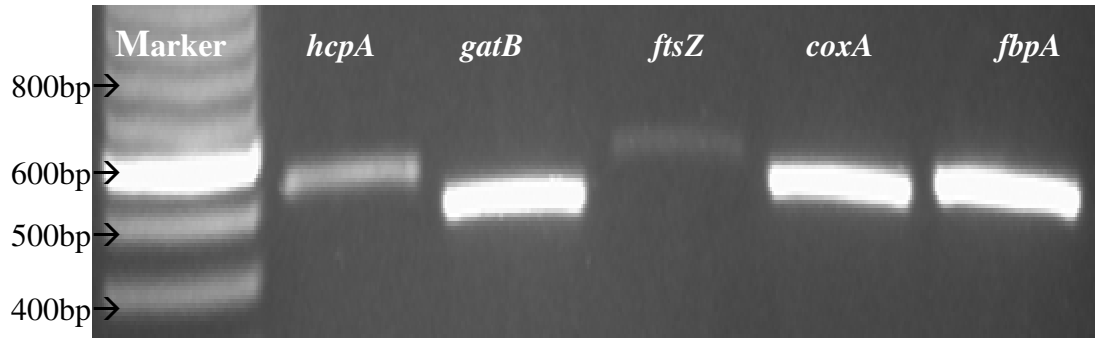

Figure 4.9: Amplification of *Wolbachia* MLST genes in *G. austeni*

The sequences for the Kenyan and South African isolates were identical in four of the five genes used namely *gatB, hcpA, ftsZ and fbpA*. The representative *hcpA* sequence alignment shown in figure 4.10 displays the similarity of four samples from Kenya (Shimba Hills, Arabuko-Sokoke) and South Africa (Mbazwane, Muzi) at this locus. However, there were at least four single nucleotide variations in the *coxA* sequences of the two populations. These changes were present both intra- and inter -population (Figure 4.11). The sequences obtained were compared with isolates already deposited in the MLST database (http://pubmlst.org/wolbachia/). The Kenyan samples were allocated allele numbers 128, 108, 127, 98 and 20 for the *gatB, coxA, hcpA, ftsZ, and fbpA* loci respectively. The South African *coxA* sequence was assigned number 127. The novel strains identified in this study were assigned number 197 (Kenya) and 231 (South Africa) which were new allelic profiles or ST according to the data available on the MLST database (Table 4.7). The MLST-based phylogeny of *G. austeni Wolbachia* strains in relation to strains found in other *Glossina* and arthropod species is detailed in figure 4.12.

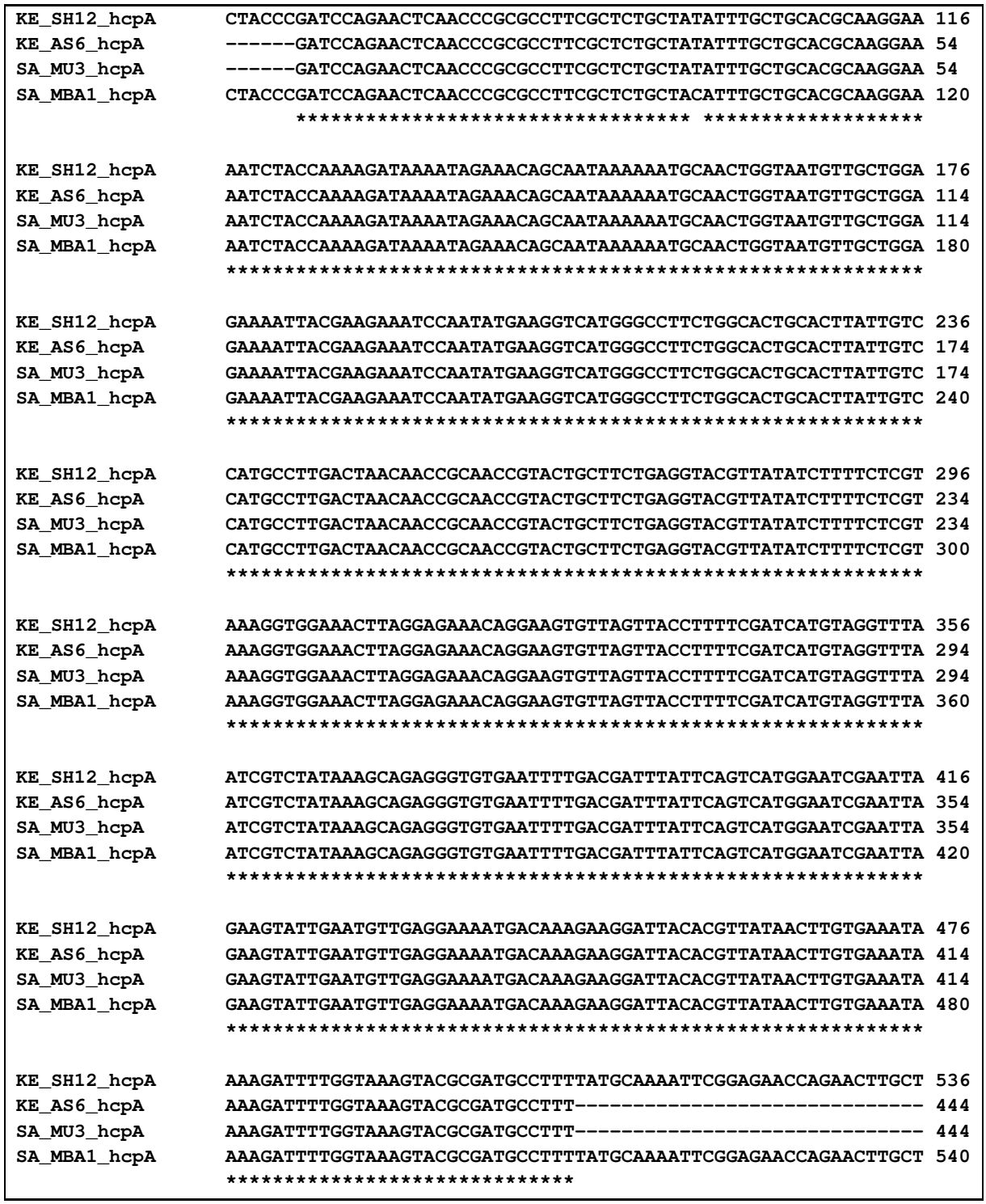

Figure 4.10: Multiple sequence alignment of *hcpA* from *G. austeni Wolbachia*

Four representative samples are shown: SA-South Africa, KE-Kenya, SH-Shimba Hills, MBA-Mbazwane, MU-Muzi, AS-Arabuko-Sokoke

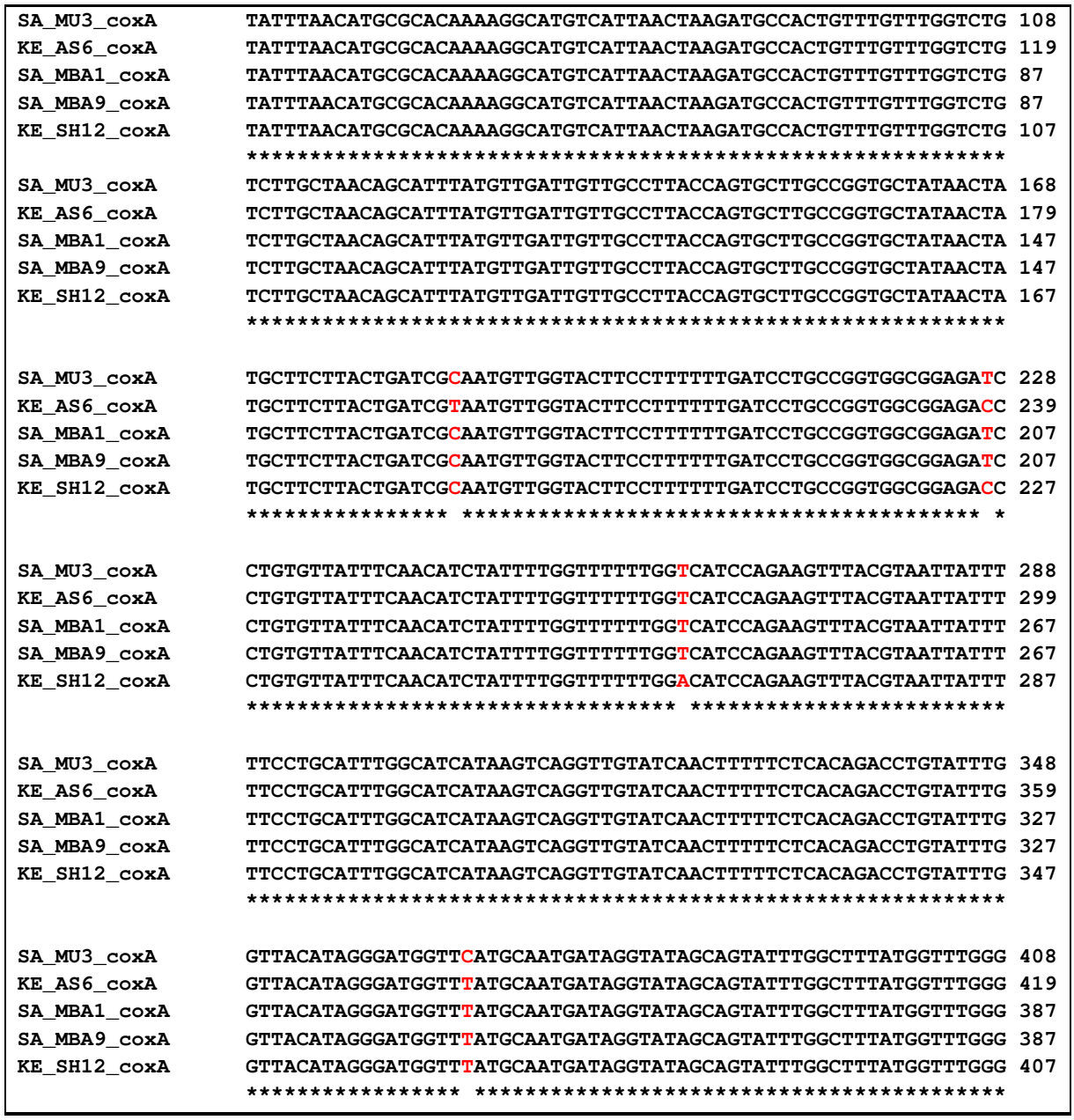

Figure 4.11: Multiple sequence alignment of *coxA* from *G. austeni Wolbachia*

Five representative samples are shown, with four regions of sequence divergence highlighted in red colour. SA-South Africa, KE-Kenya, SH-Shimba Hills, MBA-Mbazwane, MU-Muzi, AS-Arabuko-Sokoke

Table 4.7: *G. austeni Wolbachia* MLST allelic profiles

Species (Origin)		MLST				
	Sequence Type (ST)	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*
G. austeni (Kenya)	**197**	128	108	127	98	20
G. austeni S. Africa	**231**	128	109	127	98	20
G. m. morsitans (Kenya)	**229**	142	128	23	113	15

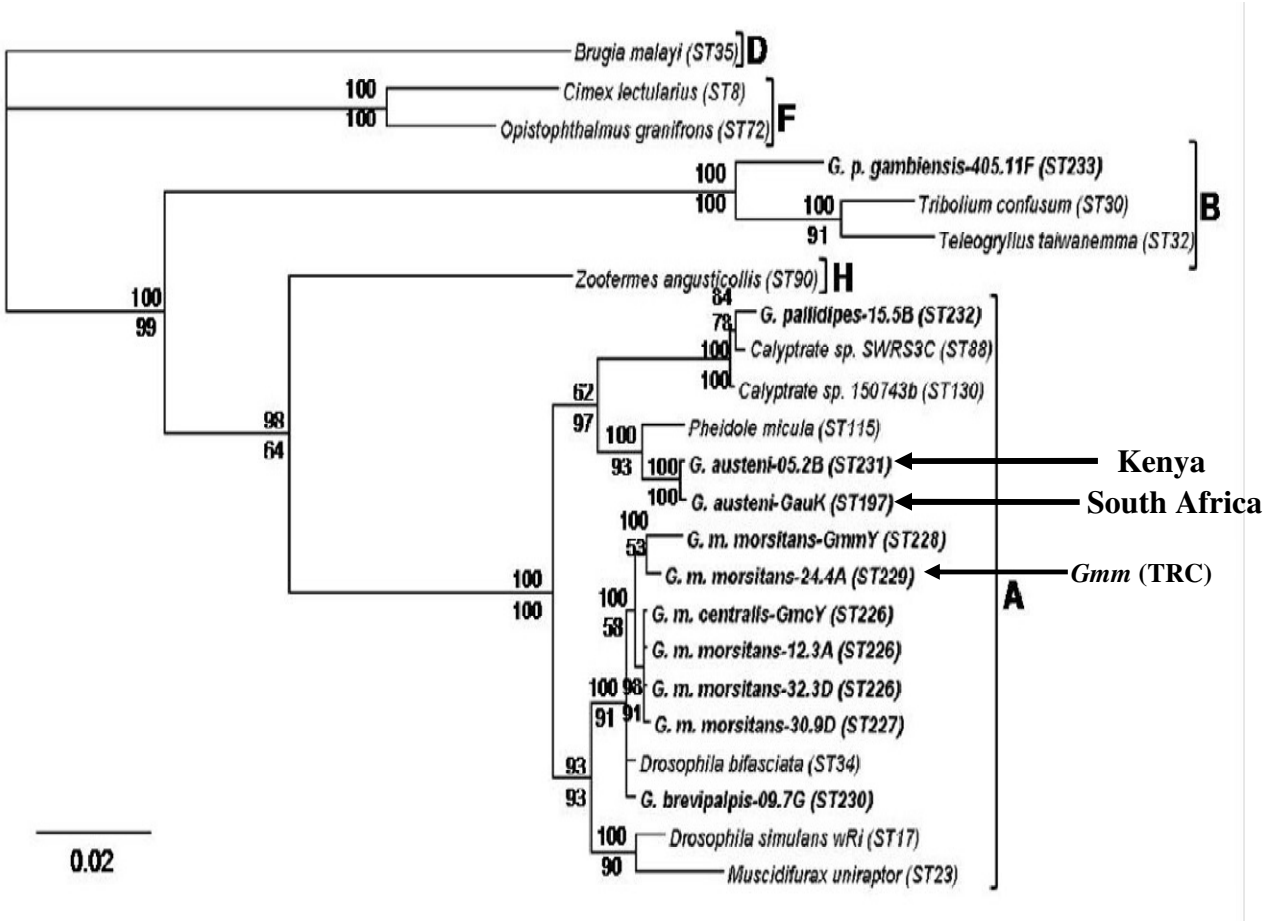

Figure 4.12: Bayesian inference phylogeny based on the concatenated MLST data

The 11 *Wolbachia* strains present in *Glossina* are indicated in bold letters, and the other strains represent supergroups A, B, D, F and H. The *G. austeni* and *G. m. morsitans* isolates used in this study are indicated by the arrows. Adapted from Doudoumis *et al.,* (2012).

