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DISSERTATION

Interaction between tsetse symbiont, pathogens on trypanosome infection in wild tsetse populations

ausgeführt zum Zwecke der Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften unter der Leitung von

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Table of contents

List of Abbreviation

Abbreviation/ Term	Definitions
AAT	African Animal Trypanosomiosis
AICc	Akaike information criterion
ANOVA	Analysis of Variance
AW-IPM	Area Wide Integrated Pest Management
BKF	Burkina Faso
BLAST	Basic Local Alignment Search Tool
CI	Cytoplasmic Incompatibility
CIRDES	Centre International de Recherche-Développement sur l'Elevage en zone Subhumide
CCM	Citri-Chrysopicola-Mirum
DDT	Dichlordiphenyltrichlorethan
DNA	Deoxyribonucleic Acid
ETH	Ethiopia
FAO	Food and Agricultural Organization of the United Nations
fruR	*Spiroplasma* fructose repressor
fliC	Flagellin gene
GLM	General Linear Model
GpSGHV	Glossina pallidipes Salivary Gland Hypertrophy Virus
GmmIV	Glossina morsitans morsitans Iflavirus
GmmNegeV	Glossina morsitans morsitans Negevirus
GHA	Ghana
GUI	Guinea
GpCAG	*Glossina pallidipes* microsatellite
Ga	*Glossina austeni*
Gb	*G. brevipalpis*
Gff	*G. fuscipes fuscipes*
Gmm	*G. morsitans morsitans*
Gmsm	*G. m. submorsitans*
Gpg	*G. palpalis gambiensis*
Gpp	*G. palpalis palpalis*
HAT	Human African Trypanosomiasis
IAEA	International Atomic Energy Agency
IPCL	Insect Pest Control Laboratory

Abbreviation/ Term	Definitions
ITM	Institute of Tropical Medicine
ITS1	Internal Transcribed Spacer 1
KEN	Kenya
kbp	Kilo base pair
m	meter
ml	Milliliter
mMDS	Metric multidimensional scaling
MLI	Mali
MOZ	Mozambique
MLST	Multi locus sequencing typing
NCBI	National Center for Biotechnology Information
nm	Nanometer
NAFA	Nuclear Applications in Food and Agriculture
PERMANOVA	Permutational multivariate analysis of variance
parE	*Spiroplasma* DNA Topoisomerase 4 subunit B
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
rpoB	RNA polymerase subunit beta
RNA	Ribonucleic Acid
rRNA	Ribosomal ribonucleic Acid
+ssRNA	positive-sense single-stranded RNA
SGHV	Salivary Gland Hypertrophy Virus
SIT	Sterile Insect Technique
SAF	South Africa
SWA	Eswatini
thiC	Thiamine biosynthesis gene
Tz	*Trypanosoma brucei* sspp
Tc	*Trypanosoma congolense*
Tv	*Trypanosoma vivax*
VSG	Variant Specific Glucoprotein
ZAI	Democratic Republic of the Congo
ZAM	Zambia
ZIM	Zimbabwe

Abstract

Tsetse flies serve as the solely vectors of African trypanosomes, causing diseases such as Human African Trypanosomiasis (HAT, sleeping sickness), and African Animal Trypanosomosis (AAT, Nagana). Given the absence of effective vaccines, affordable drugs, coupled with the increasing resistance to trypanocidal drugs, employing vector control becomes a compiling approach to manage this neglected zoonosis. The Sterile Insect Technique (SIT) is an environmentally friendly and sustainable method for controlling tsetse flies. Implemented as a part of Area-Wide Integrated Pest Management (AW-IPM) programms, SIT has demonstrated high efficiency in suppressing and/or eradicating tsetse fly populations. SIT involves releasing sterile males of the targeted tsetse species into a defined area to mate with wild virgin females, resulting in no offspring. However, the success of the SIT heavily relies on the mass-rearing of high-quality sterile males. Yet, the performance of the males intended for SIT programmes can adversely affected by pathogenic infection such as *Glossina pallidipes* salivary gland hypertrophy virus, which reduces the reproductive capacity of the infected flies. Moreover, releasing of large numbers of sterile males, while maintaining their vector competence in area where HAT occurs might elevate disease transmission risks. Additionally, the microbial fauna of tsetse flies, including symbiotic bacteria like *Sodalis glossinidius*, *Wolbachia pipientis*, *Spiroplasma* can influence the vectorial competence of tsetse flies. Considering these constraints faced by SIT programmes, it become crucial to evaluate the interactions among tsetse symbionts, pathogens and trypanosome infections in wild tsetse populations. The research focussed on: (i) evaluation of the prevalence of *Sodalis* and *Trypanosoma* spp. infections in wild population of tsetse flies and exploring their interactions; (ii) determination of the potential association between *Wolbachia* and GpSGHV co-infection with *Wigglesworthia* and *Sodalis* in field samples; (iii) assessing the prevalence of *Spiroplasma* in natural tsetse population and investigating the different strains of the bacterium circulating in *G. tachinoides* population. Lastly, evaluating of the interactions between *Spiroplasma* with the trypanosome and *Wigglesworthia* in *G. tachinoides*. The results indicate variable prevalence of *Sodalis* and *Trypanosoma* spp. infections, with significant correlations observed in certain tsetse species, emphasizing their potential influence on vector competence. Investigations into GpSGHV and *Wolbachia* interactions revealed species-specific co-infection patterns, suggesting a protective role of *Wolbachia* against GpSGHV. These findings underscore the dynamic nature of interactions between tsetse flies and their associated microbes, emphasizing the need for a nuanced understanding under field conditions. Moreover,

Spiroplasma, found in *Glossina tachinoides*, demonstrated a significant reduction in trypanosome density, indicating a potential enhancement of tsetse refractoriness to trypanosome infections. This discovery holds practical implications for mitigating risks associated with the release of sterile males during SIT implementation in trypanosome-endemic areas. In conclusion, the combined research underscores the intricate relationships between tsetse flies, microbial symbionts, and pathogens. Understanding these interactions is crucial for refining and optimizing SIT programs, offering valuable insights for sustainable and effective vector control strategies in the absence of vaccines and cost-effective drugs for trypanosomiasis.

Keys words: Tsetse flies, SIT, Trypanosomiasis, Symbiotic interaction, vector competence

Zusammenfassung

Tsetsefliegen sind die einzigen Überträger von afrikanischen Trypanosomen, die Krankheiten wie die Afrikanische Trypanosomose beim Menschen (HAT, Schlafkrankheit) und die Afrikanische Tier-Trypanosomose (AAT, Nagana) verursachen. Angesichts des Fehlens wirksamer Impfstoffe und erschwinglicher Medikamente sowie der zunehmenden Resistenz gegen Trypanozide ist die Kontrolle des Vektors ein Ansatz zur Bekämpfung dieser Zoonosen. Die Sterile Insect Technique ist eine umweltfreundliche und nachhaltige Methode zum Management von Tsetsefliegen. Die SIT wird im Rahmen von Programmen zur flächendeckenden Schädlingsbekämpfung (Area-Wide Integrated Pest Management, AW-IPM) eingesetzt und hat sich bei der Unterdrückung und/oder Ausrottung von Populationen der Tsetsefliege als äußerst effizient erwiesen. Bei der SIT werden sterile Männchen der betreffenden Tsetse-Arten in einem bestimmten Gebiet freigelassen, um sich mit wilden Weibchen zu paaren, was keine Nachkommen zur Folge hat. Der Erfolg der SIT hängt jedoch stark von der Massenaufzucht (mass rearing) hochwertiger steriler Männchen ab. Die Leistung der für SIT-Programme bestimmten Männchen kann jedoch durch pathogene Infektionen wie „*Glossina pallidipes* salivary gland hypertrophy virus" (GpSGHV) beeinträchtigt werden, was die Fortpflanzungsfähigkeit der infizierten Fliegen verringert. Darüber hinaus könnte die Freisetzung einer großen Anzahl steriler Männchen unter Beibehaltung ihrer Vektorkompetenz in Gebieten, in denen HAT auftritt, das Risiko der Krankheitsübertragung erhöhen. Auch die mikrobielle Fauna der Tsetsefliegen, einschließlich symbiotischer Bakterien wie *Sodalis glossinidus, Wolbachia pipientis und Spiroplasma*, kann die Vektorkompetenz der Tsetsefliegen beeinflussen. In Anbetracht dieser Einschränkungen, mit denen SIT-Programme konfrontiert sind, ist es von entscheidender Bedeutung, die Wechselwirkungen zwischen Tsetse-Symbionten, Pathogenen und Trypanosomen-Infektionen in wilden Populationen zu bewerten. Diese Forschungsarbeit konzentriert sich auf: (i) die Erhebung der Prävalenz von *Sodalis*- und *Trypanosoma* spp.-Infektionen in wilden Populationen der Tsetsefliege und die Erforschung ihrer Wechselwirkungen; (ii) die Bestimmung des möglichen Zusammenhangs zwischen *Wolbachia*- und *Gp*SGHV-Koinfektionen mit *Wigglesworthia* und *Sodalis* in Feldproben; (iii) die Bewertung der Prävalenz von *Spiroplasma* in natürlichen Populationen und die Untersuchung der verschiedenen Stämme des Bakteriums, die in Populationen von *G. tachinoides* zirkulieren. Schließlich werden die Wechselwirkungen zwischen *Spiroplasma* und Trypanosomen und *Wigglesworthia* in *G. tachinoides* untersucht. Die Ergebnisse deuten auf eine variable Prävalenz von *Sodalis*- und *Trypanosoma* spp.-

Infektionen hin, wobei bei bestimmten Arten signifikante Korrelationen erfasst wurden, was ihren potenziellen Einfluss auf die Vektorkompetenz unterstreicht. Untersuchungen der Wechselwirkungen zwischen *Gp*SGHV und *Wolbachia* ergaben artspezifische Koinfektionsmuster, was auf eine schützende Rolle von Wolbachia gegen *Gp*SGHV hindeutet. Diese Ergebnisse unterstreichen den dynamischen Charakter der Interaktionen zwischen Tsetsefliegen und den mit ihnen assoziierten Mikroben und machen weiters deutlich, dass eine Kenntnis der Gegebenheiten unter Feldbedingungen erforderlich ist. Darüber hinaus resultierte die Infektion von *Spiroplasma* in *G. tachinoides* in einer signifikanten Verringerung der Dichte an Trypanosomen, was auf eine potenzielle Verbesserung der Widerstandsfähigkeit der Tsetsefliegen gegenüber Trypanosomeninfektionen hinweist. Diese Erkenntnisse haben praktische Auswirkungen auf die Verringerung der Risiken, die mit der Freisetzung steriler Männchen während der SIT in endemischen Gebieten verbunden sind. Zusammenfassend lässt sich feststellen, dass die Summe der Forschungsarbeiten die komplexen Interaktionen zwischen Tsetsefliegen, Symbionten und Krankheitserregern unterstreichen. Das Verständnis dieser Wechselwirkungen ist für die Optimierung von SIT-Programmen von entscheidender Bedeutung und bietet wertvolle Erkenntnisse für nachhaltige und wirksame Vektorkontrollstrategien, solange es keine effektiven Impfstoffe und kostengünstige Medikamente gegen Trypanosomosis gibt.

Stichwörter: Tsetsefliegen, SIT, Trypanosomosis, symbiotische Interaktion, Vektorkompetenz

Declaration

I hereby declare that this doctoral thesis was performed for the degree of Doctor of Philosophy in English in Doctoral programme in Natural Sciences: Technical Chemistry under the guidance and supervision of Prof. Robert L. Mach from the Institute of Chemical, Environmental and Biological Engineering and Prof. Adly M. M. Abdalla from the Insect Pest Control Laboratory, Joint FAO/IAEA Center of Nuclear Techniques in Food and Agriculture. The dissertation which I drafted in the term of the thesis entitled "**Interaction between tsetse symbiont, pathogens on trypanosome infection in wild tsetse populations**". I confirm as well that this thesis is the result of original research work obtained at the Insect Pest Control Laboratory, Joint FAO/IAEA Center of Nuclear Techniques in Food and Agriculture, Seibersforf, Austria. Moreover, I guarantee that this work does not infringe copyright law from other sources and such work has been mentioned and acknowledged. I have not submitted the doctoral thesis for any other degree or professional qualification. All the experiments were almost entirely carried out by myself; the collaborative contributions have been indicated clearly and recognized.

List of Publications

1- **Mouhamadou M. Dieng**, Kiswend-sida M. Dera, Percy Moyaba, Gisele M. S. Ouedraogo, Guler Demirbas-Uzel, Fabian Gstöttenmayer, Fernando C. Mulandane, Luis Neves, Sihle Mdluli, Jean-Baptiste Rayaisse, Adrien M. G. Belem, Soumaïla Pagabeleguem, Chantel J. de Beer, Andrew G. Parker, Jan Van Den Abbeele, Robert L. Mach, Marc J. B. Vreysen & Adly M. M. Abd-Alla. Prevalence of *Trypanosoma* and *Sodalis* in wild populations of tsetse flies and their impact on sterile insect technique programmes for tsetse eradication. Sci Rep. 2022;12: 3322. doi:10.1038/s41598-022-06699-2.

2- Kiswend-sida M. Dera, **Mouhamadou M. Dieng**, Percy Moyaba, Gisele M. S. Ouedraogo, Soumaïla Pagabeleguem, Flobert Njokou, François S. Ngambia Freitas Chantel J. de Beer, Robert L. Mach, Marc J. B. Vreysen & Adly M. M. Abd-Alla. Prevalence of *Spiroplasma* and interaction with wild *Glossina tachinoides* microbiota (Parasite, accepted 2023)

3- **Mouhamadou M. Dieng**, Augustinos AA, Guler Demirbas-Uzel, Vangelis Doudoumis, Andrew G. Parker, George Tsiamis, Robert L. Mach, Kostas Bourtzis and Adly M. M. Abd-Alla. Interactions between *Glossina pallidipes* salivary gland hypertrophy virus and tsetse endosymbionts in wild tsetse populations. Parasit Vectors. 2022;15: 447. doi:10.1186/s13071-022-05536-9

Chapter 1

General Introduction

1. General introduction

The general introduction of this thesis highlights relevant information on tsetse flies. Indeed, literature regarding their taxonomy, anatomy, biology and Trypanosomiasis disease are provided. In addition, the current, past methods and the SIT vector controls for the management of this zoonosis disease are inventoried. The success of SIT is always facing on new challenges regarding the holistic factors involved in his implementation. In fact, the ethical issue is regularly emphasized due to the disease transmission risk associated with the application of SIT. Therefore, it is important to study the interaction between tsetse symbiont, pathogens on trypanosome infection. The following section discussed the different interaction of: *Trypanosoma* infection and *Sodalis*; virus infection (GpSGHV) and tsetse symbionts; in wild tsetse populations; *Spiroplasma* and the parasite specifically in *Glossina tachinoides*.

1.1   Tsetse fly vector of Trypanosomiasis diseases

Tsetse flies have a significant impact on sub-Saharan Africa due to their blood-feeding behaviour and their role as vectors of trypanosomes (*Trypanosoma* spp). This protozoan parasite is responsible of Human African Trypanosomiasis (HAT, sleeping sickness), and African Animal Trypanosomosis (AAT, Nagana) [1–3]. Trypanosomes are transmitted cyclically between reservoir and vertebrate hosts or from host to host by tsetse flies (*Glossina* spp.). Within the tsetse flies, the parasite completes one part of its life cycle [4,5]. HAT and AAT causes death if left untreated and are listed among the group of neglected tropical diseases (NTDs) in Africa [6–8].

Two trypanosome species and subspecies are pathogen in human. The acute form of HAT is caused by *Trypanosoma brucei rhodesiense* in East and South Africa, while *Trypanosoma brucei gambiense* is responsible of the chronic disease in Western and Central Africa [9–11]. HAT was first detected over 200 years ago [12], the disease saw significant progress between 1900-1910 before declining [13], largely due to coordinated campaigns efforts in affected areas [6,7,13]. However, in the beginning of the 21th century, it was estimated that 300.000 people were infected [14], representing 10-15% of the 60-70 million people living in risk areas under control [14,15]. This suggests that the actual number of infected individuals may have been underestimated.

In livestock several trypanosome species, including *Trypanosoma congolense*, *T. vivax*, *T. brucei. brucei*, *T. simiae* and *T. godfreyi* provoke AAT in animals such as ruminants, equids,

camelids and suids [16–18]. *T. evansi* is pathogenic but not transmitted by tsetse flies. Nagana continues to be problematic and has a substantial negative economic impact on livestock and agricultural improvement efforts [19]. In the 37 Africa's countries where it occurs, more than 50 million cattle are at risk [20], and 35 million doses of trypanocide are injected for prevention per year [21] leading to annual losses in agriculture estimated at $ 4,75 billion [22,23].

Prior tsetse control tactic included the use of chemicals methods (baits, aerial spray, spraying of animals, live bait or "pour-ons"); and prophylaxis with frequent treatment of animals and human to manage AAT and HAT. These methods have been recognized as harmful to the environment, for non-target insects and costly. Nowadays the increasing resistance of the parasite to available trypanocidal drugs [24–28], along with the lack of effective vaccines to protect against trypanosomiasis [29,30], makes tsetse vector control an interesting approach to reduce the diseases in Africa.

One of the most effective methods for controlling tsetse fly vector is the sterile insect technique (SIT) when implemented as a part of an area-wide integrated pest management (AW-IPM) approach [31,32]. Elucidating the molecular interactions between tsetse-symbiome-pathogen in trypanosome infection constitute a major objective for improving SIT [33]. Understanding the interactions between tsetse symbionts, pathogens on trypanosome infections is essential for developing strategies to control trypanosome transmission and reduce the prevalence of African trypanosomiasis.

1.1.1 Taxonomy, distribution and habitat

Taxonomy

Tsetse flies belong into the class of *Insecta*, subclass *Pterygota*, Order *Diptera*, Suborder *Cycporrhapha*, family *Glossinidae* [11]. Their single genus *Glossina* spp includes 33 species and subspecies divided into 3 groups: *Austenina* (*fusca* group), *Nemorhina* (*palpalis* group) and *Glossina* (*morsitans* group) [34,35]. However only 8-10 species of tsetse fly are veterinary and medical importance [36].

The most important unique feature in tsetse fly is their blood feeding for both males and females on vertebrate host [37]. As well viviparity is the unique reproductive mode of tsetse flies consisting that female delivers a third instar larva every 9-10 days [34,38]. Furthermore tsetse flies develop *k*-strategy reproduction system meaning that female produce low number of offspring and provide high care on the larva to enable better survival of the offspring [34,39].

Another distinctive feature is their posture at rest, where they fold their wings over their back. Additionally, they can be identified by the characteristic "hatchet" shape of the discal cell in their wings [40–42].

The current taxonomy depends on the morphological differences in the structure of the superior and inferior claspers of the male genitalia and the number of plates formed on the external armature of female **(Figure 1)**. The distinct configurations of the head of the inferior clasper are valuable for identifying species and subspecies within the *palpalis* group. Furthermore, the absence of the signum in both the *morsitans* and *palpalis* groups is a key anatomical feature located in the uterus, which plays a crucial role in identifying female tsetse flies belonging to the *fusca* subspecies[34].

The taxonomy rely as well as on DNA sequence data including the mitochondrial DNA [43] and the16S rDNA of the inherited primary endosymbiont *Wigglesworthia glossinidia* [44]. Certainly, the habitats and the geographical distribution of tsetse flies are another important factor used to distinguish between the three major groups of tsetse flies. The ecological preferences and geographical distribution of these flies can vary significantly between groups, aiding in their differentiation [42].
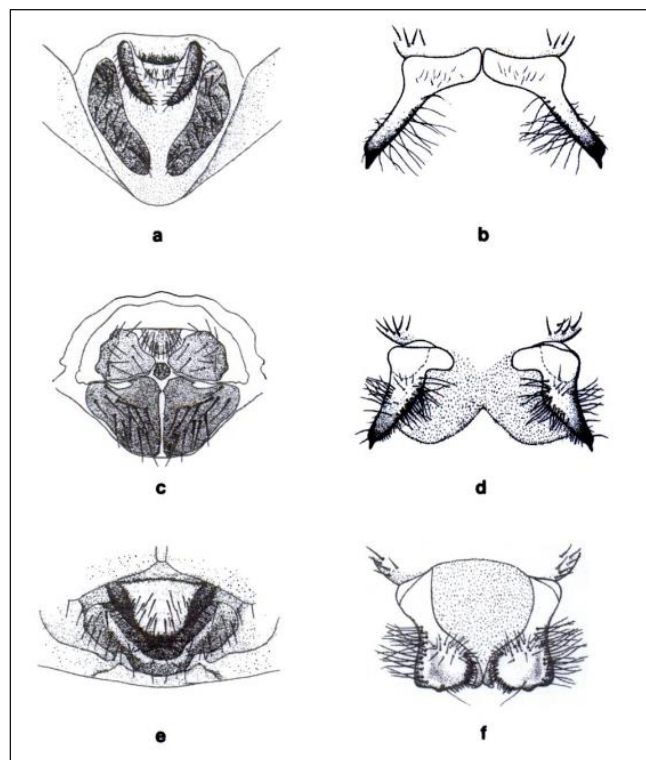

Figure 1: Identification features of *Glossina* genitalia for subgeneric classification.
(a) Female external armature – *fusca* group; (b) male superior claspers (*fusca* group);
(c) female external armature – *palpalis* group; (d) male superior claspers – *palpalis* group; (e) female external armature – *morsitans* group; (f) male superior claspers – *morsitans* group (source [45]).

Distribution and habitat

Tsetse flies are distributed in sub-Saharan Africa between the 15th North Latitude and 26th South Latitude [35]. The repartitioning of tsetse species occurs in the borderline of the Sahara and Somali desert in the north and the Kalahari and Namibian desert in the south and the eastern part of Africa [5] **(Figure 2)**.

Flies of the *morsitans* group prefer the woodlands savannah with *Brachystegia* **(Figure 2B)** and *Isoberlinia* **(Figure 2C)** light forest in central, eastern and western Africa respectively. These species can also thrive along the arid Sudanian vegetation in the north and the Mopane savanah of eastern Africa [46]. *G. austeni*, previously classified as species member of the *morsitans* group, now belongs to the subgenus of *Machadomyia* [47]. This species is dis-continuously distribution along the east African coast from Kwazulu-Natal (South Africa) to Somalia [48].
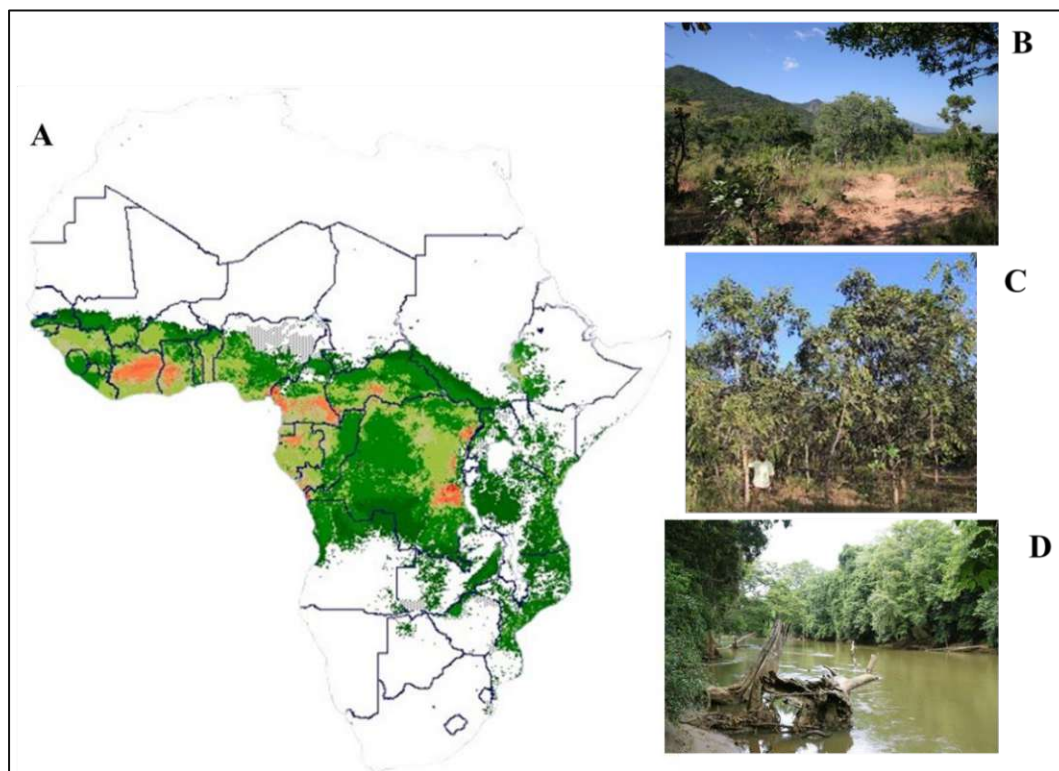
Figure 2: Distribution of tsetse species in Africa (Image credit: http://ergodd.zoo.ox.ac.uk/tseweb/distributions.htm)
(A) Distribution of tsetse species, ***morsitans* group: (B)** *Brachystegia* forest: (Image credit:
https://upload.wikimedia.org/wikipedia/commons/b/b7/Nyika_miombo.jpg
and **(C)** *Isoberlinia* forest:(Image credit: https://encrypted
tbn0.gstatic.com/images?q=tbn:ANd9GcSjWCHqg7RiNS5q4Qk8LMLKjEej8ucT8g3CMQ&usqp=CAU
palpalis group: (D) Riverine:(Image credit:
https://www.researchgate.net/publication/274928854/figure/fig3/AS:667813714882574@1536230605883/Riverine-Forest-SA.jpg

Tsetse flies of the *morsitans* and *palpalis* groups are most predominant species epidemiologically in causing Trypanosomiasis [49]. *G. morsitans* subspecies. and *G. pallidipes* transmit the acute form of the disease to human in East Africa [50]. *G. m. morsitans* is the most important subspecies in Africa and the major vector of AAT [51].