4.10 *Sodalis* Strain Diversity Based on *GPO1* and VNTRs

Sequencing of the *GPO1* gene product yielded identical sequences indicating there were no differences in *Sodalis* strains at this locus. This sequence was identical to the GenBank entry accession number AP008235 for *Sodalis glossinidius* str. 'morsitans' plasmid pSG3, complete sequence. This means that at this particular locus, *Sodalis* isolated from these two species of tsetse flies are identical to that isolated from *G. m. morsitans.* To provide further differentiation, three *Sodalis* microsatellite markers were used: ADNg 5/2, ADNg15/16 and ADNg21/22. The alleles produced were visible in the 2% gel used (Figure 4.13).

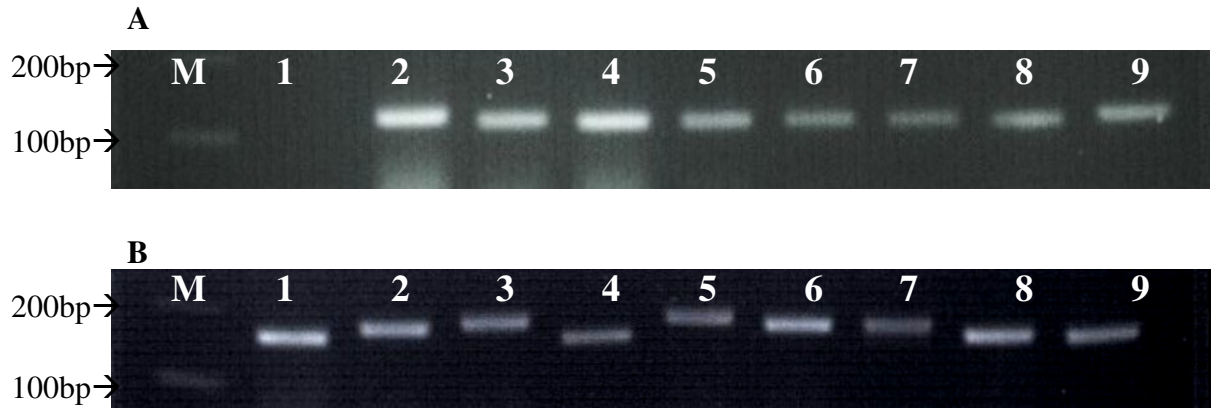

Figure 4.13: Diagnostic size differences of two *Sodalis* microsatellite loci in a 2% agarose gel
A: ADNg 15/16: M-100bp DNA marker, 1-negative control, lanes 2-8 *G. pallidipes*, Lane 9-*G. austeni.* **B**: ADNg 21/22: M-100bp DNA marker, lanes 1-8 *G. pallidipes*, Lane 9-*G. austeni*

The sequences obtained at the ADNg21/22 loci products from 11 samples including two laboratory colony samples corresponded to GenBank entries for *Sodalis glossinidius* ADNg 21/22 sequence clones. A total of seven alleles were identified using this marker. These alleles carried between four and fourteen repeats of the repeat sequence CGG, as detailed in table 4.8. Sequence variation on the ADNg21/22 loci from 11 samples including two laboratory colony samples and four trypanosome-infected field samples is shown in figure 4.14. Laboratory colony samples from the two different species had the same number of repeats (6), whereas the field samples had between 4 and 14 repeats. Four of the field samples were infected with trypanosomes, but there was no obvious differentiation in repeat number between infected and non-infected samples.

Table 4.8: Number of CGG tandem repeats in the *Sodalis* ADNg21/22 loci

Species	Sample	Source	Trypanosome infection	No of CGG repeats
G. pallidipes	Gp01	F	-ve	4
	Gp02	F	-ve	9
	Gp03	F	-ve	12
	Gp04	F	+ve	4
	Gp05	F	+ve	14
	Gp06	F	+ve	11
	Gp07	F	+ve	11
	Gp08	F	-ve	7
	Gp09	C	-ve	6
G. austeni	Ga10	C	-ve	6
	Ga11	F	-ve	4

F-field; C-colony;+ve-trypanosome-infected; -ve - not infected

```
CLUSTAL O(1.2.1) multiple sequence alignment


Gp09-C      ---TAACTCGATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   57
Gp10-F      --AAGCTAAGATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   58
Gp05-F      -AAGCTTAAGATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   59
Gp08-F      -AAGCTTCTGATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   59
Gp06-F      -TACCCTATGATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   59
Gp07-F      AACCGGTCTCATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   60
Gp03-F      ----ACGTCGATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   56
Gp02-F      ---AAGCTCGATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   57
Gp04-F      --AAACTCTGATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   58
Gp01-F      AAGCCGTAAGATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   60
Gp11-C      --TGAGGTCGATGTCATTGCCGTGGCCGCGCTGGTGGCGAATATTGTGTCATTCTGGCTG   58
                     **************************************************


Gp09-C      CTGCACGGCGGCGGC------------------------GGCGGCGGCGAAAAAAATATA   93
Gp10-F      CTGCACGGCGGCGGC------------------------GGCGGCGGCGAAAAAAATATA   94
Gp05-F      CTGCACGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGAAAAAAATATA   119
Gp08-F      CTGCACGGCGGCGGCGG--------------------CGGCGGCGGCGAAAAAAATATA   98
Gp06-F      CTGCACGGCGGCGGCGGCGGCGGCGG---------CGGCGGCGGCGGCGAAAAAAATATA   110
Gp07-F      CTGCACGGCGGCGGCGGCGGCGGCGG---------CGGCGGCGGCGGCGAAAAAAATATA   111
Gp03-F      CTGCACGGCGGCGGCGGCGGCGGCGG------CGGCGGCGGCGGCGGCGAAAAAAATATA   110
Gp02-F      CTGCACGGCGGCGGCGGCGG---------------CGGCGGCGGCGGCGAAAAAAATATA   102
Gp04-F      CTGCACGGCGGC--------------------------------GGCGGCGAAAAAAATATA   88
Gp01-F      CTGCACGGCGGC--------------------------------GGCGGCGAAAAAAATATA   90
Gp11-C      CTGCACGGCGGC--------------------------------GGCGGCGAAAAAAATATA   88
            ************                                  ********************


Gp09-C      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGGAGATTTGCTCCTATCTAGCAATGCT       149
Gp10-F      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGGAGATTTGCTCCATCGG---------       141
Gp05-F      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGAAGATTTGATCAAC------------       163
Gp08-F      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGGAGATTTGCTCCAGAGAGG-------       147
Gp06-F      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGGAGATTTGCTCAATTCGGA-------       159
Gp07-F      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGGAGATTTGCTCAATTC----------       157
Gp03-F      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGGAGATTTGCTCAAAGACC---------       158
Gp02-F      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGGAGATTTGCTCAATGCTAGGCAT---       155
Gp04-F      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGGAGATTTGCTCCAGTGTAA--------       137
Gp01-F      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGGAGATTTGCTCCATTAG---------       137
Gp11-C      ACCTGCGCGCCGCGGCGCTGCATGTGCTGGGAGATTTGCTCCACT-----------       133
            ****************************** ******* **
```

Figure 4.14: Sequence variation at the *Sodalis* ADNg21/22 loci

Ga-*G. austeni*, Gp-*G. pallidipes*, F-field sample, C-laboratory colony sample. The repeat region is enclosed in box. Variation in 9 field and 2 laboratory samples is shown.

4.11 *Sodalis* - Trypanosome Co-infection in Wild *G. pallidipes*

Co-infection was determined by analysis of the same subset of samples for both *Sodalis* and trypanosome infection.

4.11.1 Incidence of trypanosome infection

A total of 787 flies were dissected in the field, comprising 235 *G. austeni* and 552 *G. pallidipes*. Additionally, 598 tsetse fly samples were subjected to PCR analysis for detection of infecting trypanosomes. This included 296 *G. austeni* composed of 149 males and 147 females. At the same time 302 *G. pallidipes* composed of 141 males and 161 females were also analyzed. In both species of tsetse flies, proportionately more females than males were infected; however infection prevalence was significantly higher in *G. pallidipes* (Table 4.9).

Table 4.9: Sex ratio of flies found infected with trypanosomes using PCR

Species	n	N	Males	Females	Statistics
G. austeni	296	38	16 (42.1)	22 (57.9)	p=0.0505
G. pallidipes	302	29	1(3.4)	28 (96.6)	p<0.0001*

N=total number of positive flies. Figure in brackets is % of infected flies only. *significantly different infection between males and females

The total incidence of trypanosome infection was 12.8% and 9.6% for *G. austeni* and *G. pallidipes,* respectively. The average infection rate realized in the three years was 14±6.4% and 9.6±3.2% for *G. austeni* and *G. pallidipes* respectively. In both fly species, the highest trypanosome infection rate was observed in 2010 (Table 4.10). However, chi-square test showed that the differences in prevalence rates for the three years was not significantly different in both *G. austeni* (χ^2=5.882, df=2, p=0.0528) and *G. pallidipes* (χ^2=2.321, df=2, p=0.3133).

Table 4.10: Annual variation of trypanosome infection in *G. pallidipes* and *G. austeni*

Species	2009	2010	2011	Total
G. austeni	14/116 (12.1)	14/66 (21.2)	10/114 (8.8)	38/296 (12.8) [9.25-17.19%]
G. pallidipes	6/100 (6)	12/100 (12)	11/102 (10.8)	29/302 (9.6) [6.53-13.50%]

% infection prevalence in round brackets, 95% confidence interval in square brackets.

The ITS1 assay was specific enough to discriminate between the different trypanosomes species infecting tsetse, as well as identify flies with mixed trypanosome infections (Figure 4.15).

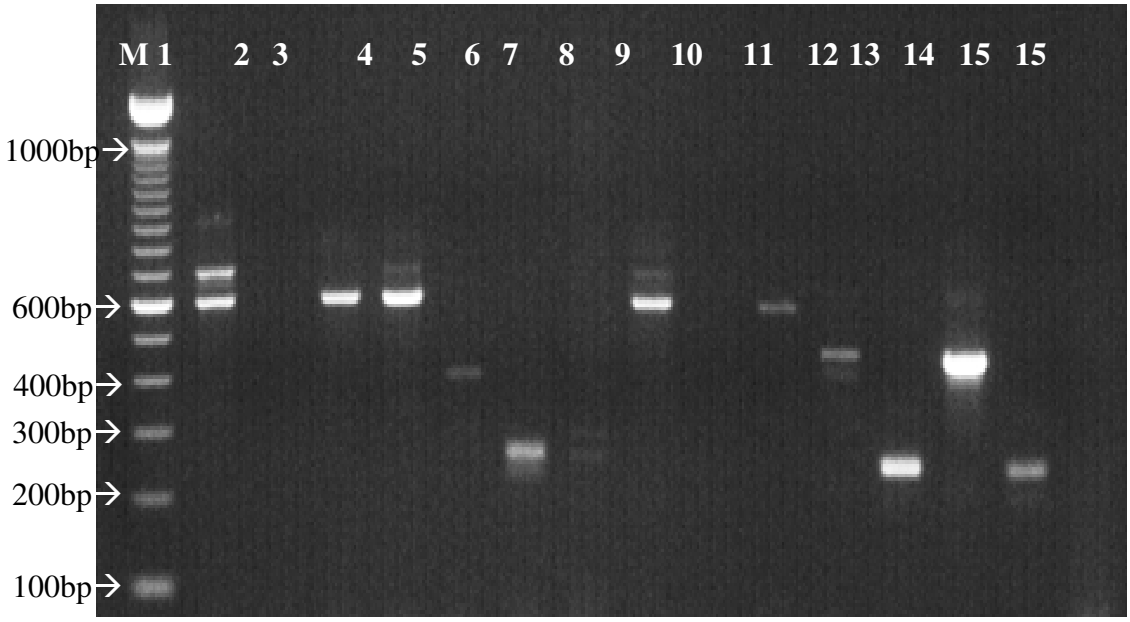

Figure 4.15: Amplification of trypanosome DNA in infected tsetse flies

M-DNA size marker: Lanes 1, 3, 4,8 and 10-*T. congolense* sub-types, Lane 5-*T. simiae*, Lane 6, 7, 12-*T. vivax*, Lanes 11,13 and 14 are positive controls for *T. brucei, T. vivax and T.evansi,* respectively, Lane 15-negative control. Infecting trypanosomes are differentiated by the size of the product. Gel image for 10 representative samples shown.

The most common species of trypanosomes found infecting wild flies (n=598) were *T. congolense* (5.18%) and *T. vivax* (4.18%). Two sub-types of *T. congolense* were identified, namely *T. congolense* Kilifi (TCK) and *T. congolense* savannah (TCS). Other trypanosomes found infecting flies were *T. brucei* (in mixed infections) and *T. simiae*. The distribution of infecting trypanosome species among the two tsetse fly species is presented in table 4.11. Statistical analysis of the total trypanosome infection prevalence indicted that observed values were not significantly different between the two tsetse species (p=0.2435). Considering both fly species, similar trypanosome infection patterns that were observed include (a) *T. congolense* was the most common trypanosome at every sampling point (b) mixed infections were identified in only the 2009 sample set and (c) *T. simiae* was absent in the 2011 sample set (Figure 4.16).

Table 4.11: Species of trypanosomes found infecting Kenyan *G. pallidipes* and *G. austeni*.