Tsetse flies of the *palpalis* group are primarily found in riverine and lacustrine habitat **(Figure 2D)**. This group includes *G. fuscipes fuscipes* and various *G. palpalis* subspecies (*G. p. gambiensis* and *G. p. palpalis*) as well as *G. tachinoides* which are indubitably responsible of the chronic form of HAT in West and Central Africa [11,49,52]. The *fusca* group species inhabit the moist forest of West Africa and are highly susceptible to human intrusion, being specific to wild host. The *Fusca* group generally has little epidemiological impact, except for *G. brevipalpis* in East and South Africa which can be an competent vector of AAT [49,53,54].

1.1.2 Anatomy and biology

Anatomy

A basic anatomy of tsetse fly is showed in **Figure 3** [34]. In all insects, the exoskeleton is an external tegument formed from a cuticle composed with chitin. The cuticle is still soft in teneral fly, but become rigid after a few hours [55]. The morphology of the tsetse's head is characteristic of the of *Muscidae* family, but it has a long and slender proboscis, with a long biting mouthpart that has a bulb-like structure at the base of the head. The maxillary palps are as long as the proboscis [56].

The digestive tract of tsetse flies extends from the proboscis to the anus and is consists of several parts: the oesophagus, the proventriculus, the midgut, and the hindgut. The proboscis is composed of the labrum, labium, and hypopharynx, which are extended by two salivary ducts. Each duct leads to a salivary gland. In *G. m. morsitans* species, each salivary gland measures approximately 15 mm and is located in the dorso-lateral part of the abdomen [57,58].

The midgut of tsetse flies extends from the proventriculus to its junction with the Malpighian tubules. The anterior part of the midgut, which is more muscular than the posterior portion, forms a straight tube that crosses the thorax. The rest of the midgut is coiled within the abdomen [59]. The proventriculus, located just behind the neck in the anterior end of the thorax, is the most complex section of the midgut in tsetse flies. It's continuous function to produce the peritrophic membrane, a lengthy chitinous coat that extends over the entire length of the

midgut. The peritrophic membrane is always present and serves to prevent direct contact between the ingested blood and the epithelial cells [60].

In female tsetse flies, the abdomen houses the internal reproductive system, which includes the ovaries, spermathecal ducts, uterus, and uterine glands. Moreover, the abdomen also contains the spermathecae which are responsible for storing spermatozoa after mating [61].
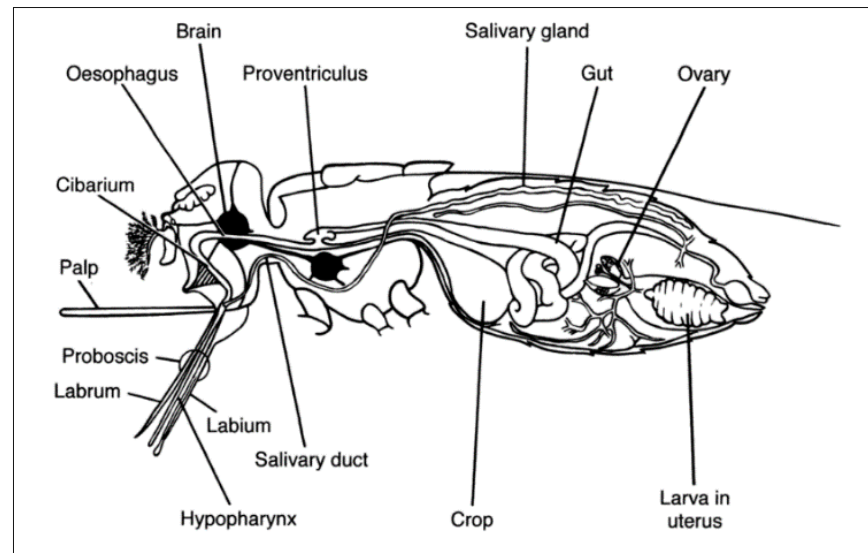
Figure 3: Anatomy of *Glossina* (female) after Mulligan (1970) (Source [34])

Male tsetse flies have two anatomical structures in their genitalia known as the superior and inferior claspers. These claspers are used by the male to grasp the female's abdomen during mating. In contrast, the female tsetse flies have external armature formed by plates. Specifically, there are five plates for the *fusca* group, six for the *palpalis* group, and two pairs of fused anal plates along with a median sternal plate for the *morsitans* group. Only female tsetse flies belonging to the *fusca* group have a unique structure called the signum, which is a chitinized structure located on the interior surface of the uterus wall (**Figure1**) [34].
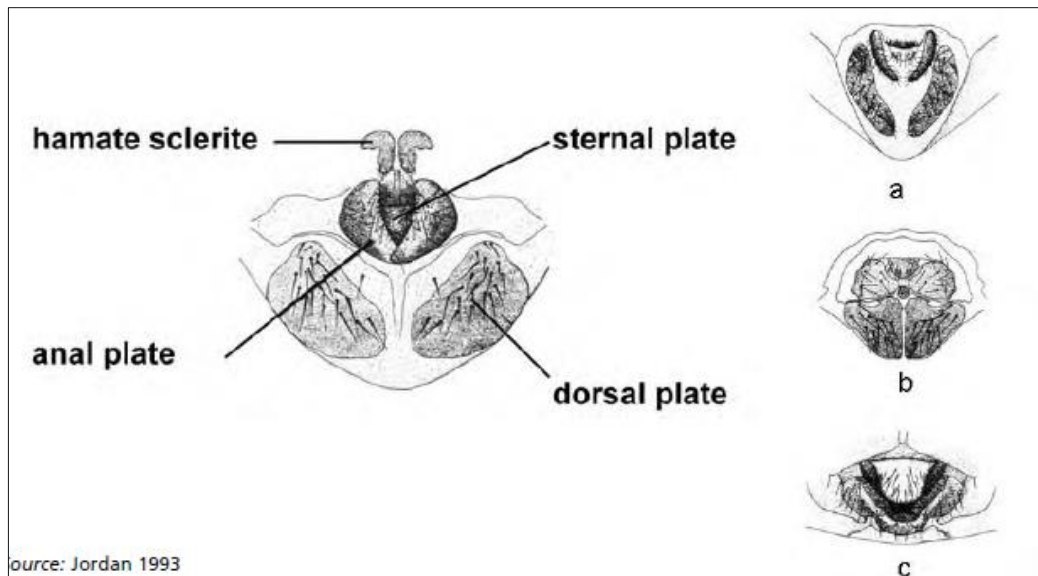
Figure 4: The external genital armature of female tsetse indicating the dorsal plates, the anal plates, the sternal plate and the hamate sclerite (after Mulligan 1970), and (right) a diagram showing the differences of the genital armature between the (a) *fusca* group, (b) *palpalis* group, and (c) *morsitans* group (Source [45])

Biology

Tsetse flies feeding

Tsetse flies, both males and females, are primarily blood-feeders and require a blood meal every 3-4 days to sustain themselves. While they exclusively feed on vertebrate blood, they can show preferences for certain hosts [34,41,62]. The feeding process of tsetse flies involves several steps, one of which includes using of their proboscis to lacerate the skin and create a superficial lesion through which they ingest blood [63].

Blood-sucking is facilitated by the vasodilator and anticoagulant proprieties of the saliva. During the blood meal, which takes 1-10 minutes, tsetse flies can ingest 0,03 ml of blood, increasing their weights 2-3 times [64]. This increased body weight can adversely affect the flight ability of tsetse, making it crucial to avoid predators after feeding. Primary excretion is a physiological strategy to remove the excess water from the blood meal through the Malpighian tubules [34].

Vertebrate blood, rich in protein, constitutes the sole source of nourishment for tsetse flies throughout their lifespan. These proteins are digested in the hindgut by six proteinases (aminopeptidase, carboxypeptidase A and B, trypsin, "trypsin- like" enzyme and "chymotrypsin-like" enzyme) [65,66].

When tsetse flies digest their blood meals, they store fats as triglycerides in their fat body. From these fats, they synthetize proline. Although proteins are their main energy source, they primarily use proline, which is converted into alanine through oxidation in the muscle, to generate the energy required for flight [63,67,68].

Tsetse reproduction

Viviparous is the known mode of the reproduction for female tsetse flies [34,47]. In fact, all pupiparia reproduce through adenotrophic viviparity where each female develops one egg at time, followed by an intrauterine embryonic and larval development. During its development, the larva feeds on nutrients provide by female as "milk" secreted by the female's reproductive accessory glands [69]. This nourishment for the larva contains proteins, lipids and symbiotics bacteria such as *Wigglesworthia* and *Sodalis* [70,71]. Theses endosymbionts are inherited from mother and are necessary to complete tsetse reproductive system [72,73].

Tsetse fly's lifecycle **(Figure 5)** begins with mating between the male and female on the first or second day after emergence. Only one mating is enough to fertilize the female for her entire lifespan. Males have the capacity to mate approximately ten times when the opportunity arises and the sperm migrate in the spermathecae for storage. Tsetse fly females may occasionally accept multiple mating to fill their spermathecae. However, in field, females most likely mate with a male once or twice [39,74,75].

After fertilization, the larva undergoes three stages of development within the uterus. At around 9 days the female deposits a third instar larva on the ground, which immediately burrows and pupate within about 15 minutes in loose or light soil. The emergence of an adult fly occurs after a puparium period of around 30 days based on environmental conditions such as humidity and temperature [47].

Tsetse flies have a slow reproduction rate due to their viviparity. Females flies in laboratory colony lines can produce 8-10 offspring at 9-11 days, compared to the field flies where females produce significantly fewer offspring [47,76]. The relative high adult survival rate of tsetse flies, may serve as a mechanism to compensate for their low reproductive rate [77,78].
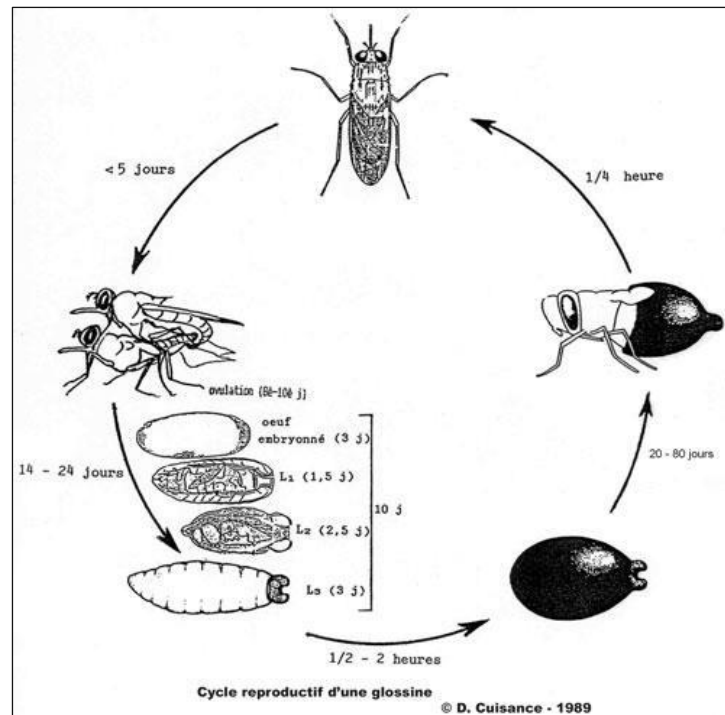
Figure 5: Tsetse flies lifecycle, (Source [79]).

1.1.3   Tsetse fly vector competence

Tsetse flies are the sole vectors capable to transmitting African trypanosome cyclically [4,5]. For the parasite to be inoculate into a vertebrate host, it needs to establish and mature in the fly's midgut and salivary gland or mouthpart depending on the trypanosome species [80,81]. However, only a few engorged trypanosome complete this cycle in the tsetse fly [82].

Vector competence can be define by the ability to acquire the parasite and facilitate its maturation and transmission to a mammalian host [83]. On the other hand, the vectorial capacity of tsetse means the inherent capability of a fly to develop an infective metacyclic form [84].

Many factors have been suggested to play a role in the vector competence. The antimicrobial peptide *attacin* found in tsetse fly tissue [85] was shown to produce an important immune response that modulates trypanosome infection in some *morsitans* subspecies. Mechanism of phagocytosis of trypanosomes by heamocytes may also intervene to suppress trypanosome development [22,86,87]. Lectin glycoproteins present in the tsetse fly midgut [88] have been reported to eliminate most of the ingested trypanosome through proto-apoptic process [89].

Similarly, tsetse flies symbionts such as *Sodalis glossinidius* and *Spiroplasma* are also suspected to be involved in the fly's vector competence [90–93].

Many field investigations have reported that the infection rate seldomly exceeds 10% of the fly population, these data supports the very low infection rate of less than 50% found in vitro [94–97]. For *T. brucei* species and subspecies, the rate of the infective form in the salivary gland is low, typically in the range of 0,1% [50,52,98].