	Area	n	*T. c.*	*T. v.*	*T. sm*	mixed	Total
G. austeni	SHNR	196	12 (6.1)	11(5.6)	2 (1.0)	0 (0)	25 (12.7)
	ASNR	100	8 (8)	3 (3)	1 (1)	1 (1)	13 (13)
G. pallidipes	SHNR	302	11 (3.6)	11 (3.6)	7 (2.3)	1 (0.3)	29 (9.6%)

The % infection is presented in brackets. T. c. - *T. congolense*, T. v. - *T. vivax*, T. sm - *T. simiae*, mixed - *T. b. brucei* / *T. congolense*

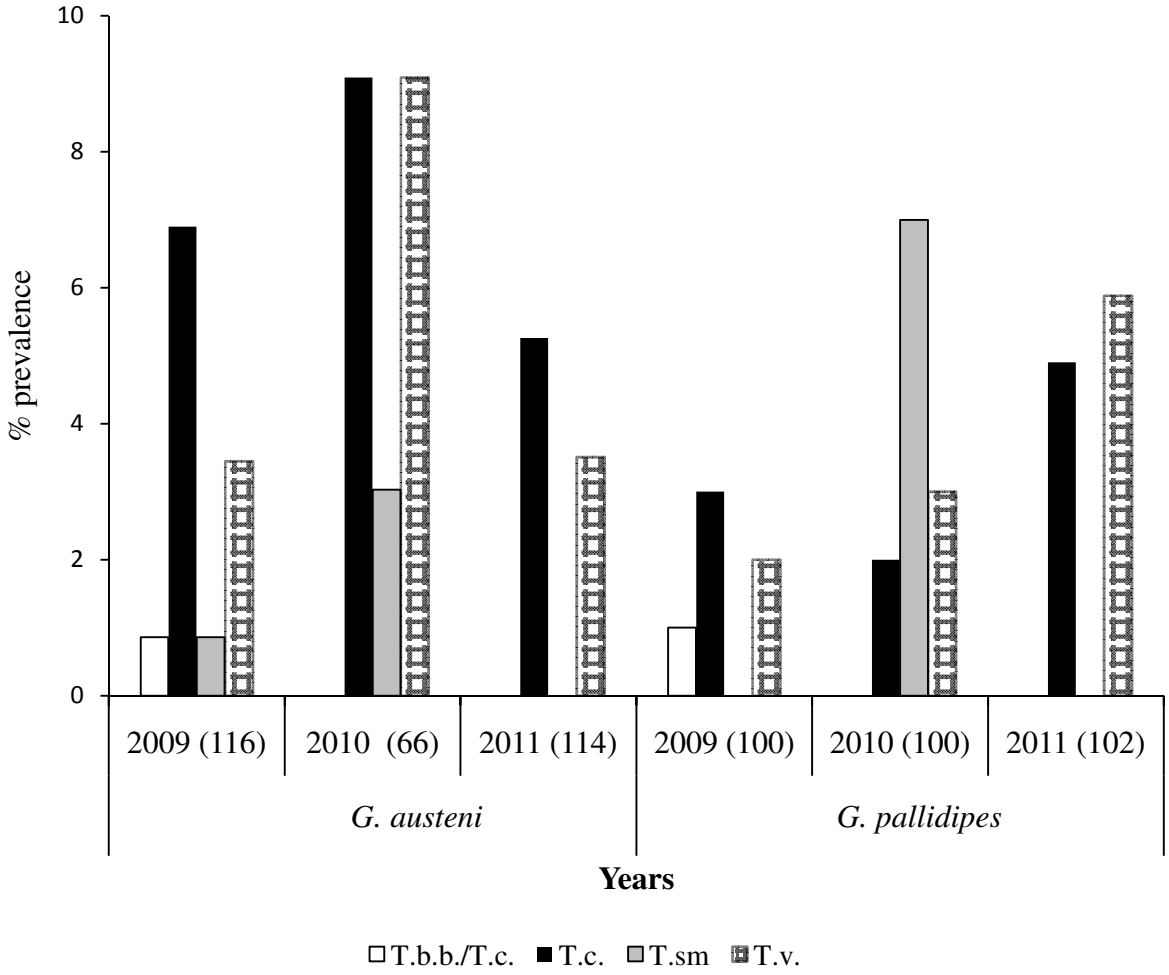

Figure 4.16: Annual variation in prevalence of different trypanosome species infecting tsetse Numbers in brackets represent total number flies analyzed for that specific year and species; Tbb-*T. b. brucei*, T.c.-*T. congolense*, T.sm-*T. simiae*, T.v.-*T. vivax*

4.11.2 Co-infection of wild flies with trypanosomes and *Sodalis*

Most of the flies sampled were found to carry neither *Sodalis* nor trypanosomes and only a small proportion (13/598) harbored both trypanosomes and *Sodalis* bacteria. Of these 10 (3.3%) were *G. pallidipes* and 3 (1%) were *G. austeni*. In flies that carried the symbiont, 20.8% and 27.3% had a concurrent trypanosome infection in *G. pallidipes* and *G. austeni* respectively. The occurrence of co-infection was most commonly associated with *T. simiae* parasites whereby, out of 7 flies found infected with trypanosomes, 6 contained the bacteria (85.7%). Correspondingly, 5/31 (8%) *T. vivax*-infected flies and 2/25 (16%) *T. congolense*-infected flies also had *Sodalis* infection. The association between trypanosomes and *Sodalis* infection was statistically significant in *G. pallidipes* but not so in *G. austeni* (Table 4.12).

Table 4.12: *Sodalis* and trypanosome co-infection in wild *G. pallidipes* and *G. austeni*

	G. pallidipes (n=302)		*G. austeni* (n=296)	
	T+	T-	T+	T-
S+	10 (3.3%) **[20.8]**	38 (12.6%)	3 (1.0%) **[27.3]**	8 (2.7%)
S-	19 (6.3%)	235 (77.8%)	35 (11.8%)	250 (84.5%)
p – value	*p* = 0.0127 *		*p* = 0.1554 (NS)	

* significant, NS-not significant. Bold values in square brackets indicates % of *Sodalis*- positive flies that were also parasite-positive

4.12 *Sodalis* - Trypanosome Co-infection in Experimentally Challenged *G. pallidipes*

In the *T. b. rhodesiense* time series experimental group, a total of 70.3% (n=118) of the flies were found to infected with the symbiont. A similar percentage of flies were able to establish the *T. b. rhodesiense* infection (Table 4.13).

Table 4.13: Total *Sodalis* and *T. b. rhodesiense* infection on various days post-infection

DPI	n	*Sodalis* infection	*T. b. rhodesiense*
7	25	20 (80.0)	15 (60.0)
14	22	19 (86.4)	16 (72.7)
21	26	17 (65.4)	18 (69.2)
28	27	20 (74.1)	25 (96.6)
35	18	7(38.9)	9 (50.0)
Total	**118**	**83 (70.3)**	**83 (70.3)**

n-number of flies tested at specific number of dpi; numbers in brackets represent % infection

Considering co-infection, 53.4% of total flies assayed were infected with both the symbiont and the parasite (S+T+), while 12.7% had neither symbiont nor parasite (S-T-). At all time points sampled viz. 7, 14, 21, 28 and 35 days post infection, a higher proportion (76.5 ±8.9%)of symbiont-infected flies were able to establish the trypanosome infection, compared to those which did not S+T- (17.9± 6 3%). There was a departure from this observation at 35 dpi, when no flies were found to have both the symbiont and the parasite (Table 4.14).

Table 4.14: Association of *Sodalis* and *T. b. rhodesiense* co-infection in individual flies

DPI	n	*Sodalis* infected flies		*Non-infected flies*	
		T+	T-	T+	T-
7	25	14 (56.0)	6 (24.0)	1 (4.0)	4 (16.0)
14	22	15 (68.2)	4 (18.2)	1 (4.5)	2 (9.1)
21	26	14 (53.8)	3 (11.5)	4 (15.4)	5 (19.2)
28	27	20 (74.1)	0 (0)	5 (18.5)	2 (7.4)
35	18	0 (0)	7 (38.9)]	9 (5.0)	2 (11.1)
Total	**118**	**63 (53.4)**	**26 (22.0)**	**20 (16.9)**	**15(12.7)**

n, number of flies tested at specific dpi; numbers in brackets represent % infection with respect to sample size, T+, no. of trypanosome infected flies; T-, no. of parasite negative flies.

In the *T. b. brucei* infection group, 15 out of 91 flies (16.5%) dissected were infected with trypanosomes. Of these, 9 were mature infections with trypanosomes found in the mouthparts, midgut and in the salivary gland. The other 6 flies had trypanosomes in their midgut and salivary gland and midgut alone, respectively. PCR analysis was carried out using the DNA extracted from the gut revealed that 50.5% and 46.2% of total flies assayed carried *Sodalis* and trypanosomes infections respectively. In this group, 23.1% of flies sampled were co-infected with both symbiont and parasite. In the *T. congolense* infection group (n=98), the dissection procedure identified 8 infections in 160 flies dissected (5%). Subsequently, PCR assays determined infection with the symbiont and *T. congolense* to be 32.7% and 61.2% respectively. In the *T. congolense* group, 26.5% of the samples were co-infected with both microbes (Table 4.11). *Sodalis*-infected flies of both species developed higher trypanosome infection rates than the *Sodalis*-negative flies, regardless of fly and trypanosome species. For example, using *T. congolense* an infection rate of 74.3% was detected in *Sodalis*-positive flies, whereas in *Sodalis*-negative flies, an infection rate of 53.1% was realized. Fisher's exact test revealed that the association between the presence of *Sodalis* and concurrent trypanosome infection was statistically significant in *T. b. rhodesiense* and *T. congolense* but not in *T. b.* brucei (Table 4.15).

Table 4.15: *Sodalis* and parasite co-infection in experimentally-infected *G. pallidipes*

	T. b. rhodesiense (n=118)		*T. b. brucei* (n=91)		*T. congolense* (n=98)	
	T+	*T-*	*T+*	*T-*	*T+*	*T-*
S+	63 (53.4%) **[75.9]**	20 (16.9%)	21 (23.1%) **[45.7]**	25 (27.5%)	26 (26.5%) **[81.3]**	6 (6.1%)
S-	20 (16.9%)	15(12.7%)	21 (23.1%)	24 (26.4%)	34 (34.7%)	32 (32.7%)
p value	$p = 0.0493$		$p = 1.0000$ (NS)		$p = 0.0074$	

NS - not significant (Fisher's exact test). Bold values in square brackets indicates % of *Sodalis*-positive flies that were also parasite-positive, S+, total number of flies harboring symbiont; T+, total number of infected flies; T+, parasite- positive; T-, parasite negative

4.13 Effect of Ampicillin on *G. pallidipes* Survival and Symbiont Clearance

In general, the mean survival of *G. pallidipes* treated with ampicillin was significantly lower than that of untreated controls. The proportion of flies surviving at day 22 post-eclosion in the experimental group was 10± 2.8% and 79±2.8% in the control group (Figure 4.17). *Sodalis* was detected in flies that had fed on ampicillin diet for at most three feeds. After that period, *Sodalis* was no longer detectable in the midgut tissues. In the case of *Wigglesworthia*, no sample was found positive using the *thiC* primers.

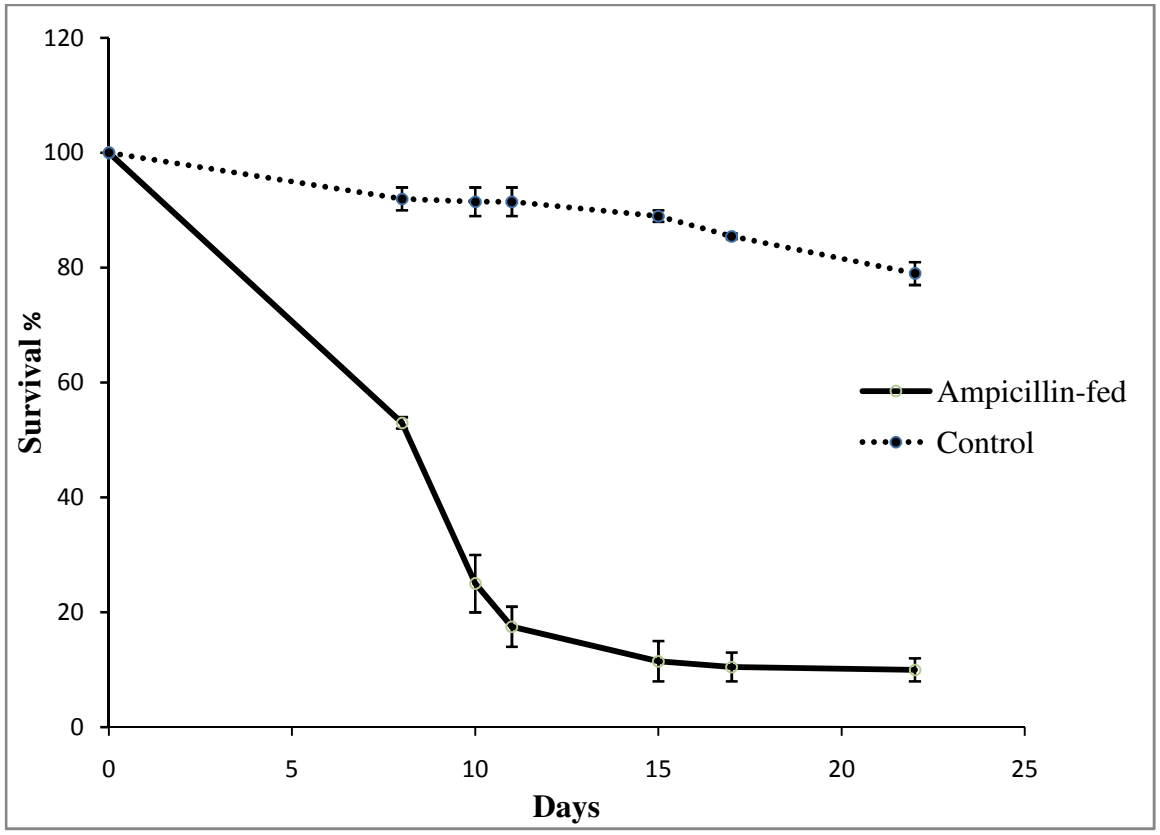

Figure 4.17: Survival of *G. pallidipes* maintained on ampicillin-spiked blood.

Error bars represent the standard error of the mean (SEM). Two replicates of each treatment were conducted.