1.2 Development of *Trypanosoma* spp in tsetse fly

In a taxonomic view, trypanosomes are flagella protozoa classified into the order *Kinetoplastidae*, family *Trypanosomatidae*, and genus of *Trypanosoma* [99]. Based on the transmission mode, the genus *Trypanosoma* is divided into two distinct groups: *Stercoraria* and *Salivaria*. The *Stercoraria* group is mechanically transmitted with feces by blood-sucking bugs (e.g *Rhodnius* spp). These insects are the vectors of *Trypanosoma cruzi,* the causative agent of the "Chagas disease" in human in south America [100].

The *Salivaria* group is cyclically transmitted with saliva and includes four subgenus with their respective species: *Dutonella* (*T. vivax* and *T. uniformis*); *Nannomonas* (*T.* congolense, *T. simiae, T. godfreyi*); *Pyctomonas* (*T. suis*) and *Trypanozoon* (*T. b. brucei*, *T. b. gambiense*, *T. b. rhodensiense*) [101].

The development of trypanosomes involves that tsetse flies ingesting the short stumpy form from an infective mammalian host. Once established in the midgut, the stumpy form differentiates into procyclic trypomastigotes, a process suggested to be induced by trypsin in the midgut [102]. These procyclic trypomastigotes then developed into epimastigotes, which mature either in salivary gland or mouthpart for *Trypanozoon* and *nannomonas* respectively [34,80,81]. In the case of *T. brucei* species and subspecies, the epimastigote forms undergo division into two asymmetrical forms, long and short epimastigote, in the lumen of the salivary gland [80]. The short epimastogote further replicates and attach to the epithelium [103], producing metacyclic forms with VSG-free coats by a second asymmetric division [104]. These forms represent the unique adaptative form for mammalian and are injected into a new host through a bite of an infected tsetse fly [105].

For the *Dutonella* subgenus, the development of *T. vivax* is the simplest in tsetse and occur in the proboscis and sometimes in the cibarium [106].
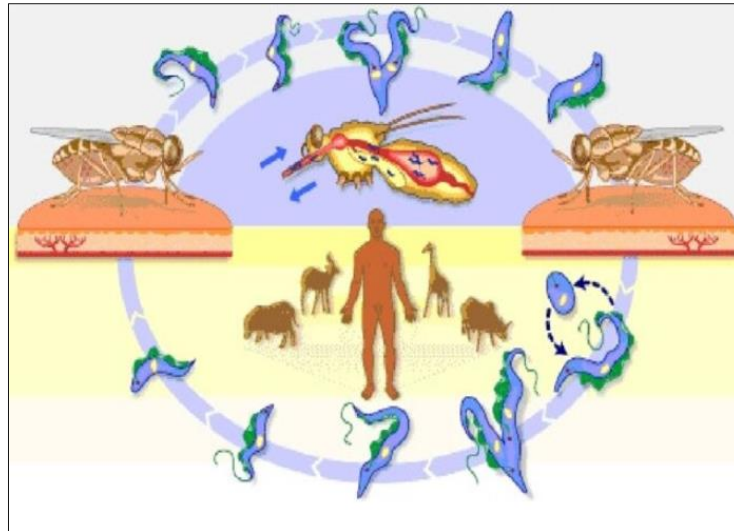
Figure 6: Life cycle of the *Trypanosome brucei* (Source: http://www.who.int/tdr/diseases/tryp/lifecycle.htm) (Source [107])

1.3 Tsetse's fly fours symbionts

According to de Bary (1879) [108], symbiosis can be defined as "the permanent association between two or more specifically distinct organisms, at least during a part of the life cycle."

In symbiotic relationships, typically, one organism is larger than the other(s) and is referred to as "the host," while the smaller organisms are known as "symbiotes" or "symbionts". Symbiosis encompasses various aspects: ectosymbiosis, where the partners live externally to each other, with the smaller partner called the ectosymbiote; and endosymbiosis, where the smaller associates, called endosymbiotes, reside inside the host but remain extracellular. Endosymbiotes are often located in the digestive tract or specialized organs. From an epistemological perspective, the term "symbiote" is the more appropriate designation rather than "symbiont." [108].

Symbiosis between insect and bacteria can be obligate in cases where the survival of host depends to the endosymbiont. Unlike facultative endosymbionts which are not necessary for survival, although they could contribute positively to the host [109].

Insect symbiotic relationship can be classified into three categories: mutualism, where both the insect and the bacteria derive benefits from their association; parasitism, where the parasite benefits at the expense of the host. The host is typically harmed in these interactions, and the parasite may negatively impact the host's fitness and reproduction. Finally, commensalism represents relationships where the bacteria benefit without causing any damage to the host [110].

Considering today's updated research, in addition to the three mains symbiotic microbes (*Wigglesworthia glossinidia*, *Sodalis glossinidius* and *Wolbachia pipientis*) **(Figure 7)**, tsetse fly harbour a fourth discovered symbiont, characterized as *Spiroplasma* [72,93,110,111].
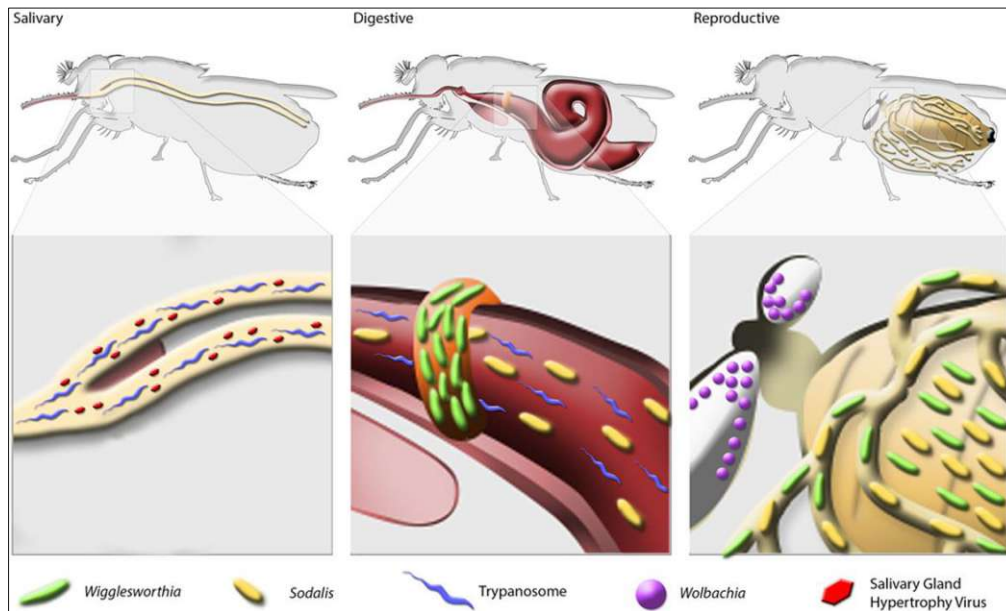
Figure 7: Localization of symbionts, pathogens and trypanosome in tsetse fly (Source [112])

The interaction between vectors, their microbiota and the parasites are complex [110], symbiotic microorganisms appear to influence various aspects of the insect physiology, reproduction, and defence against naturals threats [113].

1.3.1 *Wigglesworthia*

The first discovery of bacteria in digestive tract of *G. fuscipes* and *G. tachinoides* was made by Robert Koch in Stuhlmann (1907). Later, Roubaud (1919) suggested that the bacteria were symbiotic and involved in blood digestion by tsetse fly. These authors postulated the presence of the bacteria living intracellularly in a tissue. The symbiont was further characterized in details reported by Wigglesworth (1929).

Wigglesworthia glossinidius is the obligate mutualist primary (P) symbiont of tsetse fly belonging to the *Enterobacteriaceae* class γ Proteobacteria [114,115]. All tsetse flies analysed today harbour an obligate symbiont of the genus *Wigglesworthia* [44,111]. It's a Gram-negative bacterium based on their membrane composition, with a rod-shape measuring 8µ in length and 1-1,14 µ in width [116]. This symbiont resides intracellularly in a special organ called bacteriome, located in the anterior section of the tsetse midgut [117], as well extracellularly within milk gland secretion, from which it is maternally transmitted to offspring [70,118,119].

The primary symbiont in tsetse flies serves several vital functions. One of its main functions is to contribute to the fitness and reproductive success of tsetse fly by supplementing essential nutrients, particularly B vitamins, which are limited in the fly's blood diet [120–122]. Another critical function of *Wigglesworthia*, is its presence during intrauterine larval development, which is necessary for the proper functioning of the immune response in adult flies. [123]. Conversely, the absence of this symbiont during the larval stage can render adult tsetse flies more susceptible to trypanosome infection [124].

1.3.2   *Sodalis*

Sodalis glossinodius, the secondary commensal symbiont of tsetse fly, is found in all laboratory colonized flies and in some wild populations [125]. This Gram-negative bacterium belongs to the *Enterobacteriaceae* family within the γ-3 Proteobacteria [90,126]. Initially, It was described as a Rickettsia-like organism, ant its isolation from hemolymph was accomplished by culturing it in *Aedes albopictus* cells [127].

Sodalis exhibits a broad host tissue tropism [125], as it can be found in larval stage via milk secretion and is vertically transmitted to the progeny [128]. In adult stage, the symbiont resides both intracellularly and extracellularly in the midgut, fat body, uterus, oviduct, hemolymph, salivary and milk glands [119,125,129]. It has been demonstrated that *Sodalis* can be transmitted during mating by the male [130]. *Sodalis* show genotypic traits similar to those found in several free-living microbes and can be cultivated in vitro [127,131].

The precise functional role that *Sodalis* plays in tsetse fly remain unclear. The symbiont appears to be present in all laboratory colonies, but its prevalence in wild tsetse populations is estimated to range from 0% to75% [132,133]. Experimental elimination of *Sodalis* in *G. morsitans* morsitans specie by treatment with streptozotocin has no impact in fly fecundity, as the obligate symbiont *Wigglesworthia* remain intact. However, a significant reduction of fly's lifespan was observed along with increased susceptibility to trypanosome infection [91].

It has been suggested that *Sodalis* may modulate the ability of trypanosomes to establish an infection, this effect depends on a specific genotype of the symbiont [91,133,134]. *Sodalis* symbiont possesses several desirable characteristics that make it a promising candidate for expressing effector molecules to reduce the tsetse fly's capacity to transmit trypanosome infections. Notably, this bacterium can be isolated, cultured and manipulated in vitro **(Figure 8A and 8B)**, resides in close proximity to trypanosome, and is vertical transmitted. This

approach, known as paratransgenesis, offers a promising alternative to directly genetically engineering the vector species itself [135,136].
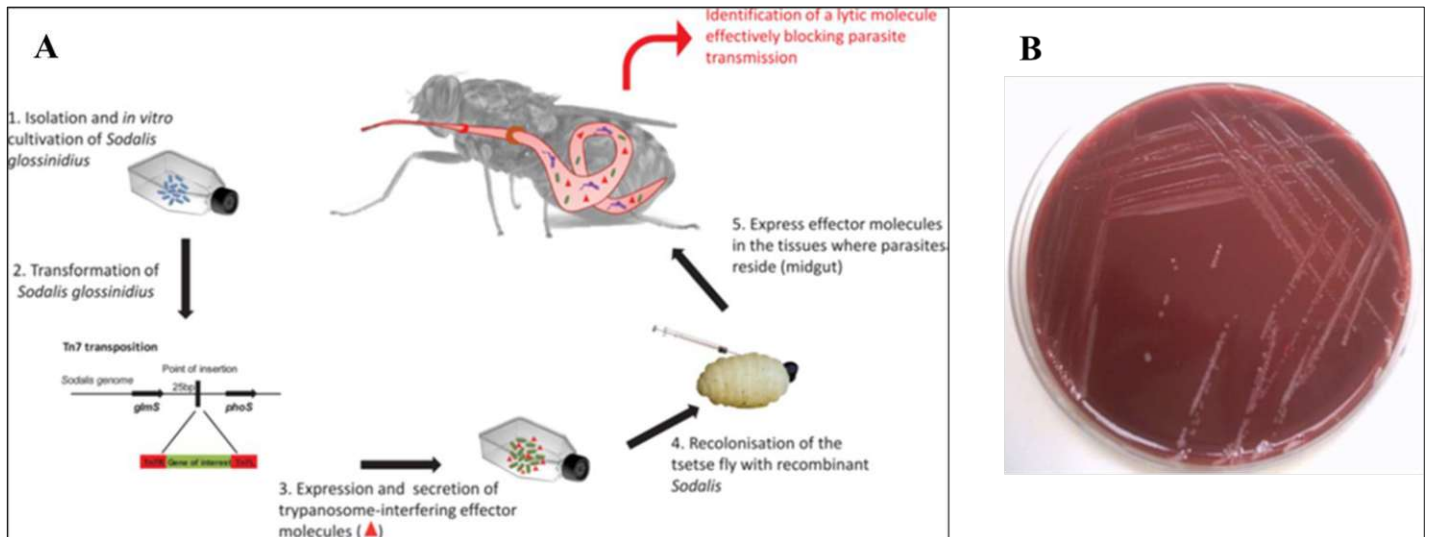
Figure 8: (A) Current status on tsetse paratransgenesis (Source [137]) **(B)** *Sodalis glossinidius* cultured on 10% packed horse blood Mitsuhashi and Maramorosch insect (MMI) medium agar (1%) in microaerobic atmosphere at 26.5 °C (day 4 post inoculation) (Source [138]

1.3.3 *Wolbachia*

The genus of *Wolbachia* was initially identified in *Culex pipiens* and classified in the Order *Rickettsiales* [139]. It is an α-Proteobacterium, Gram-negative bacterium that fall into the category of the third facultative and parasitic endosymbiont occuring in some natural tsetse populations [114]. Numerous studies have reported that *Wolbachia* is widely distributed in arthropods, being found in every insect order, terrestrial crustaceans, spiders, scorpions, with the infection rate of insect species to be as high as 65% [140–143].