4.14 Trypanosome Establishment in Ampicillin-Treated *G. pallidipes*

Dissection of *G. pallidipes* male flies that had been fed on ampicillin and subsequently offered an infective blood meal at the age of 10 days, did not reveal any parasites in either the *T. b. brucei* group (n=102) at 40 dpi nor the *T. congolense* group at 30 dpi (n=153). The samples were also confirmed to be *Sodalis* negative. In the control groups, 3.0% (n=67), 8.0% (n=50) of the flies showed a *T. b. brucei* or a *T. congolense* infection upon dissection, respectively. The difference in infection rate between ampicillin-treated and non-treated controls was significant with *T. congolense* (χ^2=7.092, df=1, p=0.0077) but not significant in *T. b. brucei* (χ^2=1.057, df=1, p=0.3038)

CHAPTER FIVE

DISCUSSION

5.1 Tsetse Fly Density

Three species of tsetse *G. pallidipes, G. austeni* and *G. brevipalpis* were captured in SHNR, while only *G. austeni* and *G. brevipalpis* were captured in ASNR. The mixed type of vegetation found in SHNR provides suitable habitat for all three species which belong to the savannah and forest groups. However, the absence of *G. pallidipes* in ASF can be attributed to the fact that this species prefers open savannah grassland habitats, whereas the ASF habitat is composed mainly of dense indigenous forest. The number of flies captured, in decreasing order, was *G. pallidipes*>*G. austeni*>*G. brevipalpis* which is similar to that reported in previous studies (Ohaga *et al.*, 2007). This relative abundance could also have been influenced by the type of trap used. The present study used biconical and Ngu traps. Among the three fly species present in the study area, these traps are considered to be most suitable for *G. pallidipes.* In an area with the same three fly species in Mozambique, the biconical traps were found to be very effective for the capture of *G. pallidipes* and *G. brevipalis*, but not for *G. austeni* since not all flies attracted to the trap entered the collecting device (Takken, 1984). The use of these traps could therefore have biased the catches toward *G. pallidipes*. However, comparison of traps was not an objective and the two trap types used sufficed to produce adequate fly numbers for the study.

5.2 *Wolbachia* Infections in Wild and Laboratory Populations of *G. austeni*

All individual flies analyzed from Kenyan *G. austeni* field populations were shown to be infected with *Wolbachia* while the South African fly population infection rate was 98%. An infection rate of 83% was recorded in the laboratory population from Tanzania. The present results indicate an increase in the Kenyan *G. austeni Wolbachia* infection rate of 48% obtained in 1996 (Cheng *et al.*, 2000), and therefore indicate an increase in infection prevalence towards fixation. This increase is due to the characteristic of *Wolbachia* to perpetuate its own spread via infected females; an asymmetry in fertility which causes the proportion of *Wolbachia*-infected insects to increase and spread into a population (Rasgon and Scott, 2003). This results in the number of *Wolbachia*-infected in a given population increasing from low levels to complete fixation in as little as three years (Turelli and Hoffmann, 1991). It may also be the effect of

immigration of infected flies into this population (Hancock and Godfray, 2012). It is postulated that the lower infection rate observed in the laboratory population may be as a result of a lower fidelity of maternal transmission of the bacteria. One limitation in this study was that only male flies were available for the assessment of prevalence in *G. austeni* laboratory colony. However, studies with other arthropods show no significant differences in *Wolbachia* infection rates between males and females (Albuquerque *et al.*, 2011; Zouache *et al.*, 2011). Therefore, it would be concluded that the laboratory prevalence found in this study would be representative of the whole colony although confirmation of this result by analyzing both sexes is required.

5.3 *Wolbachia* Infections in Wild and Laboratory Populations of *G. pallidipes*

Wolbachia infection was not detected in the *G. pallidipes* populations sampled. This result is consistent with previously reported zero infection rates for *G. pallidipes* flies from Kenya and Uganda (Cheng *et al*., 2000). However, recent work has reported a prevalence of 1.2% in 1,823 samples originating from Ethiopia, Tanzania, Zambia and Zimbabwe (Doudoumis *et al.*, 2012). The present study is in agreement with previous findings that *Wolbachia* infection is low or undetectable in *G. pallidipes.* This fly species is a major vector for both human and animal trypanosomosis in Kenyaand has been the target species in previous control programmes established in Nguruman, Busia and Lambwe Valley (Barrett and Okali, 1998). If *Wolbachia*-based control strategies are to be attempted, a method of introducing the bacteria into this species would have to be developed. There are currently no methods developed for propagating *Wolbachia in vivo*. That fact, coupled with the very low natural prevalence of *Wolbachia* in *G. pallidipes* and the inherent difficulty of mass-rearing this fly species (Abd-Alla *et al.*, 2011), the establishment of a sufficiently large self-sustaining laboratory colony of *Wolbachia*-infected *G. pallidipes* for large scale intervention seems quite unlikely.

Another factor that may have influenced the results obtained in this study with regard to *Wolbachia* prevalence in *G. pallidipes* could be the sensitivity of the diagnostic test used. This study used the *wsp* and the 16S rRNA assays, which have both been used successfully to amplify *Wolbachia* DNA in several tsetse species (Cheng *et al.*, 2000; Doudoumis *et al.*, 2012). These assays are suitable for analysis of a large sample set as was used in the present study. However, it is probable that samples which are described as “non-infected” may often simply have low levels of the organism of interest. The detection of such low titre infections could be enhanced by the

use of highly sensitive and discriminative tools, which can detect *Wolbachia* infections in species or samples that have been deemed uninfected through standard PCR methods (Miller and Schneider, 2010). Thus, it can be postulated that *G. pallidipes* may harbour low titre *Wolbachia* infections. This would also explain why *Wolbachia* prevalence in this species using the conventional PCR protocols was reported to be very low (Doudoumis *et al.*, 2012). The density of *Wolbachia* may have a role to play in expression of CI (Ikeda *et al.*, 2003). It would be of further interest to determine if CI is expressed in *G. pallidipes* which has low titre *Wolbachia* infections.

5.4 *Sodalis* Infection in *G. austeni* and *G. pallidipes*

Secondary symbionts are generally found at intermediate frequencies within host populations (Russel and Moran, 2006). In the present study, *Sodalis* was detected in field populations of both *G. austeni* and *G. pallidipes,* with a prevalence of 3.7% and 15.9%, respectively. *Sodalis* infection in nature shows great variation in prevalence between populations and species. For example, while *G. f. fuscipes* has been shown to have undetectable *Sodalis* infections (Lindh and Lehane, 2011; Alam *et al.*, 2012), the infection rate in *G. p. palpalis* may be as high as 54.9% (Farikou *et al*., 2010). Contrastingly, low infection frequencies of 9.3% have been reported for *G. p. palpalis* in Liberia (Maudlin *et al.*, 1990; Dale and Maudlin, 1999). Variation in symbiont infection frequencies may be influenced by environmental factors, such as geographical location and climatic conditions, as well as by biological factors, such as parasitism pressure, the arthropod's host and population genetics, competition between symbionts, increased virulence and bottlenecks experienced by symbionts during vertical transmission (Mira and Moran, 2002; Hansen *et al.*, 2007; Toju and Fukatsu, 2011). This would explain the variation in infection frequencies of sympatric populations, field and laboratory populations.

5.5 Annual Variation of *Sodalis* Infection in *G. austeni* and *G. pallidipes*

In the present study, the prevalence of *Sodalis* infection in both species more than doubled in the year 2010 as compared to the previous year. Temporal variations in symbiont infection prevalence are correlated with climatic and ecological factors. In weevils, higher infections with *Sodalis*, *Wolbachia* and *Rickettsia* have been reported at localities of higher temperature (Toju and Fukatsu, 2011). *Sodalis* is also known to increase the tolerance of heat stress in pea aphids

(Montllor *et al.*, 2002). Weather data obtained from the Kenya coast indicates that the year 2010 was characterized by poor long and poor short rainy seasons (GoK, 2011). In the short rainy season, depressed rainfall was coupled with higher temperatures as the dry weather conditions extended into traditional short rainy season of October-December. The samples analyzed in this study were collected during the months of October-November. It may be deduced that during this time, the flies were exposed to higher than normal temperatures. In contrast both years 2009 and 2011 were characterized by near-normal weather conditions. We postulate that the higher temperatures experienced during that period might have contributed to an increase in the *Sodalis* prevalence in *G. austeni* and *G. pallidipes* during the year 2012.

5.6 *Wolbachia* Prevalence Based on 16S rRNA Analysis

All *Wolbachia* infections detected by *wsp* analysis were confirmed by 16S rRNA analysis. The 16S rRNA analysis revealed only one band in *G. austeni*, as opposed *G. morsitans* which produces two distinct bands. Sequence analysis of these three bands identifies them as all being of *Wolbachia* origin. The 438bp band in *G. austeni* represents cytoplasmic *Wolbachia*. Tetracycline treatment of *Wolbachia*-infected *G. morsitans* females results in loss of this band. However, the 296bp chromosomal copy present in all tissues remains detectable. The antibiotic removal of the cytoplasmic *Wolbachia*, as evidenced by use of both the 16S rRNA and *fbpA* genes provides evidence of a horizontal gene transfer event (Doudoumis *et al.*, 2012). Lateral transfer of bacterial genes into their multicellular hosts has been reported in several species including *Drosophila* and *Aedes* (Dunning Hotopp *et al.*, 2007). Lateral gene insertions are evolutionary events. The present results may therefore point to possible divergence of *G. m. morsitans* and *G. austeni* species prior to initial infection with *Wolbachia* and subsequent lateral transfer of some *Wolbachia* genes. This is supported by the postulation that *G. austeni* is a primitive or ancient tsetse species (Rogers and Robinson, 2004). An analysis of *Wolbachia* strain types infecting tsetse has shown that they represent independent acquisitions by different species (Zhou *et al.*, 1998) and that *Wolbachia* strains infecting *G. m. morsitans* are phylogenetically distant from those infecting *G. austeni* (Cheng *et al*., 2000). In contrast, the primary tsetse symbiont *Wigglesworthia* displays concordant evolution with its host signifying that the initial association with the tsetse fly was established before divergence of the tsetse species (Chen *et al.*, 1999).

The density of *Wolbachia* was apparently greater in the Kenyan samples than in the South African samples, based on band intensity. *Wolbachia* density is affected by factors including age but there are no significant differences in *Wolbachia* density in the different developmental stages of the tsetse fly (Rio *et al.*, 2006). Although the individual ages of the flies assayed in this study were not known and therefore not age-matched, it can be concluded that the density variation noted between the Kenyan and the South African flies was not due to age variations but could be presumed to be population-specific.

The bacterial density of *Wolbachia* in the host influences the level of CI expression with higher densities associated with stronger CI expresion (Clancy and Hoffmann, 1998). The *G. austeni* distribution belt covers several defined populations along the East African coast. Having noted variations in *Wolbachia* density between just two of these populations, further studies to compare more of these populations with reference to presence and level of CI expression would augmentexisting knowledge on feasibility of CI-mediated vector control interventions.

5.7 Tissue Distribution of *Wolbachia* and *Sodalis* in *G. austeni* and *G. pallidipes*

Whereas obligate mutualists display strict intracellular localization within host tissues, secondary symbionts exhibit a wider range of tissue tropism (Zouache *et al.*, 2009). Wide tissue tropism of *Wolbachia* is inferred in many insects (Dobson *et al.*, 1999). In *G. austeni*, *Wolbachia* was present in the head, thorax, carcass (including gut), ovaries and legs. The apparent bacterial density was greater in females than in males, with the former showing stronger bands on the gel. This higher density is explained by the fact that *Wolbachia* is concentrated mainly in gonad tissues, and females have more volume of the similar structures. In the current study *Sodalis* was detected in both tsetse species in the reproductive tract, the midgut and the carcass, but not in the salivary gland. Similar results were obtained by Cheng and Aksoy (1999) who detected *Sodalis* infecting *G. austeni* midgut, hemolymph and muscle but not the salivary gland. However *Sodalis* was found in the salivary gland of *G. m. morsitans*, *G. p. palpalis* and *G. brevipalpis.* These variations in tissue distribution maybe due to symbiont strain or fly species (Cheng *et al.*, 2000). The organs analyzed were obtained by dissection, which could have been a limitation in that it may have been possible for cross-contamination to take place between organs. However, minimal cross-contamination was observed, given that the present findings correspond to results

obtained from studies conducted using specific and precise localization techniques such as fluorescent *in situ* hybridization (Balmand *et al.*, 2013).

5.8 Age Structure of the Sympatric *G. austeni* and *G. pallidipes*

Age structuring by wing fray analysis revealed a younger *G. austeni* male population. In both species, females survived more than twice the number of days as did the males. However, ovarian ageing did not support the hypothesis of younger *G. austeni* population. One of the more interesting phenotypes of *Wolbachia* is the life-shortening effect of some strains on their hosts. In a laboratory study, the *w*MelPop-CLA strain was shown to halve the lifespan of *A. aegypti* (Bian *et al.*, 2010). It has been hypothesized that this *Wolbachia* strain may be similar to the one found in *G. austeni* (Aksoy, 2000). Against this background, and considering the absence of *Wolbachia* in the sympatric *G. pallidipes* population, it may also be hypothesized that *Wolbachia* infection could be one of the factors contributing to shorter lifespan in *G. austeni* males. The basis of this life-shortening effect being apparent only in males but not in females requires further study. The availability of a comparative *Wolbachia*-free line would be instrumental in confirming this observation.

5.9 Strain Variation in *G. austeni Wolbachia*

The MLST analysis of the Kenyan *G. austeni* population revealed only one strain of *Wolbachia*, which is virtually identical to the South African *G. austeni* strain (Doudoumis *et al.*, 2012). This finding is significant in relation to application of *Wolbachia*-mediated vector control interventions. *Wolbachia* infection confers various effects upon its hosts, many of them affecting reproduction (Werren, 1997). The most important phenotype CI has been demonstrated to occur in *G. morsitans* (Alam *et al.*, 2011). However, the presence of CI in *G. austeni* remains to be confirmed. The present study identified a homogenous population of *Wolbachia* in *G. austeni* from the Kenyan coast. For tsetse control applications, it would be first necessary to subject this population to laboratory adaptation and subsequently to develop a *Wolbachia*-free *G. austeni* line to confirm presence of CI. This would then provide the possibility of controlling *G. austeni* in this region through *Wolbachia*-mediated CI. Noting that *G. austeni Wolbachia* strains are homogenous in populations from Kenya and South Africa, it would be of further interest to

genotype the various tsetse populations in order to correlate host genetic diversity with *Wolbachia* diversity in the geographic area.