In tsetse flies, *Wolbachia* displays a tropism for ovarian tissue, suggesting the transovarial transmission as a primary transmission mode of symbiont transmission [114]. The bacterium is intracellular and transmitted to progeny through the egg cytoplasm [44]. It was shown to infect not only the trophocytes and the oocytes in the ovaries but also embryos and larvae [119,144]. However, hybridization method allowed the detection of *Wolbachia* in the lumen and secretory cells of the milk glands in *G. m. morsitans* [145].

Wolbachia has been suggested to induce sperm-egg incompatibility between the gametes of infected males and uninfected females, commonly referred to as unidirectional cytoplasmic

incompatibility (CI). This phenomenon can play a role in the divergence and speciation of host [146]. Studies on the functional role of *Wolbachia* in tsetse colonies have shown that this symbiont is capable of supporting the expression of cytoplasmic incompatibility [147]. The bacterium can manipulate many aspects of the biology, physiology, ecology, and evolution of their hosts [148,149]. However, females expressing cytoplasmic incompatibility have shown a loss of fecundity due to the early embryogenic failure [147].

The role of this symbiont on trypanosome infection in tsetse fly is still a topic of debate. Available data indicate a negative association between *Wolbachia* and trypanosome infections in *G. f. fuscipes*, suggesting that this symbiont may help prevent trypanosome infections [150]. Others investigations have shown that *Wolbachia* infection appears to have no impact on the establishment of trypanosomes in some tsetse species [151,152].

The CI features exhibited by *Wolbachia* are an intriguing tool for controlling vector-borne diseases and agriculture pests. One method is to release *Wolbachia*-infected males that are incompatible with females, thereby exploiting cytoplasmic incompatibility phenotypes [153]. Moreover, *Wolbachia* induces strong cytoplasmic incompatibility in tsetse [154], supporting the idea that it could be used as a tool to control tsetse populations in the field.

1.3.4 *Spiroplasma*

Spiroplasma is a genus of wall-less bacteria classified within the *Mollicutes* and it has been described in plants and arthropods [155]. Recently, in tsetse flies microbiota communities, *Spiroplasma glossinidia* has been identified as the fourth symbiont in some wild and laboratory fly using the 16S rRNA [111,156]. A multi-locus sequencing typing (MLST) analysis was used to identify two distinct strains exclusively within the *palpalis* group, specifically in *G. f. fuscipes* and *G. tachinoides*. Additionally, an in situ hybridization method was employed to locate *Spiroplasma* in various tissues, including ovaries, testes, larvae, digestive tissue, and hemolymph [111].

The transmission route of the fourth symbiont has been determined as maternally to the larva. The presence of *Spiroplasma* in male reproductive tissue also indicates paternal transmission. [111,157]. Possible horizontal transmission of the symbiont has been reported in *G. f. fuscipes* maintained in colony [93].

In other insects, the bacterium has been known to confer resistance to pathogens. for example, *Spiroplasma* infections in *Drosophila* protect against nematode infections [158]. However, symbiont infections can sometimes lead to reproductive abnormalities in *Drosophila* females [159]. In the case of *G. f. fuscipes* laboratory colonies, it has been shown that the symbiont may protect flies against trypanosome infections [93].

1.4   Tsetse pathogens

In addition to tsetse flies microbial communities present in tsetse flies, several species can also be infected by a known as salivary gland hypertrophy virus (SGHV) [33]. Until recently, the *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) was the only known pathogenic virus infecting tsetse flies [160,161]. However, more recently, Meki *et al*. [162] characterized and identified two +ssRNA viruses (*Iflaviruses* and *Negeviruse*) in *G. m. morsitans* colony.

1.4.1   Salivary Gland Hypertrophy Virus (SGHV)

Several tsetse fly species are susceptible to GpSGHV infection, which belongs to the *Hytrosaviridae* family. These viruses have a distinctive rod-shaped structure, measuring approximately 70 nm x 640 nm, and they contain a single circular double-stranded DNA genome [160,163]. Infection with GpSGHV significantly reduces the reproductive capacity of the infected flies. Additionally, it causes abnormalities in the gonads of host. When the virus infection becomes symptomatic, it leads to the hypertrophy of the salivary glands (resulting in SGH), causing them to swell [164–166].

The transmission dynamics of GpSGHV within natural populations are believed to be primarily maternally transmitted to offspring through either transovum or via infected milk gland secretion [167–169]. In laboratory-bred tsetse colonies, the infection spreads horizontally, mainly through membrane feeding, and also from infected females to their progeny [164]. While the infection rate of GpSGHV in wild tsetse populations is generally low, ranging from 0,2% to 5%, PCR detection has revealed a widespread asymptomatic infection, affecting up to 100% of individuals. Interestingly, only a small proportion, around 5%, of these infected individuals develop symptomatic infections. It is worth noting that symptomatic infections have been linked to the collapse of *G. pallidipes* colonies [170].

The precise mechanism governing the individual variation in expressing symptomatic or asymptomatic infection is not yet fully understood. Indeed, it is crucial to investigate the

intricate interactions between the virus and the microbiota housed by tsetse flies [171,172]. Certainly, laboratory studies have revealed that GpSGHV infection does not significantly affect the symbiont density in certain conditions. However, a positive correlation between the GpSGHV and *Sodalis* density was observed in *Glossina fuscipes* species. In contrast, other taxa displayed a negative correlation between GpSGHV and symbiont densities. Particularly striking was the observation of the lowest *Wigglesworthia* density in *G. pallidipes*, the species most severely affected by GpSGHV infection [173].

Recent research regarding the third symbiont in tsetse flies revealed that the absence or low densities of *Wolbachia* are positively correlated with SGHV outbreaks in *G. pallidipes* colonies. This trend differs from other tsetse species that infrequently display pronounced SGH symptoms. [174]. These findings highlight the potential influence of *Wolbachia* on the susceptibility of tsetse fly to SGHV infections.

1.4.2 Iflaviruses and Negeviruses

Iflaviruses and Negeviruses have been successfully characterized in different insects. These viruses belong to different families, such as the *Iflaviridae* family, and some have even been newly discovered and classified under the *Negevirus* taxon [175,176].

The *Iflaviridae* family consists of positive-sense single-stranded RNA (+ssRNA) viruses belonging to the order *Picornavirales*. Within this family, there is a single genus known as *Iflavirus*. The genome of *Iflavirus* is comprised of +ssRNA and encodes a single, large polyprotein [175].

Viruses classified within the newly described *Negevirus* taxon are enveloped and have a positive-sense single-stranded RNA (+ssRNA) genome. [177]. Within the *Negevirus* taxon, two major distinct clades have been identified: *Nelorpivirus* which is more closely related to plant and *Sandewavirus* [178].

In tsetse flies, these two viruses have been isolated and identified from *Glossina m. morsitans* colony. These viruses have been phylogenetically categorized into the genera of *Ifavirus* (named as Glossina morsitans morsitans Iflavirus: GmmIV) and *Negevirus* (named as Glossina morsitans morsitans Negevirus: GmmNegeV). Further it has been found that both viruses are present in the host's brain, fat bodies, reproductive organs, milk glands, and salivary glands. These findings suggest the possibility of horizontal and/or vertical transmission for these

viruses. Although the potential impact of GmmIV and GmmNegeV in tsetse rearing remains unknown, it is noteworthy that none of the tsetse species currently infected with these viruses are showing any apparent symptoms [162].

1.5   Control of African Trypanosomiasis

The primary approach for controlling African Trypanosomiasis is to eliminate the parasite from the host's bloodstream, and second, managing the vector responsible for transmitting the disease [179].

1.5.1   Control in host vertebrate and limits

The control of *trypanosoma* parasites in the vertebrate hosts is widely relies in the use of trypanocidal drugs [180]. Indeed, the control strategies of Trypanosomosis in livestock involve both chemotherapy and chemoprophylaxis, which use drugs for curative and preventive purposes, respectively. The available drugs currently for chemotherapy are the phenanthridine, isometamidium and homidium, the aromatic diamidine, diminazene [34]. Diminazene aceturate is injected to treat animals already infected with trypanosomes [181], whereas isometamidium chloride is administered to protect livestock from trypanosome infections [182].

Trypanocidal drugs used to treat or prevent trypanosomiasis can sometimes be harmful and cause severe side effects even death [29]. Additionally, the emergence of resistance to trypanocidal drugs lead to treatment or prevention failures [183]. These methods are not sustainable, however, the removal of tsetse fly vector would be the most effective alternative for managing this disease [184].

1.5.2   Vector control

The ability of the trypanosome to constantly change his VSG-coated (variant-specific surface glycoproteins) hinders the development of an effective vaccine to protect both human and livestock [185]. Further, the development of drugs resistance among trypanosome [28,183] complicates the management of these diseases. In response to these challenges, vector control measures are considered more sustainable approach for the disease management.

1.5.3 Past and current methods

Historically tsetse control employed the clearing of brush and extermination of native mammals which served both as source of blood meal for tsetse fly and reservoir hosts for trypanosomes. These methods were effective for reducing tsetse fly populations, however, it can disrupt the ecosystems and affect biodiversity [186,187].

In addition, tsetse fly control had relied heavily on the use of chemical insecticides, including ground spraying, helicopter residual spraying, application of low dosage aerosols from fixed-wing aircraft and chemically impregnated traps as well as the live bait or "pour-on" techniques. However, this approach has faced several challenges and limitations, including the problem of reinvasion [188,189]. The primary insecticide compounds used for tsetse fly control were indeed DDT, dieldrin, and endosulfan. However, these products have shown to be toxic to the environment and posed risks to non-target insects. Due to these environmental and health concerns, there has been a shift toward the use of synthetic pyrethroids, which are more environmentally safe [34,190]. Moreover, efforts have been made to develop more sustainable and environmentally-friendly tsetse fly control methods, such as sterile insect technique (SIT) to minimize the ecological impact of traditional approaches.

1.5.4 The Sterile Insect Technique

The sterile insect technique (SIT) is a promising non-chemical method for tsetse fly control, especially in situations where tsetse populations are at low densities [184]. The SIT principle is based on the mass-rearing of males sterilised by ionizing radiation. Subsequently, these sterile males are released in large number in target area to outcompete their wild conspecifics in mating with wild virgin females, resulting to no offspring [191–193]. SIT is environmentally friendly and efficient when used as a part of an area-wide integrated pest management (AW-IPM) approach [31,32].

To employ the SIT technique successfully for tsetse fly control, it is crucial to ensure the production of large number of high quality males in mass-rearing facilities [194,195]. Therefore, the quality control in tsetse mass-rearing is important and involves the evaluation of various parameters such as the mating competitiveness of sterile males [196].

The SIT control tactic has been successfully implemented in Unguja Island (Zanzibar) for the eradication of *G. austeni* Newsteat and AAT in 1997 [197], also in Burkina Faso for *G. p. gambiense* and *G. tachinoides* [198] and in Ethiopia for *G. pallidipes* [199].

While the SIT can be effective in controlling tsetse fly populations, several obstacles and challenges can be encountered during SIT campaigns. For instance, long-term mass-rearing can increase the susceptibility of insects used for SIT to pathogenic infections [200], such as GpSGHV virus which can lead to a decline of *G. pallidipes* colonies within a few generations [201]. In addition symbiotic bacteria (*Wigglesworthia*, *Sodalis*, *Wolbachia* and *Spiroplasma*) can influence the performance of these flies [110,124]. Furthermore, radiation has no significant effect on the vectorial competence of *G. m. morsitans* mass-reared tsetse fly [202] and the sterilized males used in SIT may transmit the trypanosome during blood feeding [113].

The fact that sterile males maintaining their vector competency and are intended for release in areas where human sleeping sickness is occurring might increase the risk of disease transmission. This represents an ethical issue associated with the SIT approach. Hence the proposal of paratransgenesis approach to produce tsetse fly refractory to trypanosome infection was investigated [113].

1.6   Development of tsetse paratransgenesis

Paratransgenesis is a genetic method that involves modifying the symbiotic organisms associated with insect vectors using DNA recombinant technologies. The goal of paratransgenesis is to manipulate these symbiotic organisms to express effector molecules that can potentially block the development of parasites/pathogens within the insect vector [136,203]. This approach was primarily used in the context of vector-borne diseases, where insects like mosquitoes act as vectors that transmit pathogens such as parasites or viruses to humans. In tsetse fly, paratransgenesis can be an alternative approach to be combine in the SIT program to manage Trypanosomiasis [113,204,205].