5.10 *Sodalis* Strain Diversity in *G. austeni* and *G. pallidipes*

Analysis of the *GPO1* sequences from different *Sodalis*- infected flies did not reveal any inter- or intra-specific sequence divergence. Previously reported phylogenetic studies which used a single locus approach also found limited inter-population and inter-specific divergence in *Sodalis* strains (Aksoy *et al.*, 1997; Weiss *et al.*, 2006; Snyder *et al.*, 2011). These findings have provided additional evidence of a recent symbiotic association between *Sodalis* and its tsetse host (Akman *et al.*, 2001).

Methods with a higher discriminative power such as amplified fragment length polymorphism (AFLP), restricted fragment length polymorphism (RFLP) and VNTR methods have been used in *Sodalis* phylogeny studies (Geiger *et al.*, 2005). The current study used an adaptation of the VNTR approach to differentiate seven *Sodalis* strains. The study was however limited by the unavailability of polyacrylamide gel electrophoresis (PAGE) facilities which would have enabled the separation of the alleles into their specific sizes. However, the sequence data obtained serves to provide complementary evidence of polymorphism. It is noteworthy that previous work using the same loci noted the repeat sequence as GCC, whereas sequence data generated in this study noted the repeat sequence to be CGG (Farikou *et al.*, 2011). This may be attributed to the different species of tsetse flies used in the two studies, with the latter study reporting on *G. p. gambiensis*. This implies that there could be variation in the *Sodalis* strains infecting the different species. Therefore, along this line, further work on a larger data set is required in order to unravel any relationships that may exist between the infecting *Sodalis* strain and trypanosome maturation.

5.11 Trypanosome Infection in Tsetse Flies from the Coastal Tsetse Belt

Tsetse flies are the main vectors of trypanosomes although biting flies have been implicated in the mechanical transmission of the parasite, especially in the case of *T. evansi* (Leak, 1999). Using the dissection method, an infection rate of 6% and 4.9% was realized in *G. austeni* and *G. pallidipes,* respectively. Using PCR, the corresponding trypanosome infection rates were 12.8% and 9.6%. The estimates of trypanosome infection in flies are often an underestimate, and often,

higher infections rates are found in livestock inhabiting the same area. For example, cattle infections around SHNR have been reported to be as high as 18% (Ohaga *et al.*, 2007). The rates observed by dissection were lower than those of PCR, indicating the higher sensitivity of PCR (Thumbi *et al.*, 2008), as well as the fact that a single infected fly may feed, and thereby infect, several hosts (Van den Abeele *et al.*, 2010). *T. congolense* was the most common infection encountered, followed by *T. vivax* and *T. simiae*. These results are consistent with those reported by Tarimo *et al*. (1984) who found *G. pallidipes* infection prevalence in the same area to average 8.7% and the order of abundance of infecting trypanosome species to be *T. congolense* (62.5%), *T. vivax* (35.5%) and *T. brucei* (2%).

In the present study, the level of trypanosome infection in *G. austeni* was consistently higher than that found in *G. pallidipes.* This result concurs with previous findings that in areas of mixed tsetse infestation, despite lower abundance of *G. austeni* relative to other sympatric species, *G. austeni* is often found with higher levels of trypanosome infection (Motloang *et al.*, 2012).While this may signify a higher vectorial capacity in *G. austeni* than in its sympatric species, account has to be taken of the multiple undefined factors affecting field situations. A controlled experimental study comparing the ability of *G. austeni* and *G. pallidipes* to pick up and establish trypanosomes infections would suffice to increase our understanding of transmission dynamics in these two species.

It was also notable that very few multiple trypanosome infections were found in both fly species. This is in contrast to results obtained from Rufiji district along the Tanzanian coast whereby more than half of all *G. pallidipes* and *G. austeni* flies analyzed were infected with between two and five different species of trypanosomes (Malele *et al.*, 2011). It is inferred that the presence of a wide spectrum of potential tsetse hosts, both domestic and wild animals as well as frequent feeding upon these hosts may contribute to an abundance of mixed trypanosome infections (Simo *et al.*, 2012). However, in both countries, samples were collected in or around wildlife game reserves having a wide variety of wild animals. Therefore the difference in prevalence of mixed infections cannot be wholly attributed to host variety and may be a factor of the relative abundance of trypanosomes circulating in each given area.

5.12 *Sodalis* and Trypanosome Co-infection in Field Samples

The presence of *Sodalis* has been postulated to increase the susceptibility of tsetse fly to trypanosomes infection (Geiger *et al.*, 2007). However, in the present study, the proportion of *Sodalis*-infected flies with a concurrent trypanosome infection was lower than the proportion without the trypanosome infection. In part, this can be attributed to the relatively small number of co-infections detected. The low co-infection rate obtained in this study is in contrast with the rates of 32.2% reported in *G. p. palpalis* (Farikou *et al.*, 2010). It is however worth noting that in both the present study and the *G. p. palpalis* study, the trypanosomes species infecting *Sodalis*-positive flies belong to the sub-groups *Trypanozoon* and *Nannomonas*. These two sub-groups have a similar development cycle whereby maturation takes place in the midgut (Leak, 1999). The synergistic effect of *Sodalis* on trypanosome establishment and maturation applies to trypanosomes species that pass through a midgut stage in fly and therefore includes *T. congolense, T. brucei* and *T. simiae* but excludes *T. vivax* (Welburn *et al.*, 1993). The positive co-relation between the presence of *Sodalis* and trypanosome infection evident in *G. pallidipes* is consistent with previously reported findings which postulate that the presence of *Sodalis* supports the infection of the fly with various trypanosomes (Farikou *et al.*, 2010). It would therefore seem that in nature, co-infection is dependent upon the specific identity of both fly and trypanosome.

5.13 *Sodalis* and Trypanosome Co-infection in Experimentally Challenged *G. pallidipes*

A significant association was observed between the presence of *Sodalis* and the establishment of infection with *T. b. rhodesiense* and *T. congolense* where more than 75% of *Sodalis*-infected flies established infections with these trypanosome species. In contrast, only 45.7% of *Sodalis*-infected flies established a *T. b. brucei* infection. Previous workers have found that an existing *Sodalis* infection strongly favours the establishment of *T. b. gambiense* and *T. b. brucei* in *G. p. palpalis* (Geiger *et al.*, 2007). These divergent results indicate that the inter-action between *Sodalis* infection and parasitism appears to vary depending on the species of infecting trypanosome and therefore highlights the considerable influence of vector-trypanosome species pairings on successful establishment of infection (Moloo *et al.*, 1992).

5.14 Effect of Ampicillin on *G. pallidipes* Survival, Symbiont Clearance and Trypanosome Establishment

The ampicillin-fed *G. pallidipes* male flies experienced higher mortality than untreated controls. This finding concurs with previous work using female *G. m. morsitans* whereby a slight reduction in survival of antibiotic-treated flies was observed as compared to untreated controls This decreased longevity was attributed to inefficient digestion of the bloodmeal in the fly midgut (Pais *et al.*, 2008). The present finding that *Sodalis* is cleared from the midgut within about 5-7 days of ampicillin feeds, agree with previous studies which documented that continuous exposure of *G. pallidipes* to ampicillin-treated blood caused a partial decrease in the *Sodalis* titres (Pais *et al.*, 2008). Dissection did not reveal any infections in either the *T. b. brucei* or *T. congolense* group. Control groups infected simultaneously but which had not been treated with ampicillin had higher infection rates of 3.0% and 8.0%, respectively. Although the number of trypanosome infections that developed was very few, these results indicate that *G. pallidipes* flies pre-treated with ampicillin could become refractory to trypanosome infection. The reasons for this could be two-fold. The administration of an ampicillin-spiked diet to tsetse flies results in the elimination of extracellular bacteria in the flies, including *Sodalis* and *Wigglesworthia* from the midgut. The elimination of *Wigglesworthia* in *G. m. morsitans* increases susceptibility to infection (Pais *et al.*, 2008). However, in this study, we were unable to amplify *Wigglesworthia thiC* using the primers used to amplify this gene in other tsetse species such as *G. m. morsitans* (Alam *et al.*, 2012). Therefore, it may also be possible that although the *Sodalis* infection was cleared, *Wigglesworthia* was not completely eliminated and may have contributed to the apparent refractoriness observed. Another contributing factor may have been the age of the flies at the infective feed. In tsetse flies, it is the presence of a bloodmeal which stimulates secretion of the peritrophic membrane (PM) (Leak, 1999). In teneral flies which have not had a single bloodmeal, the immature state of the PM and immature immune responses, among other factors, contribute to the "teneral phenomenon" which renders newly emerged flies to be most susceptible to infection. Thus, the timing of the infective bloodmeal affects the establishment of trypanosomes in the tsetse midgut and the possibility of establishing an infection decreases with the number of bloodmeals that the fly has consumed (Walshe *et al.*, 2011). In the laboratory, susceptibility of tsetse flies to *T. congolense* and *T. brucei* infections typically falls rapidly from as high as 70% to less than 10% following the third bloodmeal (Welburn and Maudlin, 1992).

Therefore, the fact that the infective feed was offered to “old” flies which had already fed three times prior to infective feed, may have caused this result. Further work to develop a *Sodalis*-free line that would then be infected as tenerals would help to resolve this issue. In addition, availability of alternative primers for the detection of *Wigglesworthia* in *G. pallidipes* will permit further analysis.

CHAPTER SIX

CONCLUSIONS AND RECOMENDATIONS

6.1 Conclusions

This study sought to characterize tsetse-endosymbiont interactions in *G. austeni* and *G. pallidipes* populations from the Kenya coast. Specifically, it determined the prevalence, tissue distribution, temporal and strain variations of *Sodalis* and *Wolbachia* in *Glossina austeni* and *G. pallidipes.* Further, the study examined the co-relation between trypanosome and symbiont infection in naturally and experimentally-infected flies, as well as the effect of ampicillin on *G. pallidipes* survival, symbiont clearance and trypanosome establishment.

The prevalence, tissue distribution and temporal variations of *Wolbachia* and *Sodalis* endosymbionts in *Glossina austeni* and *G. pallidipes* were determined. The *Wolbachia* infection frequency was observed to be 100% in *G. austeni*, but absent or undetectable in the *G. pallidipes* populations studied, including the TRC laboratory population. *Sodalis* prevalence was generally low in both species, but higher in laboratory than in field populations. Localization of symbionts influences their inter-specific interactions, in particular with trypanosomes. In this study, localization of both *Sodalis* and *Wolbachia* in the gut was confirmed. This is also the organ of trypanosome maturation and therefore a strong co-existence, and possibly interaction, is to be expected. The absence of *Sodalis* in the salivary gland of *G. austeni* was significant, as the symbiont was present in the same organ in other *Glossina* species. Over the 3-year study period, the infection frequency of *Sodalis* was observed to co-related with the average temperature.

The variation of symbiont strains from different geographical populations was determined. For *Wolbachia*, *wsp* strain homogeneity was observed in *G. austeni* populations from Kenya (Shimba, Arabuko-Sokoke) and South Africa. Using MLST, sequence variation was observed in the *coxA* locus, sufficient to differentiate the *Wolbachia* strains originating from these two countries. For *Sodalis*, multiple VNTR strain variants were identified in both *G. austeni* and *G. pallidipes*. These strains were however, not differentiated by species, geographical origin or trypanosome infection status. Therefore, the present study was unable to draw any relationship between specific *Sodalis* strains and susceptibility to trypanosome infection.

The co-relation between symbiont infection and trypanosome establishment/ maturation was investigated. This study revealed a low rate of *Sodalis*-trypanosome co-infection under natural conditions. However, in experimental studies, *Sodalis*-trypanosome association was found to be significant in *T. b. rhodesiense and T. congolense* infections but not for *T. b. brucei.*

The study also determined the effect of administration of ampicillin on *G. pallidipes* survival, symbiont clearance and trypanosome establishment. It was observed that ampicillin-spiked blood caused reduced longevity in *G. pallidipes*. Clearance of *Sodalis* bacteria from tsetse midgut was achieved with continuous administration of three antibiotic feeds. Further, flies that had fed on antibiotic blood demonstrated reduced susceptibility to infection with *T. b. brucei* and *T. congolense*.

6.2 Recommendations

This study revealed several opportunities for further research into tsetse-symbiont interactions. The apparent absence of *Wolbachia* infection in *G. pallidipes* requires further analysis for verification. Therefore, it is recommended that diagnostic tools/methods with higher sensitivity be applied to verify the absence of *Wolbachia* in *G. pallidipes.*

Some tsetse individuals may harbor all the three endosymbionts, thus compounding cross-interactions between the symbionts themselves and between tsetse and trypanosomes. It is recommended that future work be carried out to develop aposymbiotic *G. pallidipes* lines which will allow independent determination of the impact of specific symbionts on acquisition of trypanosome infection.

The presence and level of CI in *G. austeni* still remains unknown. The present study recommends that to explore the possibility of using *Wolbachia*-mediated CI for the control of *G. austeni*, a *Wolbachia*-free line be developed and cross-mating experiments carried out.

The correlation between symbiont status and trypanosome infection may be determined by the vectorial capacity of the tsetse species involved. In order to fully unravel this relationship, it is recommended that laboratory-based studies be conducted to confirm the observed differences in vectorial capacity of *G. austeni* and *G. pallidipes* in mixed populations.

REFERENCES

Abd-Alla, A.M.M., Parker, A.G., Vreysen, M.J.B. and Bergoin, M. (2011). Tsetse salivary gland hypertrophy virus: Hope or hindrance for tsetse control? *PLoS Neglected Tropical Diseases*, **5**: e1220.

Akman, L., Rio, R., Beard, C. and Aksoy, S. (2001). Genome size determination and coding capacity of *Sodalis glossinidius*, an enteric symbiont of tsetse flies, as revealed by hybridization to *Escherichia coli* gene arrays. *Journal of Bacteriology*, **183**: 4517-4525.

Aksoy, S. (1995). *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidae* sp. nov., taxa consisting of the mycetocyte-associated primary endosymbionts of tsetse flies. *International Journal of Systematic Bacteriology*, **45**: 848-851.