It has been demonstrated that *Sodalis* can be genetically modified to express nanobodies targeting the trypanosome surface epitope in various tsetse tissues [206]. Additionally, the symbiont can also recovered over time after radiation treatment offering the opportunity to combine SIT and paratrangenesis [202].

Objectives of the Thesis

The goal of this thesis is to evaluate the interactions between tsetse symbionts, pathogens on trypanosome infections in wild tsetse populations. This research aims to enhance our understanding of the dynamics of trypanosome infections associated with tsetse fly bacterial symbionts and pathogens, to optimize effectiveness and safety of SIT.

Chapter 1, gives an insight into the existing literature in Trypanosomiasis, tsetse flies systematics and taxonomy, their biology and anatomy. It also presents the currents state of knowledge regarding the roles of bacterial endosymbiont, and relevant pathogens in trypanosome infections in tsetse wild tsetse populations. The available methods of vector control and the SIT technique are as well reviewed in detail. Finally, this chapter offers an overview of advanced research in paratransgenesis and the potential for integration with SIT.

In chapter 2, the prevalence of *Trypanosoma* and *Sodalis* in wild tsetse populations were evaluated in order to determinate their impact on SIT programmes. The screen of several tsetse taxa from different countries and localities revealed the evolution of the status of the *Trypanosoma* and *Sodalis* infections in a continent-wide scale. The evaluation of the association between the parasite and the symbiont has proposed some interesting guidelines for decision-makers to enhance the SIT safety.

In chapter 3, it was challenging to evaluate the prevalence and the interactions between the newly discover tsetse fly fourth symbiont and trypanosome infection in wild tsetse populations. *G. tachinoides,* in particular, hosts a phylogenetically characterized *Spiroplasma,* and this tsetse specie is of extreme concern in the transmission of HAT in west and central Africa. The information from this study reinforces that this symbiont may improve tsetse fly refractoriness to trypanosome infection and could serve as a potential candidate for paratransgenesis.

In chapter 4, the prevalence of virus infections such as *Glossina pallidipes* salivary gland hypertrophy virus is known to be low in wild tsetse populations. Evaluating the status of the GpSGHV infection in different tsetse species was crucial to gain insights into the interactions between the virus and tsetse symbionts in field condition. The findings may provide guidance for preventing virus infections in tsetse mass-reared for SIT programs.

In chapter 5, the conclusion drafts a general discussion of the findings in all the chapters in relation to the thesis scope. Additionally, it synthetizes the involvement of the thesis in SIT programmes follows up by the recommendation for further studies.

Chapter 2

Prevalence of *Trypanosoma* and *Sodalis* in wild populations of tsetse flies and their impact on Sterile Insect Technique Programmes for tsetse eradication

scientific reports

Prevalence of *Trypanosoma* and *Sodalis* in wild populationsof tsetse flies and their impact on sterile insect technique-programmes for tsetse eradication

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The sterile insect technique (SIT) is an environment friendly and sustainable method to manage insect pests of economic importance through successive releases of sterile irradiated males of the targeted species to a defined area. A mating of a sterile male with a virgin wild female will result in no offspring, and ultimately lead to the suppression or eradication of the targeted population. Tsetse flies, vectors of African *Trypanosoma,* have a highly regulated and defined microbial fauna composed of three bacterial symbionts that may have a role to play in the establishment of *Trypanosoma* infections in the flies and hence, may influence the vectorial competence of the released sterile males. *Sodalis* bacteria seem to interact with *Trypanosoma* infection in tsetse flies. Field-caught tsetse flies of ten different taxa and from 15 countries were screened using PCR to detect the presence of *Sodalis* and *Trypanosoma* species and analyse their interaction. The results indicate that the prevalence of *Sodalis* and *Trypanosoma* varied with country and tsetse species. Trypanosome prevalence was higher in east, central and southern African countries than in west African countries. Tsetse fly infection rates with *Trypanosoma vivax* and *T. brucei* sspp were higher in west African countries, whereas tsetse infection with *T. congolense* and *T. simiae, T. simiae* (*tsavo*) and *T. godfreyi* were higher in east, central and south African countries. *Sodalis* prevalence was high in *Glossina morsitans morsitans* and *G. pallidipes* but absent in *G. tachinoides.* Double and triple infections with *Trypanosoma* taxa and coinfection of *Sodalis* and *Trypanosoma* were rarely observed but it occurs in some taxa and locations. A significant Chi square value (< 0.05) seems to suggest that *Sodalis* and *Trypanosoma* infection correlate in *G. palpalis gambiensis*, *G. pallidipes* and *G. medicorum*. *Trypanosoma* infection seemed significantly associated with an increased density of *Sodalis* in wild *G. m. morsitans* and *G. pallidipes* flies, however, there was no significant impact of *Sodalis* infection on trypanosome density.

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Abbreviations

SIT	Sterile insect techniques
qPCR	Quantitative polymerase chain reaction
BKF	Burkina Faso
ETH	Ethiopia
GHA	Ghana
GUI	Guinea
KEN	Kenya
MLI	Mali
MOZ	Mozambique
SAF	South Africa
SWA	Eswatini
ZAI	Democratic Republic of the Congo
ZAM	Zambia
ZIM	Zimbabwe
Ga	*Glossina austeni*
Gb	*G. brevipalpis*
Gff	*G. fuscipes fuscipes*
Gmm	*G. morsitans morsitans*
Gmsm	*G. m. submorsitans*
Gpg	*G. palpalis gambiensis*
Gpp	*G. p. palpalis*
Tc	*Trypanosoma congolense*
Tv	*Trypanosoma vivax*
Tz	*T. brucei sspp.*

Tsetse flies (Diptera: Glossinidae) are distributed in sub-Saharan Africa between 15° north and 26° south latitude[1]. *Glossina* spp. are the cyclic vectors[2] of unicellular protozoa of the genus *Trypanosoma* that cause African animal trypanosomosis (AAT) or nagana and human African trypanosomosis (HAT) or sleeping sickness[3,4]. Nagana in cattle is mainly caused by *T. congolense*, *T. vivax* and *T. brucei brucei*[5] and causes annual losses to agriculture estimated at $4.75 billion[6]. In addition, around 35 million doses of trypanocidal drugs are administered to live-stock per year for managing AAT[7]. Human African trypanosomosis is fatal without treatment[8] and is caused by two *Trypanosoma* subspecies, i.e. *T. brucei rhodesiense* responsible for the acute form of HAT in East Africa and *T. b. gambiense* for the chronic form of HAT in western and central Africa[9]. The lack of effective vaccines and the development of resistance to the available trypanocidal drugs makes the control of AAT in the vertebrate host unsustainable[10,11]. Consequently, an effective tool to reduce *Trypanosoma* transmission would be the control of the tsetse vector. One effective method to manage populations of tsetse flies is the sterile insect technique (SIT) when used as part of an area-wide integrated pest management (AW-IPM) approach[12,13]. The SIT method relies on the mass-production and sterilization of male flies by ionizing radiation. The sterile males are released in the target area for mating with wild females and the absence of offspring will gradually reduce the density of the targeted tsetse populations[14].

The biological transmission of the *Trypanosoma* species requires the parasite to undergo a series of proliferation and differentiation steps in the tsetse alimentary tract and finally mature into an infective form in the mouthparts (*T. congolense*) or salivary glands (*T. brucei* spp.)[15]. However, tsetse flies are refractory to *Trypanosoma* infection meaning that the probability that *Trypanosoma* ingested during a blood meal complete their developmental cycle in the fly to result in a mature infection is rather low[16–18]. The endogenous bacterial microbiome seems important in providing tsetse flies the natural ability to mitigate *Trypanosoma* infections[19]. Three major endosymbiotic bacteria have been identified in tsetse flies, i.e. *Wigglesworthia glossinidia*, *Sodalis glossinidius* (hereafter mentioned as *Sodalis*) and *Wolbachia pipientis*[20]. Some studies suggested that the obligate mutualist *Wigglesworthia* must be present in the larval stage during the development of a mature tsetse fly to properly develop a well-functioning immune system contributing to a refractory phenotype against *Trypanosoma*[5,19].

Sodalis, the second mutualistic symbiont, can be found in the midgut, hemolymph, muscles, fat body, milk glands, and salivary glands of certain tsetse species and is inherited by the progeny through transovarial transmission[21]. The biological role/importance of *Sodalis* for tsetse remain unclear and needs to be clarified[22]. This symbiont might provide some benefits to the host as flies without *Sodalis* have a significantly shorter lifespan as compared with flies with it[23], however the establishment of a *Sodalis* free colony was feasible[24]. *Sodalis* also presents many ideal characteristics to be used for expressing molecular effectors in paratransgenic tsetse[25]. In addition, previous work suggested that *Sodalis* may modulate the ability of *Trypanosoma* to establish an infec-tion in the tsetse midgut as some studies reported that the elimination of this bacterial endosymbiont results in an increased tsetse fly refractoriness to *Trypanosoma* infection[23,26,27]. Moreover, Geiger et al.,[28] suggested that specific genotypes of *Sodalis* presents in *G. p. gambiensis* from insectary colonies facilitate *Trypanosoma* infection. Soumana et al.,[29] revealed that a variation in the *Sodalis* population caused by a hosted prophage can influence the trypanosome infections. In contrast, a recent study demonstrated that the absence or presence of *S. glossinidius* in the tsetse fly does not affect the fly's susceptibility toward *Trypanosoma* infection[24]. In conclusion, from the above-described results, it is clear that our knowledge on the impact of *Sodalis* on *Trypanosoma* infection in tsetse remains limited and fragmented and is still under debate[23]. Moreover, exploring on a large scale the occurrence and possible association between *Sodalis* and *Trypanosoma* infection in wild flies is highly required. The above-described potential impact of *Sodalis* to facilitate *Trypanosoma* infection in tsetse, and the fact that *Sodalis* is

Region	Country	*Sodalis* prevalence (%)*	Trypanosome prevalence (%)
	Ethiopia	94/459 (20.48)[a,b,e]	92/459 (20.04)[a,d,e]
East, central and southern Africa	Kenya	288/1008 (28.57)[a,b]	448/1008 (44.44)[a,b,e]
	Democratic R. of Congo	4/35 (11.43)[a,b,e]	1/35 (2.86)[a,e]
	Mozambique	7/100 (7.00)[a,b,e]	80/526 (15.21)[a,e]
	South Africa	9/526 (1.71)[a,c,e]	0/30 (0.00)[a,e]
	Eswatini	0/30 (0.00)[a,b,c,e]	8/100 (8.00)[a,e]
	Tanzania	227/338 (67.16)[a,d,c]	128/338 (37.87)[a,e]
	Uganda	91/210 (43.33)[d]	19/210 (9.05)[a,c,e]
	Zambia	11/210 (5.24)[a,b,e]	97/210 (46.19)[a,d,e]
	Zimbabwe	39/211(18.48)[a,b,e]	113/211 (53.55)[a,e]
	Subtotal	770/3127 (24.62)	986/3127 (31.53)
West Africa	Burkina Faso	11/2274 (0.48)[a,e]	498/2274 (21.90)[a,e]
	Ghana	0/234 (0.00)[a,e]	143/234 (61.11)[a,d]
	Guinea	90/314 (28.66)[a,e]	7/314 (2.22)[a,c]
	Mali	0/364 (0.00)[a,e]	25/364 (6.86)[a,c,e]
	Senegal	0/547 (0.00)[a,e]	78/547 (14.25)[a,e]
	Subtotal	101/3733 (2.70)	750/3733 (20.09)
	Total (average)	871/6860 (12.69)	1736/6860 (25.30)

Table 1. Global prevalence of *Sodalis* and Trypanosomes in tsetse samples analyzed per country. *Values indicated by the same lower-case letter do not differ significantly at the 5% level.

found in all laboratory-reared tsetse colonies and some wild populations[21] indicates that mitigating action, such as feeding the flies 2–3 times on blood supplemented with trypanocidal drugs before release, is required in SIT programs to minimize the risk of disease transmission by the large number of released males that harbour *Sodalis*. Field studies in two HAT foci in Cameroon used PCR to detect *Trypanosoma* and *Sodalis* in *G. palpalis pal-palis* and the results indicate that the presence of *Sodalis* favours *Trypanosoma* infections especially by *T. brucei* s.l.[30]. Furthermore, in the wildlife-livestock-human interface in the Maasai Mara National Reserve in Kenya, itwas shown that *G. pallidipes* infected with *Sodalis* was associated with increased *Trypanosoma* infection rates[31]. However, other studies have found no strong association between trypanosome and *Sodalis* in some tsetse species collected in four locations in Kenya[32]. Channumsin et al.,[33] suggested that the association between *Trypanosoma* infection and the presence of *Sodalis* will vary depending on tsetse and *Trypanosoma* species. Similarly, studies carried out in the Fontem focus in Cameroon did not find a relationship between the endosymbiont and the parasite in *G. p. palpalis*[34], and no significant *Sodalis-Trypanosoma* infection association was found in *G. tachi- noides* in two sites of the Faro and Déo Division in Adamawa region of Cameroon[35]. Likewise, no association between the presence of the parasite and *Sodalis* was found in *G. brevipalpis*, *G. m. morsitans* and *G. pallidipes* in the Luambe National Park of Zambia[36].