Aksoy, S. (2000). Tsetse: A haven for microorganisms. *Parasitology Today*, **16**: 114-118.

Aksoy, S., Chen, X. and Hyspa, V. (1997). Phylogeny and potential transmission routes of midgut-associated endosymbionts of tsetse (Diptera: Glossinidae). *Insect Molecular Biology*, **6**: 183-190.

Aksoy, S., Maudlin, I., Dale, C., Robinson, A.S. and O´Neill, S.L. (2001). Prospects for control of African trypanosomiasis by tsetse vector manipulation. *Trends in Parasitology*, **17**: 29-35.

Aksoy, S., Weiss, B. and Attardo, G. (2008). Paratransgenesis applied for control of tsetse transmitted sleeping sickness. In Aksoy, S. (Ed) *Transgenesis and the management of vector-borne disease*. Landes Bioscience Springer Science, pp 35-45.

Alam, U., Hyseni, C., Symula, R.E., Brelsfoard, C., Wu, Y., Kruglov, O., Wang, J., Echodu, R., Alioni, V., Okedi, L.M., Caccone, A. and Aksoy, S. (2012). Microfauna-host interactions: Implications for trypanosome transmission dynamics in *Glossina fuscipes fuscipes* in Uganda. *Applied Environmental Microbiology*, **78**: 4627-4637.

Alam, U., Medlock, J., Brelsfoard, C., Pais, R., Lohs, C., Balmand, S., Carnogursky, J., Heddi, A., Takac, P., Galvani, A. and Aksoy, S. (2011). *Wolbachia* symbiont infections induce strong cytoplasmic incompatibility in the tsetse fly *Glossina morsitans. PLoS Pathogens*, **7**: e1002415.

Albuquerque, A.L., Magalhães, T. and Ayres, C.F. (2011). High prevalence and lack of diversity of *Wolbachia pipientis* in *Aedes albopictus* populations from Northeast Brazil. *Memorias do Institudo Oswaldo Cruz*, **106**: 773-776.

Allsopp, R. (2001) Options for vector control against trypanosomosis in Africa. *Trends in Parasitology*, **17**: 15-19.

Allsopp, R., Bauer, B., Oloo, F., Feldmann, H. and Mattioli, R. (2010). Tools and Strategies for Area-wide Tsetse Fly Suppresion. *PAAT Information Service*. pp 41.

Anderson, N.E., Mubanga, J., Fevre, E.M., Picozzi, K., Eisler, M.C., Thomas, R. and Welburn, S. (2011) Characterisation of the wildlife reservoir community for human and animal trypanosomiasis in the Luangwa Valley, Zambia. *PLoS Neglected Tropical Diseases*, **5**: e1211.

Arthofer, W., Riegler, M., Avtzis, D.N. and Stauffer, C. (2009a). Evidence for low-titre infections in insect symbiosis: *Wolbachia* in the bark beetle *Pityogenes chalcographus* (Coleoptera, S*colytinae*). *Environmental Microbiology*, **11**: 1923-1933.

Arthofer, W., Riegler, M., Schneider, D., Krammer, M. and Miller, W.J. (2009b). Hidden *Wolbachia* diversity in field populations of the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). *Molecular Ecology*, **18**: 3816-3830.

Baldo, L., Dunning Hotopp, J.C., Jolley, K.A., Bordenstein, S.R., Biber, S.A., Choudhury, R.R., Hayashi, C., Maiden, M.C.J., Tettelin, H. and J.H, Werren. (2006). Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis Applied and Environmental Microbiology*, **72**: 7098-7110.

Balmand, S., Lohs, C., Aksoy, S. and Heddi, A. (2013). Tissue distribution and transmission routes for the tsetse fly endosymbionts. *Journal of Invertebrate Pathology*, **112**: 116-122.

Barclay, H. (2005). Mathematical models for the use of sterile insects. In Dyck, V.A., Hendrichs, J. and Robinson, A.S. (Eds), *Sterile Insect Technique, Principles and Practice in Area-Wide Integrated Pest Management*. pp 121-156.

Barrett, K. and Okali, C. (1998). Partnerships for tsetse control: Community participation and other options. *World Animal Review (FAO)*, **90**: 39-46.

Beard, C., O´Neill, S., Tesh, R., Richards, F. and Aksoy, S. (1993). Modification of arthropod vector competence via symbiotic bacteria. *Parasitology Today*, **9**: 179-183.

Beard, C.B., Cordon-Rosales, C. and Durvasula, R.V. (2002). Bacterial symbionts of the Triatomine and their potential use in control of Chagas disease transmission. *Annual Review of Entomology*, **47**: 123-141.

Beerntsen, B.T., James, A.A. and Christensen, B.M. (2000). Genetics of mosquito vector competence. *Microbiology and Molecular Biology Reviews*, **64**: 115-137.

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acid Research*, **27**: 573-580.

Bian, G., Lu, P., Xie, Y. and Xi, Z. (2010). The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS Pathogens*, **6**: e1000833.

Blagrove, M.S.C., Arias-Goeta, C., Failloux, A.B. and Sinkins, S.P. (2011). *Wolbachia* strain *w*Mel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. *Proceedings of the National Academy of Science USA*, **109**: 255-260.

Bonomi, A., Bassetti, F., Gabrieli, P., Beadell, J., Falchetto, M., Beadell, J., Scolari, F., Gomulski, L.M., Regazzini, E., Ouma, J.O., Caccone, A., Okedi, L.M., Attardo, G., Guglielmino, C., Aksoy, S. and Malacrida, A. (2011). Polyandry is a common event in wild populations of the tsetse fly *Glossina fuscipes fuscipes* and may impact population reduction measures. *PLoS Neglected Tropical Diseases*, **5**: e1190.

Bourn, D., Reid, R., Rogers, D., Snow, B. and Wint, W. (2001). *Environmental changes and the autonomous control of tsetse and trypanosomosis in sub-Saharan Africa: Case histories from Ethiopia, The Gambia, Kenya and Zimbabwe*. Information Press, Oxford, UK.

Braig, H.R., Zhou, W., Dobson, S. and O´Neill, S. (1998). Cloning and characterisation of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *Journal of Bacteriology*, **180**: 2373-2378.

Brelsfoard, C.L. and Dobson, S.L. (2011). An update on the utility of *Wolbachia* for controlling insect vectors and disease transmission. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, **19**: 85-92.

Brightwell, R., Dransfield, R.D., Kyorku, C., Golder, T.K., Tarimo, S.A. and Mungai, D. (1987). A new trap for *Glossina pallidipes*. *Tropical Pest Management*, **33**: 151-189.

Brownstein, J.S., Hett, E. and O'Neill, S.L. (2003). The potential of virulent *Wolbachia* to modulate disease transmission by insects. *Journal of Invertebrate Pathology*, **84**: 24-29.

Bruce, D. (1895). Preliminary report on the tsetse fly disease or Nagana in Zululand. In Bennett and Davis (Eds). Durban, pp. 28.

Casiraghi, M., Bordenstein, S.R., Baldo, L., Lo, N., Beninati, T., Wernegreen, J.J., Werren, J.H. and Bandi, C. (2005). Phylogeny of *Wolbachia pipientis* based on *gltA*, *groEL* and *ftsZ*

gene sequences: Clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the *Wolbachia* tree. *Microbiology and Molecular Biology Reviews*, **151**: 4015-4022.

Castellani, A. (1903). On the discovery of a species of *Trypanosoma* in the cerebro-spinal fluid of cases of sleeping sickness. *Proeedings of the Royal Society of London*, **71**: 501-508.

Cattand, P., Simarro, P., Jannin, J., Ly, C., Fall, A., Shaw, A. and Mattioli, R. (2009). *Linking sustainable human and animal African trypanosomiasis control with rural development strategies*. PAAT Technical and Scientific Series. Rome.

Chen, X., Li, S. and Aksoy, S. (1999). Concordant evolution of a symbiont with its host insect species: Molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. *Journal of Molecular Evolution*, **48**: 49-58.

Cheng, Q. and Aksoy, S. (1999). Tissue tropism, transmission and expression of foreign genes *in vivo* in midgut symbionts of tsetse flies. *Insect Molecular Biology*, **8**: 125-132.

Cheng, Q., Ruel, T.D., Zhou, W., Moloo, S.K., Majiwa, P., O'Neill, S.L. and Aksoy, S. (2000). Tissue distribution and prevalence of *Wolbachia* infections in tsetse flies, *Glossina* spp. *Medical Veterinary Entomology*, **14**: 44-50.

Clancy, D.J. and Hoffmann, A.A. (1998). Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia*-infected *Drosophila simulans*. *Entomologia Experimentalis et Applicata*, **86**: 13-24.

Clerinx, J., Vlieghe, E., Asselman, V., Van de Casteele, S., Maes, M. and V, Legon. (2012). Human African trypanosomiasis in a Belgian traveller returning from the Masai Mara area, Kenya. *Euro Surveillance*, **17**: 12-14.

Cox, F.E.G. (2004). History of sleeping sickness (African trypanosomiasis). *Infectious Disease Clinics of North America*, **18**: 231-245.

d'Ieteren, G.D., Authié, E., Wissocq, N. and Murray, M. (1998). Trypanotolerance, an option for sustainable livestock production in areas at risk from trypanosomosis. *Revue Scientifique et Technique de l`Office International des Epizootics*, **17**: 154-175.

Dale, C. and Maudlin, I. (1999). *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. *International Journal of Systematic Bacteriology*, **49**: 267-275.

De La Rocque, S., Augusseau, X., Guillobez, S., Michel, V., De Wispelaere, G., Bauer, B. and Cuisance, D. (2001). The changing distribution of two riverine tsetse flies over 15 years in an increasingly cultivated area of Burkina Faso. *Bulletin of Entomological Research*, **91**: 157-166.

Desquenes, M., McLaughlin, G., Zoungrana, A. and Davila, A.M. (2001). Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. *International Journal of Parasitology*, **31**: 610-614.

Desquesnes, H. and Dia, M. (2004). Mechanical transmission of *Trypanosoma vivax* in cattle by African tabanids *Alylotus fuscipes*. *Veterinary Parasitology*, **119**: 9-19.

Dobson, S., Bourtzis, K., Braig, H.R., Jones, B., Zhou, W., Rousset, F. and O´Neill, S.L. (1999). *Wolbachia* infections are distributed througout insect somatic and germ line tissues. *Insect Biochemistry and Molecular Biology*, **29**: 153-160.

Doudoumis, V., Tsiamis, G., Wamwiri, F., Brelsfoard, C., Alam, U., Aksoy, E., Dalaperas, S., Abd-Alla, A.M.M., Ouma, J., Takac, P., Aksoy, S. and Bourtzis, K. (2012). Detection and characterization of *Wolbachia* infections in laboratory and natural populations of different species of tsetse flies (genus *Glossina*). *BMC Microbiology*, **12**, S3

Dunning Hotopp, J.C., Clark, M.E., Oliveira, D.C., Foster, J.M., Fisher, P., Torres, C., Giebel, J.D., Kumar, N., Ishmael, N., Wang, S., Ingram, J., Nene, R.V., Shepard, J., Tomkins, J., Richards, S., Spiro, D., David J. , Ghedin, E., Slatko, B.E., Tettelin, H. and Werren, J.H. (2007). Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. *Science*, **317**: 1753-1756.

Dutton, J.E. (1902). Preliminary note upon a trypanosome occurring in the blood of man. *Thompson Yates Laboratory Report*, **4**: 455-468.

Etchegorry, M., Helenport, J., Pecoul, B., Jannin, J. and Legros, D. (2001). Availability and affordability of treatment for human African trypanosomosis. *Tropical Medicine and International Health*, **6**: 957-959.

Farikou, O., Njiokou, F., Cuny, G. and Geiger, A. (2011). Microsatellite genotyping reveals diversity within populations of *Sodalis glossinidius*, the secondary symbiont of tsetse flies. *Veterinary Microbiology*, **150**: 207-210.

Farikou, O., Njiokou, F., Mbida, J.A.M., Njitchouang, G.R., Djeunga, H.N., Asonganyi, T., Simarro, P.P., Cuny, G. and Geiger, A. (2010). Tripartite interactions between tsetse flies, *Sodalis glossinidius* and trypanosomes - An epidemiological approach in two historical human African trypanosomiasis foci in Cameroon. *Infections Genetics and Evolution,* **10**: 115-121.

Feldmann, H.U. (1994). Guidelines for the rearing of tsetse flies using the membrane feeding technique. In Ochieng'-Odero, J.P.R. (Ed), *Techniques for the Development of Intergrated Pest and Vector Management Strategies*. ICIPE Science Press, Nairobi, Kenya, pp. 449-471.

Fevre, E., Magona, J., Coleman, P., Woolhouse, M. and Welburn, S. (2003). Livestock demography and the risk of spread of *T. b. rhodesiense* in Uganda: Implications for policy. *Integrated Control of Pathogenic Trypanosomes and their Vectors*, **8**: 21-22.

Fondon, J.W. and Garner, H.R. (2004). Molecular origins of rapid and continous morphological evolution. *Proceedings of National Academy of Sciences USA*, **101**: 18058-18063.

Ford, L. (2007). Civil conflict and sleeping sickness in Africa in general and Uganda. *Conflict and Health*, **1**: 6.

Forde, R.M. (1902). Some clinical notes on a European patient in whose blood a trypanosome was observed. *Journal of Tropical Medicine*, **5**: 261-263.

Geiger, A., Cuny, G. and Frutos, R. (2005). Two tsetse fly species, *Glossina palpalis gambiensis* and *Glossina morsitans morsitans*, carry genetically distinct populations of the secondary symbiont *Sodalis glossinidius*. *Applied and Environmental Microbiology*, **71**: 8941-8943.

Geiger, A., Fardeau, M., Njiokou, F. and Ollivier, B. (2013). *Glossina* spp. gut bacterial flora and their putative role in fly-hosted trypanosome development. *Frontiers in Cellular and Infection Microbiology*, **3:** 34

Geiger, A., Ravel, S., Mateille, T., Janelle, J., Patrel, D., Cuny, G. and Frutos, R. (2007). Vector competence of *Glossina palpalis gambiensis* for *Trypanosoma brucei s.l.* and genetic diversity of the symbiont *Sodalis glossinidius*. *Molecular and Biological Evolution*, **24**: 102-109.

Gibson, W., Backhouse, T. and Griffiths, A. (2002). The human serum resistance associated gene is ubiquitous and conserved in *Trypanosoma brucei rhodesiense* throughout East Africa. *Infection Genetics and Evolution*, **1**: 207-214.

GoK (2011). The 2010 Short Rains Season Assessment Report. Government of Kenya, Nairobi, pp. 40.

Haines, L.R., Hancock, R.E.W. and Pearson, T.W. (2003). Cationic antimicrobial peptide killing of African trypanosomes and *Sodalis glossinidius*, a bacterial symbiont of the insect vector of sleeping sickness. *Vector-Borne and Zoonotic Diseases*, **3**: 175-186.

Hancock, P.A. and Godfray, H.C.J. (2012). Modelling the spread of *Wolbachia* in spatially heterogeneous environments. *Journal of The Royal Society Interface*, **9**:3045-3054.

Hansen, A.K., Jeong, G., Paine, T.D. and Stouthamer, R. (2007). Frequency of secondary symbiont infection in an invasive psyllid relates to parasitism pressure on a geographic scale in California. *Applied Environmental Microbiology*, **73**: 7531-7535.

Hargrove, J.W. (2003). *Tsetse eradication: sufficiency, necessity and desirability*. Research Report, DFID Animal Health Programme. University of Edinburgh, UK.

Hendrichs, J., Kenmore, P., Robinson, A. and Vreysen, M. (2007). Area-wide integrated pest management (AW-IPM): Principles, practice and prospects. In Vreysen, M.J.B., Robinson, A.S. and Hendrichs J., (Eds) *Area-wide Control of Insect Pests: From Research to Field Implementation*. Springer, Dordrecht, The Netherlands. pp 3-33.

Herbert, W.J. and Lumsden, W.H. (1976). *Trypanosoma brucei*: a rapid "matching" method for estimating the host's parasitemia. *Experimental Parasitology*, **40**: 427-431.

Hernández, P., Martín-Parras, L., Martínez-Robles, M.L. and Schvartzman, J.B. (1993). Conserved features in the mode of replication of eukaryotic ribosomal RNA genes. *The EMBO Journal*, **12**: 1475-1485.

Hoffmann, A.A. and Turelli, M. (1997). Cytoplasmic incompatibility in insects. In O'Neill, S.L., Hoffmann, A.A. and Werren, J.H. (Eds), *Influential passengers: Inherited Microorganisms and Arthropod Reproduction*. Oxford University Press. pp 42-80.

Hotez, P.J. (2008). *Forgotten people, forgotten diseases: The Neglected Tropical Diseases and their Impact on Global Health and Development*. ASM Press. pp 215

Hurwitz, I., Hillesland, H., Fieck A, Das, P. and Durvasula, R. (2011). The paratransgenic sand fly: a platform for control of Leishmania transmission. *Parasites and Vectors*, **19**: 82.

Ikeda, T., Ishikawa, H. and Sasaki, T. (2003). Infection density of *Wolbachia* and level of cytoplasmic incompatibility in the Mediterranean flour moth, *Ephestia kuehniella*. *Journal of Invertebrate Pathology*, **84**: 1-5.

Jackson, C.H.N. (1946). An artificially isolated generation of tsetse flies (*Diptera*). *Bulletin of Entomological Research*, **37**: 291-299.

KETRI (1997). Proceedings of the national workshop to revise the tsetse distribution map of Kenya. KETRI, Muguga. pp 32.

Kgori, P.M., Modo, S. and Torr, S.J. (2006). The use of aerial spraying to eliminate tsetse from the Okavango Delta of Botswana. *Acta Tropica*, **99**: 184-199.

Knipling, E.F. (1955). Possibilities of insect control through the use of sexually sterile males. *Journal of Economic Entomology*, **48**: 459-462.

Knipling, E.F. (1979). *The basic principles of Insect Population Suppression and Management*. US Department of Agriculture, Washington DC. pp 659.

Kristjanson, P., Swallow, B., Rowlands, G., Kruska, R. and De Leeuw, P. (1999). Measuring the costs of African animal trypanosomosis: The potential benefits of controls and returns to research. *Agricultural Systems*, **59**: 79-98.

Laven, H. (1967). Eradication of *Culex pipiens fatigans* through Cytoplasmic Incompatibility. *Nature*, **216**: 383 - 384.

Leak, S.G.A. (1999). *Tsetse Biology and Ecology: Their Role in the Epidemiology and Control of Trypanosomosis*. CABI, UK. pp 568.

Lee, S.H., Stephens, J.L. and Englund, P.T. (2007). A fatty-acid synthesis mechanism specialized for parasitism. *Nature Reviews Microbiology*, **5**: 287-297.

Legros, D., Fournier, C., Gastellu, Etchegorry, G.F., Maiso, F. and Szumilin, E. (1999). Therapeutic failure of melarsoprol among patients treated for late stage *T. b. gambiense* human African trypanosomosis in Uganda. *Bulletin de la Societe de Pathologie Exotique*, **92**: 171-172.

Lindh, J.M. and Lehane, M.J. (2011). The tsetse fly *Glossina fuscipes fuscipes* (*Diptera: Glossina*) harbours a surprising diversity of bacteria other than symbionts. *Antonie van Leeuwenhoek*, **99**: 711-720.

Lloyd, L. and Johnson, W.B. (1924). The trypanosome infections of tsetse flies in Northern Nigeria and a new method of estimation. *Bulletin of Entomological Research*, **14**: 265-288.

Lo, N., Casiraghi, M., Salati, E., Bazzocchi, C. and Bandi, C. (2007). How many groups of *Wolbachia* supergroups exist? *Molecular and Biological Evolution*, **19**: 341-346.

Malele, I.I., Magwisha, H.B., Nyingilili, H.S., Mamiro, K.A. and Rukambile, E.J. (2011). Multiple *Trypanosoma* infections are common amongst *Glossina* species in the new farming areas of Rufiji district, Tanzania. *Parasites and Vectors*, **4**: 217.

Masiga, D.K., Smyth, A.J., Hayes, P., Bromidge, T.J. and Gibson, W.C. (1992). Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *International Journal of Parasitology*, **22**: 909-918.

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

McMeniman, C.J., Lane, R.V., Cass, B.N., Fong, A.W., Sidhu, M., Wang, Y.F. and O'Neill, S.L. (2009). Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science*, **323**: 141-144.

Miller, W.J., Ehrman, L. and Schneider, D. (2010). Symbiotic speciation re-visited: Impact of symbiont-depletion on female fitness and mating behaviour of *Drosophila paulistorum* spp. *PLoS Pathogens*, **6**: e1001214.

Min, K.T. and Benzer, S. (1997). *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and death. *Proceedings of the National Academy of Sciences USA*, **94**: 10792-10796.

Mira, A. and Moran, N.A. (2002). Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. *Microbial Ecology*, **44**: 137-143.

Moloo, S., Sabwa, C. and Kabata, J. (1992). Vector competence of *Glossina pallidipes* and *G. morsitans centralis* for *Trypanosoma vivax*, *T. congolense* and *T. b. brucei*. *Acta Tropica*, **51**: 271-280.

Montllor, C.B., Maxmen, A. and Purcell, A.H. (2002). Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum*, under heat stress. *Ecological Entomology*, **27**: 189-195.

Moreira, L.A., Iturbe-Ormaetxe, I., Jeffery, J.A., Lu, G., Pyke, A.T., Hedges, L.M., Bruno, R., Hall-Mendelin, S., Day, A., Riegler, M., Hugo, L.E., Johnson, K.N., Kay, B.H., McGraw, E.A., van de Hurk, A.F., Ryan, P. and O´Neill, S.L. (2009). A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya and *Plasmodium*. *Cell*, **139**: 1268-1278.

Moser, D.R., Cook, G.A., Ochs, D.E., Bailey, C.P., McKane, M.R. and Donelson, J.E. (1989). Detection of *Trypanosoma congolense* and *Trypanosooma brucei* subspecies by DNA amplification using the polymerase chain reaction. *Parasitology*, **99**: 57-66.

Motloang, M., Masumu, J., Mans, B., Van den Bossche, P. and Latif, A. (2012). Vector competence of *Glossina austeni* and *Glossina brevipalpis* for *Trypanosoma congolense* in KwaZulu-Natal, South Africa. *Onderstepoort Journal of Veterinary Research*, **79**: e1-6.

Njiru, Z.K., Constantine, C.C., Guya, S., Crowther, J., Kiragu, J.M., Thompson, R.C. and Dávila, A.M. (2005). The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. *Parasitological Research*, **95**: 186-192.

Nogge, G. (1976). Sterility in tsetse flies *Glossina morsitans* Westwood caused by loss of symbionts. *Experientia*, **32**: 995-996.

Nogge, G. (1981). Significance of symbionts for the maintenance of an optimal nutritional state for successful reproduction in haematophagous arthropods. *Parasitology Today*, **82**: 101-104.

O'Neill, S.L., Gooding, R.H. and Aksoy, S. (1993). Phylogenetically distant symbiotic microorganisms reside in *Glossina* midgut and ovary tissues. *Medical and Veterinary Entomology*, **7**: 377-383.

O'Neill, S.L., Giordano, R., Colbert, A.M.E., Karr, T.L. and Robertson, H.M. (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibity in insects. *Proceedings of the National Academy of Science USA*, **89**: 2699-2702.

Ohaga, S.O., Kokwaro, E.D., Ndiege, I.O., Hassanali, A. and Saini, R.K. (2007). Livestock farmers' perception and epidemiology of bovine trypanosomiasis in Kwale District, Kenya. *Preventative Veterinary Medicine*, **80**: 24-33.

Okiria, R., Okuna, N.M., Magona, J.W. and Mayende, J.S. (2002). Sustainability of tsetse control by subsequent treatment of 10% of a previously treated Ugandan cattle population with 1% (w/v) deltamethrin. *Tropical Animal Health and Production*, **34**: 105-114.

Opiyo, E.A., Njogu, A.R. and Omuse, J.K. (1990). Use of impregnated targets for control of *Glossina pallidipes* in Kenya. *International Journal of Tropical Insect Science*, **11**: 417-425.

PAAT (2008). *On Target Against Poverty - The Programme Against African Trypanosomiasis 1997-2007*. PAAT Information Service Rome.

Pais, R., Lohs, C., Wu, Y., Wang, J. and Aksoy, S. (2008). The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. *Applied and Environmental Microbiology*, **74**: 5965-5974.

Paraskevopoulos, C., Bordenstein, S.R., Wernegreen, J.J., Werren, J.H. and Bourtzis, K. (2006). Toward a *Wolbachia* multilocus sequence typing system: Discrimination of *Wolbachia* strains present in *Drosophila* species. *Current Microbiology*, **53**: 388-395.

Poinsot, D., Bourtzis, K., Markakis, G., Savakis, C. and Merçot, H. (1998). *Wolbachia* transfer from *Drosophila melanogaster* into *D. simulans*: Host effect and cytoplasmic incompatibility relationships. *Genetics*, **150**: 227-237.

Pourcel, C., André-Mazeaud, F., Neubauer, H., Ramisse, F. and G, V. (2004). Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. *BMC Microbiology*, **4**: 33.

Rasgon, J.L. and Scott, T.W. (2003). *Wolbachia* and cytoplasmic incompatibility in the California *Culex pipiens* mosquito species complex: Parameter estimates and infection dynamics in natural populations. *Genetics*, **165**: 2029-2038.

Rendón, P., McInnis, D., Lance, D. and Stewart, J. (2004). Medfly (Diptera: Tephritidae) genetic sexing: Large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *Journal of Economic Entomology*, **97**: 1547-1553.

Riegler, M., Sidhu, M., Miller, W.J. and O´Neill, S.L. (2005). Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. *Current Microbiology*, **15**: 1428-1433.

Rio, R.V., Wu, Y.N., Filardo, G. and Aksoy, S. (2006). Dynamics of multiple symbiont density regulation during host development: Tsetse fly and its microbial flora. *Proceedings of the Royal Society of London B Biological Sciences*, **273**: 805-814.

Roberts, L.S. and Janovy, I. (2000). Kinetoplastida: Trypanosomes and their kin. In Schmidt, G.D. and Roberts, L.S. (Eds), *Foundations of Parasitology* McGraw-Hill, pp. 670.

Rogers, D.J. and Robinson, T.P. (2004). Tsetse Distribution. In Maudlin, I., Holmes, P. and Miles, M. (Eds), *The Trypanosomiases*. CAB International, London, UK. pp 139-180.

Rousset, F., Bouchon, D., Pintureau, B., Juchault, P. and Solignac, M. (1992). *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proceedings of the Royal Society of London*, **250**: 91-98.

Russel, J.A. and Moran, N.A. (2006). Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proceedings of the Royal Society of London*, **273**: 603-610.

Rutto, J. and Karuga, J. (2009). Temporal and spatial epidemiology of sleeping sickness and use of geographical information system (GIS) in Kenya. *Journal of Vector Borne Diseases*, **46**: 8-25.

Saunders, D.S. (1960). The ovulation cycle in *Glossina morstans* West. (Diptera: Muscidae) and a possible method of age determination of female tsetse flies by the examination of their ovaries. *Transactions of the Royal Society London*, **112**: 221-238.

Saunders, D.S. (1962). Age determination for female tsetse flies and the age compositions of samples of *Glossina pallidipes* Austen, *G. palpalis fuscipes* Newst. and *G. brevipalpis* Newst. *Bulletin of Entomological Research*, **53**: 579-595.

Simo, G., Silatsa, B., Njiokou, F., Lutumba, P., Mansinsa, P., Madinga, J., Manzambi, E., De Deken, R. and Asonganyi, T. (2012). Identification of different trypanosome species in the mid-guts of tsetse flies of the Malanga (Kimpese) sleeping sickness focus of the Democratic Republic of Congo. *Parasites and Vectors*, **5**: 201.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. and Flook, P. (1994). Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America,* **87**: 651-704.

Snyder, A.K., Adkins, K.Z. and Rio, R.V.M. (2011). Use of the internal transcribed spacer (its) regions to examine symbiont divergence and as a diagnostic tool for *Sodalis*-related bacteria. *Insects*, **2**: 515-531.

Steverding, D. (2008). The history of African trypanosomiasis. *Parasites and Vectors*, **1**:3.