The overall objective of this study was to evaluate the prevalence of *Sodalis* and *Trypanosoma* in wild tsetse populations at a continental scale, i.e. Burkina Faso, Democratic Republic of Congo (DRC), Eswatini, Ethiopia, Ghana, Guinea, Kenya, Mali, Mozambique, Senegal, South Africa, Tanzania, Uganda, Zambia, and Zimbabwe and analyse these data in the context of a possible association between the occurrence of *Sodalis* and *Trypanosoma* infection in tsetse. Such information might guide the decision maker for SIT programmes to take the appropriate action, if necessary, to minimize any potential risk of increased transmission.

Results

***Trypanosoma* prevalence.** Adult tsetse flies (n=6860) were screened for infection with *T. brucei* sspp (Tz) (*T. b. brucei, T. b. gambiense, T. b. rhodesiense)*, Tc (*T. congolense* savannah; *T. congolense* kilifi; *T. congolense* for-est); Tsg (*T. simiae*; *T. simiae* tsavo; *T. godfreyi*) and Tv (*T. vivax*). The results indicate that 1736 (25.3%) adultswere infected with one or more *Trypanosoma* taxa (Tables 1, 2 and 3), The *Trypanosoma* prevalence varied sig-nificantly between tsetse taxa ($X^2 = 750.18$, $df = 9$, $P \ll 0.001$) and between countries ($X^2 = 2038.1$, $df = 14$, $P \ll 0.001$). The Permanova analysis indicated as well significant differences between countries ($P = 0.009$) and taxa ($P = 0.041$) (Table 4). As all taxa were not collected from all countries, the interaction between taxa and countries was only analyzed where a taxon was collected from several countries.

Regardless of tsetse taxon, in west African countries the average *Trypanosoma* prevalence was 20% ($n = 3733$), with the highest prevalence recorded in Ghana (61%) and the lowest recorded in Guinea (2.2%). The prevalencein Burkina Faso, Mali and Senegal was 21.9, 6.9 and 14.2% respectively (Fig. 1, and Table 1). In east, central and southern African countries, the *Trypanosoma* infection prevalence was a bit higher than in west African countries with an averaged infection of 31.5% ($n = 3127$), with the highest prevalence (53.6%) in Zimbabwe and lowest prevalence (2.9%) in DRC. No *Trypanosoma* infection was detected in Eswatini (Fig. 1 and Table 1). Regard- less of the country, *Trypanosoma* prevalence varied from one taxon to another, and *G. m. morsitans* showed the highest *Trypanosoma* prevalence (41%) followed by *G. pallidipes* (38.5%) and the lowest prevalence was detected

27

Species	*Sodalis* prevalence (%)*	Trypanosome prevalence (%)
G. austeni	5/346 (1.44)[a]	58/346 (16.76)[a]
G. brevipalpis	14/350 (4)[a]	34/350 (9.71)[a]
G. f. fuscipes	24/183 (13.11)[a,b]	31/183 (16.93)[a]
G. medicorum	8/154 (5.2)[a]	61/154 (39.6)[a,b]
G. m. morsitans	156/369 (42.27)[b]	152/369 (41.19)[a]
G. m. submorsitans	1/343 (0.29)[a]	62/343 (18.07)[a]
G. pallidipes	567/1844 (30.74)[b]	711/1844 (38.55)[a,b]
G. p. gambiensis	92/2168 (4.24)[a]	343/2168 (15.82)[a]
G. p. palpalis	4/35 (11.4)[a,b]	1/35 (2.8)[a,b]
G. tachinoides	0/1068 (0.0)[a]	283/1068 (26.49)[b]
Total (average)	871/6860 (12.6)	1736/6860 (25.3)

Table 2. Global prevalence of *Sodalis* and Trypanosomes in tsetse samples analyzed per tsetse species. *Values indicated by the same lower-case letter do not differ significantly at the 5% level.

Species	Country	*Sodalis* prevalence (%)*	Trypanosome prevalence (%)
G. austeni	Mozambique	0/50 (0.00)	5/50 (10.00)
	South Africa	2/226 (0.88)	49/226 (21.68)
	Eswatini	0/30 (0.00)	0/30 (0.00)
	Tanzania	3/40 (7.50)	4/40 (10.00)
G. brevipalpis	Mozambique	7/50 (14.00)[a]	3/50 (6.00)
	South Africa	7/300 (2.33)[b]	31/300 (10.33)
G. f. fuscipes	Kenya	20/89 (22.47)	21/89 (23.60)
	Uganda	4/94 (4.25)	10/94 (10.63)
G. medicorum	Burkina Faso	8/154 (5.20)	61/154 (39.61)
G. m. morsitans	Kenya	54/85 (63.52)[a]	2/85 (2.35)
	Tanzania	62/81 (76.54)[a]	43/81 (53.08)
	Zambia	8/64 (12.50)[b]	31/64 (48.43)
	Zimbabwe	32/139 (23.02)[b]	75/139 (53.95)
G. m. submorsitans	Burkina Faso	1/343 (0.30)	62/343 (18.07)
G. pallidipes	Ethiopia	94/459 (20.48)[a,b,c]	92/459 (20.04)
	Kenya	214/834 (25.65)[a,c]	425/834 (50.95)
	Tanzania	162/217 (74.65)[a,b]	81/217 (37.32)
	Uganda	87/116 (75.00)[a,b]	9/116 (7.75)
	Zimbabwe	7/72 (9.72)[a,c]	38/72 (52.77)
	Zambia	3/146 (2.05)[a,b,c]	66/146 (45.20)
G. p. palpalis	Democratic R. of Congo	4/35 (11.42)	1/35 (2.86)
G. p. gambiensis	Burkina Faso	2/943 (0.21)	235/943 (24.92)[a]
	Guinea	90/314 (28.66)	7/314 (2.22)[b]
	Mali	0/364 (0.00)	25/364 (6.87)[b,c]
	Senegal	0/547 (0.00)	78/547 (14.25)[c]
G. tachinoides	Burkina Faso	0/834 (0.00)	140/834 (16.79)[a]
	Ghana	0/234 (0.00)	143/234 (61.11)[b]
Total (average)		871/6860 (12.69)	1736/6860 (25.30)

Table 3. Global prevalence of *Sodalis* and trypanosomes in tsetse samples analyzed per country and tsetse species. *Values indicated by the same lower-case letter do not differ significantly at the 5% level

in *G. brevipalpis* (9.71%) in east, central and southern Africa. In west Africa, *G. medicorum* showed the high-est *Trypanosoma* prevalence (39.5%) and the lowest prevalence was detected in *G. p. palpalis* (2.8%) (Table 2).

Some tsetse taxa were collected from several countries as presented in Fig. 2 and Table 3. The highest *Trypanosoma* prevalence was recorded in *G. tachinoides* in Ghana (61%). This was followed by high prevalence in *G. m. morsitans* collected from Zimbabwe (53.9%), Tanzania (53%) and Zambia (48.4%). *G. pallidipes* from Zimbabwe, Kenya, Zambia and Tanzania also showed high *Trypanosoma* prevalence of 52.7%, 50.9%, 45.2% and 37.3%, respectively. The lowest *Trypanosoma* prevalence was found in *G. p. gambiensis* from Guinea (2.2%). Based on the *Trypanosoma* prevalence presented in Fig. 2 and Table 3, the tested samples can be categorized as: (i) tsetse samples with high prevalence (>35%) detected in *G. tachinoides* from Ghana; *G. medicorum* from Burkina Faso,

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Countries	11	13,040	1185.4	2.6004	**0.009**	998
Species	7	7899.8	1128.5	2.4756	**0.041**	999
Residuals	5	2279.3	455.87			
Total	25	34,074				

Table 4. Permanova analysis for Countries and tsetse species for *Sodalis* and trypanosome (single and multiple) infection prevalence. Within the table, statistically significant differences (*P*<0.05) can be seen in bold values in countries and tsetse species. Perm(s)=permutations.
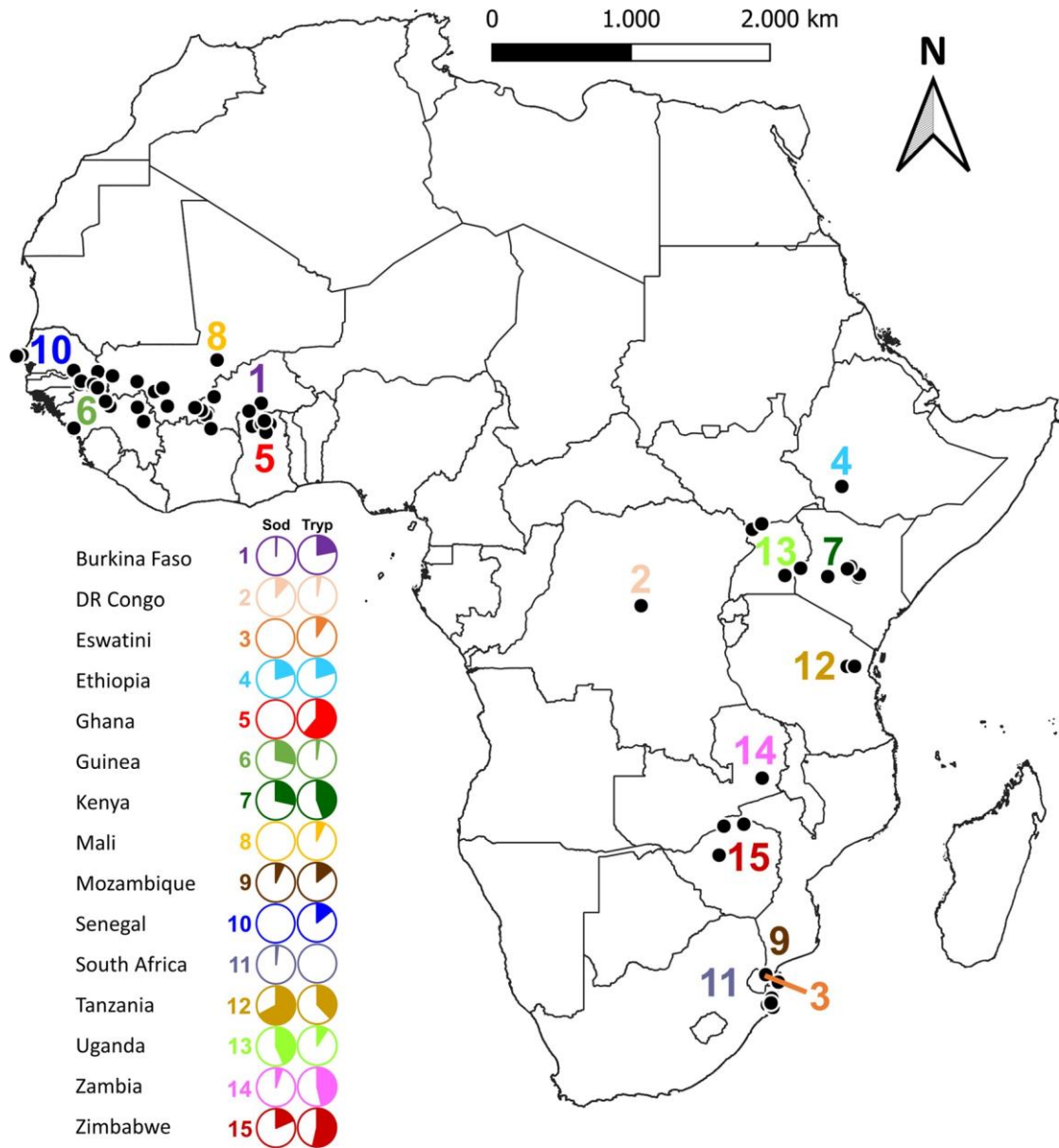
Figure 1. The geographical locations of tsetse samples in Africa. Circles indicate the total prevalence of *Sodalis* and *Trypanosoma* per country. Black dots indicate samples collection site(s) per country.