Stouthamer, R., Breeuwer, J.A. and Hurst, G.D. (1999). *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Annual Review of Microbiology*, **53**: 71-102.

Sunnucks, P. and Hales, D.F. (1996). Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus *Sitobion* (Hemiptera: *Aphididae*). *Molecular Biology and Evolution*, **13**: 510-524.

Swallow, B.M. (2000). *Impacts of Trypanosomosis on African Agriculture*. PAAT Technical and Scientific. PAAT Information Service Publication FAO, Rome.

Takken, W. (1984). Studies on the biconical trap as a sampling device for tsetse (Diptera: *Glossinidae*) in Mozambique. *International Journal of Tropical Insect Science*, **5**: 357-361.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**: 4673-4680.

Thumbi, S.M., Jung'a, J.O., Mosi, R.O. and McOdimba, F.A. (2010). Spatial distribution of African animal trypanosomiasis in Suba and Teso districts in Western Kenya. *BMC Research Notes*, **3**: 6.

Thumbi, S.M., McOdimba, F.A., Mosi, R.O. and Jung'a, J.O. (2008). Comparative evaluation of three PCR based diagnostic assays for the detection of pathogenic trypanosomes in cattle blood. *Parasites and Vectors*, **1**, 46.

Toh, H., Weiss, B.L., Perkin, S.A., Yamashita, A., Oshima, K., Hattori, M. and Aksoy, S. (2006). Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. *Genome Research*, **16**: 149-156.

Toju, H. and Fukatsu, T. (2011). Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: Relevance of local climate and host plants. *Molecular Ecology*, **20**: 853-868.

Torr, S.J., Holloway, M.T.P. and Vale, G.A. (1992). Improved persistence of insecticide deposits on targets for controlling *Glossina pallidipes* (Diptera: *Glossinidae*). *Bulletin of Entomological Research*, **82**: 525-533.

Truc, P. (2003). About *Trypanosoma brucei gambiense*, the causative agent of the chronic form of HAT: Some findings and proposals. *African Journal of Biotechnology*, **2**: 657-661.

Turelli, M. and Hoffmann, A.A. (1991). Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature*, **353**: 440-442.

Uilenberg, G. (1998). *A field guide for the diagnosis, treatment and prevention of African Animal Trypanosomosis*. Food and Agriculture Organization of the United Nations, Rome.

Van den Abeele, J., Caljon, G., De Ridder, K., De Baetselier, P. and Coosemans, M. (2010). *Trypanosoma brucei* modifies the tsetse salivary composition, altering the fly feeding behavior that favors parasite transmission. *PLoS Pathogens*, **6**: e1000926.

Venetia, Z., Clark, M.E., Zabalou, S., Karr, T.L., Savakisa, C. and Bourtzis, K. (2003). Cytoplasmic incompatibility and sperm cyst infection in different *Drosophila-Wolbachia* associations. *Genetics*, **164**: 545-552.

von Wissmann, B., Machila, N., Picozzi, K., Fèvre, E.M., Bronsvoort, B.M., Handel, I.G. and Welburn, S. (2011). Factors associated with acquisition of human infective and animal infective trypanosome infections in domestic livestock in Western Kenya. *PLoS Neglected Tropical Diseases*, **5**: e941.

Walshe, D., Lehane, M.J. and Haines, L.R. (2011). Post eclosion age predicts the prevalence of midgut trypanosome infections in *Glossina*. *PLoS ONE*, **6**: e26984.

Weiss, B.L., Mouchotte, R., Rio, R.V., Wu, Y.N., Wu, Z., Heddi, A. and S, A. (2006). Interspecific transfer of bacterial endosymbionts between tsetse fly species: Infection establishment and effect on host fitness. *Applied Environmental Microbiology*, **72:** 7013-7021.

Welburn, S., Fevre, E., Coleman, P. and Maudlin, I. (2004). Epidemiology of Human African Trypanosomiasis. In Maudlin, I., Holmes, P. and Miles, M. (Eds), *The Trypanosomiases*. CABI Publishing, Wallingford, pp. 219-232.

Welburn, S. and Maudlin, I. (1992). The nature of the teneral state *Glossina* and its role in the acquisition of of trypanosome infection in tsetse. *Annals of Tropical Medicine and Parasitology*, **86**: 529-536.

Welburn, S.C., Arnold, K., Maudlin, I. and Gooday, G.W. (1993). Rickettsia-like organisms and chitinase production in relation to transmission of trypanosomes by tsetse flies. *Parasitology*, **107**: 141-145.

Wellde, B.T., Chumo, D.A., Reardon, M.J. and Smith, D.H. (1989). A history of sleeping sickness in Kenya. *Annals of Tropical Medicine and Parasitology*, **83**: 1-11.

Werren, J.H. (1997). Biology of *Wolbachia*. *Annual Review of Entomology*, **42**: 587-609.

Werren, J.H., Baldo, L. and Clark, M.E. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, **6**: 741-751.

WHO (1998). Control and Surveillance of African Trypanosomiasis. *WHO Technical Report Series*. Geneva.

WHO (2006). African trypanosomosis or sleeping sickness, Fact sheet No. 259.

Willemse, L. (1991). A trial of odour baited targets to control the tsetse fly, *Glossina morsitans centralis* (Diptera: Glossinidae) in west Zambia. *Bulletin of Entomological Research*, **81**: 351-357.

Williams, B.I. (1996). African trypanosomiasis. In Cox, F.E.G. (Ed), *The Wellcome Trust Illustrated History of Tropical Diseases*. London, UK. pp 178-191.

Wu, M., Sun, L., Vamathevan, J., Riegler, M., Deboy, R., Brownlie, J., McGraw, E., Martin, W., O'Neill, S. and Eisen, J. (2004). Phylogenomics of the reproductive parasite *Wolbachia pipientis w*Mel: A streamlined genome overrun by mobile genetic elements. *PLoS Biology*, **2**: e64.

Zabalou, S., Riegler, M., Theodorakopoulou, M., Savakis, C. and Bourtzis, K. (2004). *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of the National Academy of Sciences USA*, **101**: 15042-15045.

Zhou, W.G., Rousset, F. and O'Neill, S. (1998). Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society of London* **265**: 509-515.

Zouache, K., Raharimalala, F.N., Raquin, V., Tran-Van, V., Raveloson, L.H., Ravelonandro, P. and Mavingui, P. (2011). Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. *FEMS Microbiology Ecology*, **75**: 377-389.

Zouache, K., Voronin, D., Tran-Van, V., Mousson, L., Failloux, A.B. and Mavingui, P. (2009). Persistent *Wolbachia* and cultivable bacteria infection in the reproductive and somatic tissues of the mosquito vector *Aedes albopictus*. *PLoS One*, **4**: e6388.

APPENDICES

Appendix 1: Sticky monopanel trap used field capture of *G. austeni*

Glass bottle containing acetone, sachet of phenols attached to bottle

The bottom half of the blue plywood panel was smeared with adhesive to capture low-flying tsetse flies

Appendix 2: Representative chromatogram of a single wsp clone from *G. austeni*

The chromatogram shows single peaks to support a single strain *Wolbachia* infection in this population. Grey bars above the base calls are quality values (quality information) about the base calls. The dotted blue-colored horizontal line is placed across the quality bars at Q=20. Bars above this line indicate base calls that have a better than 99% probability of being correct. Those below have less than a 99% probability of being correct

Appendix 3: Partial nucleotide coding sequences (cds) and GenBank accession numbers of *G. austeni Wolbachia* MLST genes

Gene	GenBank Accession Number	SEQUENCE
gatB	JF906102	gaagctgcagaatgcatgaaaaaattgaggcagattttgcgttacactggttcgtgtgatggtgatatggaaaagggatcacttcgttgtgatgcaaatgtttctg ttcgcctaaaaggcagtagtacatttggcactcgttgtgaaataaaaaatctgaactcgatacgttatattgtgcaagctatagactatgaaatacaaagacaaat tgaaatttagaaagtggggaggaaataagtcaagataccttattgtttgatgtcgctttgggaaaaacaaaagtgatgagaagcaaagaggatgcaagcgac tataggtatttccctgagcctgatttattacctgttgaggtaagccaggagaag
coxA	JF906103	atgcgcacaaaaggcatgtcattaactaagatgccactgtttgtttggtctgtcttgctaacagcatttatgttgattgttgccttaccagtgcttgccggtgctaaa ctatgcttcttactgatcgcaatgttggtacttccttttttgatcctgccggtggcggagaccctgtgttatttcaacatctattttggttttttggtcatccagaagttta cgtaattatttttcctgcatttggcatcataagtcaggttgtatcaacttttctcacagacctgtatttggttacatagggatggtttatgcaatgataggtatagcagt atttggctttatggtttgggctcatcatatgttcactgttgggcttagtgctgacgctgctgcatttttt
hcpA	JF906104	gatccagaactcaacccgcgccttcgctctgctatatttgctgcacgcaaggaaaatctaccaaaagataaaatagaaacagcaataaaaaatgcaactggta atgttgctggagaaaattacgaagaaatccaatatgaaggtcatgggccttctggcactgcacttattgtccatgccttgactaacaaccgcaaccgtactgctt ctgaggtacgttatatcttttctcgtaaaggtggaaacttaggagaaacaggaagtgttagttacctttcgatcatgtaggtttaatcgtctataaagcagagggt gtgaattttgacgatttattcagtcatggaatcgaattagaagtattgaatgttgaggaaaatgacaaagaaggattacacgttataacttgtgaaataaaagatttt ggtaaagtacgcgatgccttt
ftsZ	JF906105	ggtggtactggaaccggtgcagcaccggtaattgcaaaagcagccagagaagcaagagctgcagttaaggatagagcgccaaaagaaaaaaagatattg actgttggagttgtaactaaaccgttcggttttgaaggtgtgcgccgcatgcgcattgcagaacttggacttgaagaattgcaaaaatacgtggatacacttattg tcattccaaatcagaatttatttagaattgcaaatgaaaaaactacattttctgatgcatttaaacttgctgataatgttctgcatattggcatcagaggagtaactga cttgatggtcatgccagggcttattaatcttgacttcgctgatatagaaacagtaatgagcgagatgggcaaagcgatgatcggcaccggagaggcagaagg agaagatagagcaatcagt
fbpA	JF906106	gctggcctgctcccacttattttgaaacttaatagttccaactccttacattcaaaagatctgacttctgatcaagcaataacctcttctgtgaaagatgcactgcgtt tgggctgcttggctgttggatttactatatatcctggttctgcgaagtgttttgatatgatggaagaagcccgtgaaatcgtagctgaagccaagtcttatgggctt gcagtagtgctatggtcttatccacgtggtgaaggaatttccaaagaaggtgaaacagcagttgatattattgcctatgctgcgcacatagcagctttacttggc gctaatataatcaaagtaaaacttccaactaaatatttggaaagggagagaatagaaacagaaaatattgaatcattatctaaaagaattgaatatgttaaaagat

Wamwiri et al. Parasites & Vectors 2013, 6:232
http://www.parasitesandvectors.com/content/6/1/232

Parasites & Vectors

RESEARCH Open Access

Wolbachia, *Sodalis* and trypanosome co-infections in natural populations of *Glossina austeni* and *Glossina pallidipes*

Florence N Wamwiri[1*], Uzma Alam[2], Paul C Thande[1], Emre Aksoy[2], Raphael M Ngure[3], Serap Aksoy[2], Johnson O Ouma[1] and Grace A Murilla[1]

Abstract

Background: Tsetse flies harbor at least three bacterial symbionts: *Wigglesworthia glossinidia*, *Wolbachia pipientis* and *Sodalis glossinidius*. *Wigglesworthia* and *Sodalis* reside in the gut in close association with trypanosomes and may influence establishment and development of midgut parasite infections. *Wolbachia* has been shown to induce reproductive effects in infected tsetse. This study was conducted to determine the prevalence of these endosymbionts in natural populations of *G. austeni* and *G. pallidipes* and to assess the degree of concurrent infections with trypanosomes.

Methods: Fly samples analyzed originated from Kenyan coastal forests (trapped in 2009–2011) and South African *G. austeni* collected in 2008. The age structure was estimated by standard methods. *G. austeni* (n=298) and *G. pallidipes* (n= 302) were analyzed for infection with *Wolbachia* and *Sodalis* using PCR. Trypanosome infection was determined either by microscopic examination of dissected organs or by PCR amplification.

Results: Overall we observed that *G. pallidipes* females had a longer lifespan (70 d) than *G. austeni* (54 d) in natural populations. *Wolbachia* infections were present in all *G. austeni* flies analysed, while in contrast, this symbiont was absent from *G. pallidipes*. The density of *Wolbachia* infections in the Kenyan *G. austeni* population was higher than that observed in South African flies. The infection prevalence of *Sodalis* ranged from 3.7% in *G. austeni* to about 16% in *G. pallidipes*. Microscopic examination of midguts revealed an overall trypanosome infection prevalence of 6% (n = 235) and 5% (n = 552), while evaluation with ITS1 primers indicated a prevalence of about 13% (n = 296) and 10% (n = 302) in *G. austeni* and *G. pallidipes*, respectively. The majority of infections (46%) were with *T. congolense*. Co-infection with all three organisms was observed at 1% and 3.3% in *G. austeni* and *G. pallidipes*, respectively. Eleven out of the thirteen (85%) co-infected flies harboured *T. congolense* and *T. simiae* parasites. While the association between trypanosomes and *Sodalis* infection was statistically significant in *G. pallidipes* (P = 0.0127), the number of co-infected flies was too few for a definite conclusion.

Conclusions: The tsetse populations analyzed differed in the prevalence of symbionts, despite being sympatric and therefore exposed to identical environmental factors. The density of infections with *Wolbachia* also differed between *G. austeni* populations. There were too few natural co-infections detected with the *Sodalis* and trypanosomes to suggest extensive inter-relations between these infections in natural populations. We discuss these findings in the context of potential symbiont-mediated control interventions to reduce parasite infections and/or fly populations.

Keywords: *Glossina*, *Wolbachia*, *Sodalis*, Trypanosomes, Co-infection, Shimba Hills, Kenya

* Correspondence: F.wamwiri@gmail.com
[1]KARI-Trypanosomiasis Research Centre, P.O. Box 362-00902, Kikuyu, Kenya
Full list of author information is available at the end of the article