G. pallidipes from Kenya, Zambia, and Zimbabwe, *G. m. morsitans* from Tanzania, Zambia, and Zimbabwe; (ii) tsetse samples with medium prevalence (10–35%) detected in *G. austeni* from South Africa, *G. f. fuscipes* from Kenya and Uganda, *G. m. submorsitans* from Burkina Faso, *G. p. gambiensis* from Burkina Faso and Senegal and *G. tachinoides* from Burkina Faso; (iii) tsetse samples with low prevalence (< 10%) detected in the rest of the samples listed in Table 3 except the *G. austeni* collected from Eswatini. Despite the difference in *Trypanosoma*
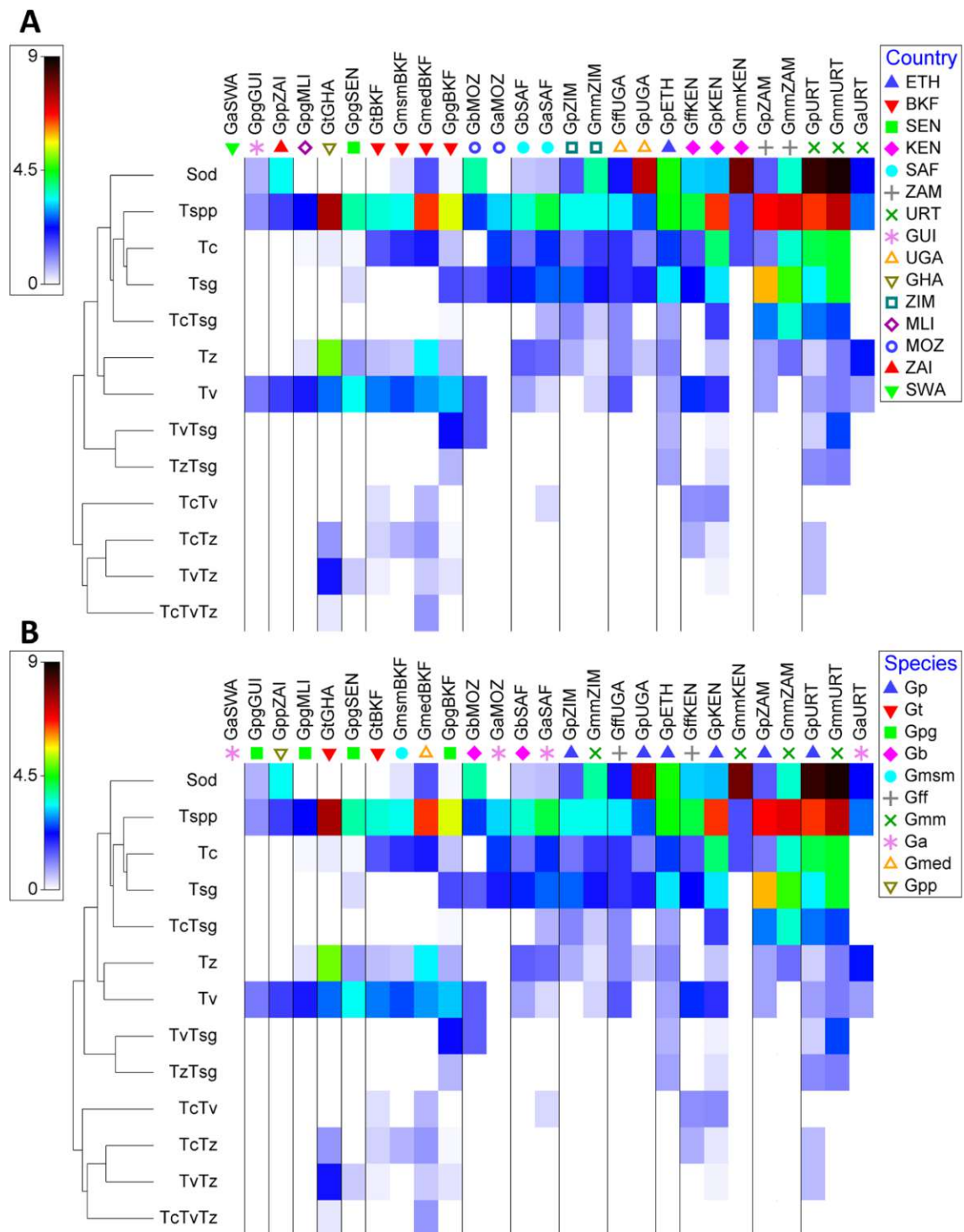
Figure 2. Prevalence of the *Sodalis* and *Trypanosoma* (single and multiple) infections per country (**A**) and tsetse species (**B**). Prevalence data were square root transformed and averaged based on country-species and the matrix display was conducted in PRIMER version 7+ software. Tree on the left of the matrix is the similaritydendrogram based on the similarity index of the square root of the prevalence values. The colour index is the square root of the prevalence values ranged 0–9 which is the square root of 0–81% prevalence. Country abbreviations follow the UNDP list of country codes https://web.archive.org/web/20060713221355/http://refgat.undp.org/genericlist.cfm?entid=82&pagenumber=1&requesttimeout=360 as follows: BKF: Burkina Faso; ETH: Ethiopia; GHA: Ghana; GUI: Guinea; KEN: Kenya; MLI: Mali; MOZ: Mozambique; SAF: South Africa; SWA: Eswatini; ZAI: Democratic Republic of the Congo; ZAM: Zambia; ZIM: Zimbabwe. Tsetse, *Sodalis* and *Trypanosoma* taxa were abbreviated as following: Ga: *Glossina austeni*; Gb: *G. brevipalpis*; Gff: *G. fuscipes fuscipes*, Gmm: *G. morsitans morsitans*; Gmsm: *G. m. submorsitans*; Gpg: *G. palpalis gambiensis*; Gpp: *G. palpalispalpalis*. Sod: *Sodalis*, Tc: *Trypanosoma. congolense* savannah; *T. congolense* kilifi; *T. congolense* forest, Tsg: *T. simiae*; *T. simiae* Tsavo; *T. godfreyi*, Tv: *T. vivax,* Tz: *T. brucei brucei, T. b. gambiense, T. b. rhodesiense*.

prevalence for each tsetse species, the differences were significant only in *G. p. gambiensis* ($X^2 = 26.71$, *df* = 4, *P* < 0.001) and *G. tachinoides*, ($X^2 = 9.38$, *df* = 1, 2, *P* = 0.002). In contrast, no significant difference was detected between countries for *G. austeni* ($X^2 = 1.47$, *df* = 4, *P* = 0.688), *G. brevipalpis* ($X^2 = 0.34$, *df* = 2, *P* = 0.559), *G. f. fuscipes* ($X^2 = 0.15$, *df* = 2, *P* = 0.702), *G. m. morsitans* ($X^2 = 1.04$, *df* = 3, *P* = 0.593) and *G. pallidipes* ($X^2 = 4.983$, *df* = 1,6, *P* = 0.418) (Table 3). No *Trypanosoma* infection was recorded in *G. austeni* from Eswatini. The best glm model (lowest AICc) selected for the overall *Trypanosoma* prevalence retained the countries as variables that fitted the data well (AICc = 1521.35) (Supplementary File 1).

Prevalence of different *Trypanosoma* taxa and mixed infections. The above-mentioned prevalence of *Trypanosoma* infection was comprised of several different *Trypanosoma* species and sub-species. Based on the size of the amplified fragment by PCR, the *Trypanosoma* infection was categorized into four groups: (i) the Tc group including the different forms of *T. congolense*; (ii) Tv group including *T. vivax* infections; (iii) *T.brucei* sspp (Tz) group including *T. b. brucei, T. b. gambiense, T. b. rhodesiense,* and infections; and (iv) Tsg group including the infections with *T. simiae, T. simiae* tsavo and *T. godfreyi*. The screening results revealed that tsetseflies could be infected with single or multiple (double or triple) taxa of *Trypanosoma*, and the proportion of theinfections with the different *Trypanosoma* taxa and the mixed infection varied with country ($X^2 = 63.56$, *df* = 14,P < 0.001) and species ($X^2 = 21.86$, *df* = 9, P < 0.001) (Supplementary File 1).

The prevalence of the different *Trypanosoma* species with respect to the above-mentioned groups, indicate that infections with the Tsg group was the highest regardless of countries or tsetse species with an average of 7.06%. The infection rate was higher (14.13%) in east, central and southern African countries than in west Africa (1.13%). Tv infection averaged at 6.75% but with higher prevalence in west African countries (10.37%) than in east, central and southern Africa (2.43%). The prevalence of Tc infection was lower than Tv and Tsg group with an average of 4.78% with higher prevalence in central and southern Africa (8.38%) than in west Africa (1.77%). The Tz group had the lowest prevalence with an average of 2.29%. Like Tv infection, the Tz prevalence was higher in west Africa (3.16%) than central and southern Africa (1.25%).

The prevalence of infection by a single *Trypanosoma* group varied significantly from one country to another and from one tsetse species to another. For Tc, Tv, Tz and Tsg the infection prevalence varied significantly with country ($X^2 = 47.74$, *df* = 14, *P* < 0.001, $X^2 = 27.40$, *df* = 14, *P* = 0.01705, $X^2 = 106.11$, *df* = 14, *P* = 0. 001 and, $X^2 = 44.74$, *df* = 14, *P* = 0.001 respectively). Regardless of tsetse species, the highest infection rate for Tc, Tv, Tz and Tsg was found in Tanzania (14.20%), Ghana (14.10%), Ghana (19.66%) and Zimbabwe (39.81%), respectively (Supplementary Table 1). Similarly, the prevalence of Tc, Tz and Tsg varied significantly with tsetse species ($X^2 = 40.364$, df = 1.9, *P* << 0.001, $X^2 = 58.253$, df = 1.9, *P* << 0.001 and $X^2 = 34.871$, df = 1.9, *P* << 0.001, respectively), however no significant difference was found in Tv prevalence between tsetse species ($X^2 = 5.475$, df = 1.9, *P* = 0.07868). Regardless of the country, the highest infection rate of Tc, Tv, Tz and Tsg was found in *G. pallidipes* (10.68%), *G. tachinoides* (12.92%), *G. medicorum* (13.64%) and *G. m. morsitans* (22.76%), respectively (Sup- plementary Table 2). No Tc infection was found in samples of *G. austeni* collected from Eswatini and Tanzania, *G. brevipalpis* from Mozambique, *G. p. palpalis* from DRC and *G. p. gambiensis* from Guinea. In addition, no Tv infection was detected in *G. austeni* collected from Eswatini and Mozambique, *G. m. morsitans* from Kenya and Zambia, *G. pallidipes* from Uganda and Zimbabwe. For Tz, *G. austeni* collected from Eswatini and Mozambique, *G. brevipalpis* from Mozambique, *G. f. fuscipes* from Kenya, *G. m. morsitans* from Kenya and Zambia, *G. p. palpalis* from DRC and *G. p. gambiensis* from Guinea did not show any infection (Fig. 2 and Supplementary Table 3).

Mixed infections of *Trypanosoma* groups (double or triple) are rare events with an average prevalence between 0.09 and 1.71% regardless of country or tsetse species. However, double infections seem to be more frequent in some countries than others($X^2 = 35.01$, *df* = 14, *P* = 0.001) for Tv–Tz and in some tsetse species than others($X^2 = 21.20$, *df* = 9, *P* = 0.012) for Tv–Tz (Supplementary File 1). The highest prevalence of the mixed infections Tv–Tz and Tc–Tz were observed in Ghana with 12.39% and 10.68%, respectively, regardless of tsetse species.Although the average Tc–Tsg prevalence was higher than that of Tv–Tz and Tc–Tz, the highest mixed infection with it was found in Zambia with 9.05%. Regardless of the country, the highest mixed infection of Tc–Tsg detected per tsetse species was ~5% in *G. m. morsitans* and *G. pallidipes*. The mixed infection of Tsg with either Tv or Tz or both was lower than 2% regardless of the country or tsetse species. Taking into account both the countryand tsetse species, the highest mixed infection of Tc–Tsg (12.5%) was detected in *G. m. morsitans* in Zambia. However, the highest prevalence of Tc–Tz (10.68%) and Tv–Tz (12.39%) was detected in *G. tachinoides* from Ghana. Although the average prevalence of Tv–Tsg was low (0.54%), a relative high infection rate of 6.17% was found in *G. m. morsitans* from Tanzania.

A triple infection of *Trypanosoma* groups (Tc–Tv–Tz) was only detected in *G. medicorum* from Burkina Faso (1.30%) and *G. tachinoides* from Ghana (1.71%) (Fig. 2 and Supplementary Table 3, Supplementary File 1).

Prevalence of *Sodalis* infection. The prevalence of *Sodalis* infection based on the PCR results varied significantly with country ($X^2 = 108.02$, df = 1, 14, *P* << 0.001) and tsetse species ($X^2 = 69.60$, *df* = 9, *P* < 0.001). Thebest glm model (lowest AICc) selected for the overall *Sodalis* prevalence retained the countries, the species andtheir interaction (where possible) as variables that fitted the data well (AICc = 1296.12). Similar to the prevalenceof *Trypanosoma*, the average *Sodalis* prevalence in east, central and southern Africa (24.6%) was higher than inwest Africa (2.70%). Regardless of tsetse species, the highest prevalence of *Sodalis* infection was found in Tanza- nia (67.1%) followed by Uganda (43.3%), Kenya (28.5%) and Ethiopia (20.48%) (Table 1). The highest prevalence of *Sodalis* infection in west Africa was found in Guinea (28.6%). No *Sodalis* infection was found in Ghana, Mali, Senegal or Eswatini. Regardless of the country, the highest *Sodalis* prevalence per tsetse species was detected in *G. m. morsitans* (42.27%) followed by *G. pallidipes* (30.74%). No *Sodalis* infection was detected in *G. tachinoides*. The prevalence of *Sodalis* infection changed when both the countries and tsetse species are taken into
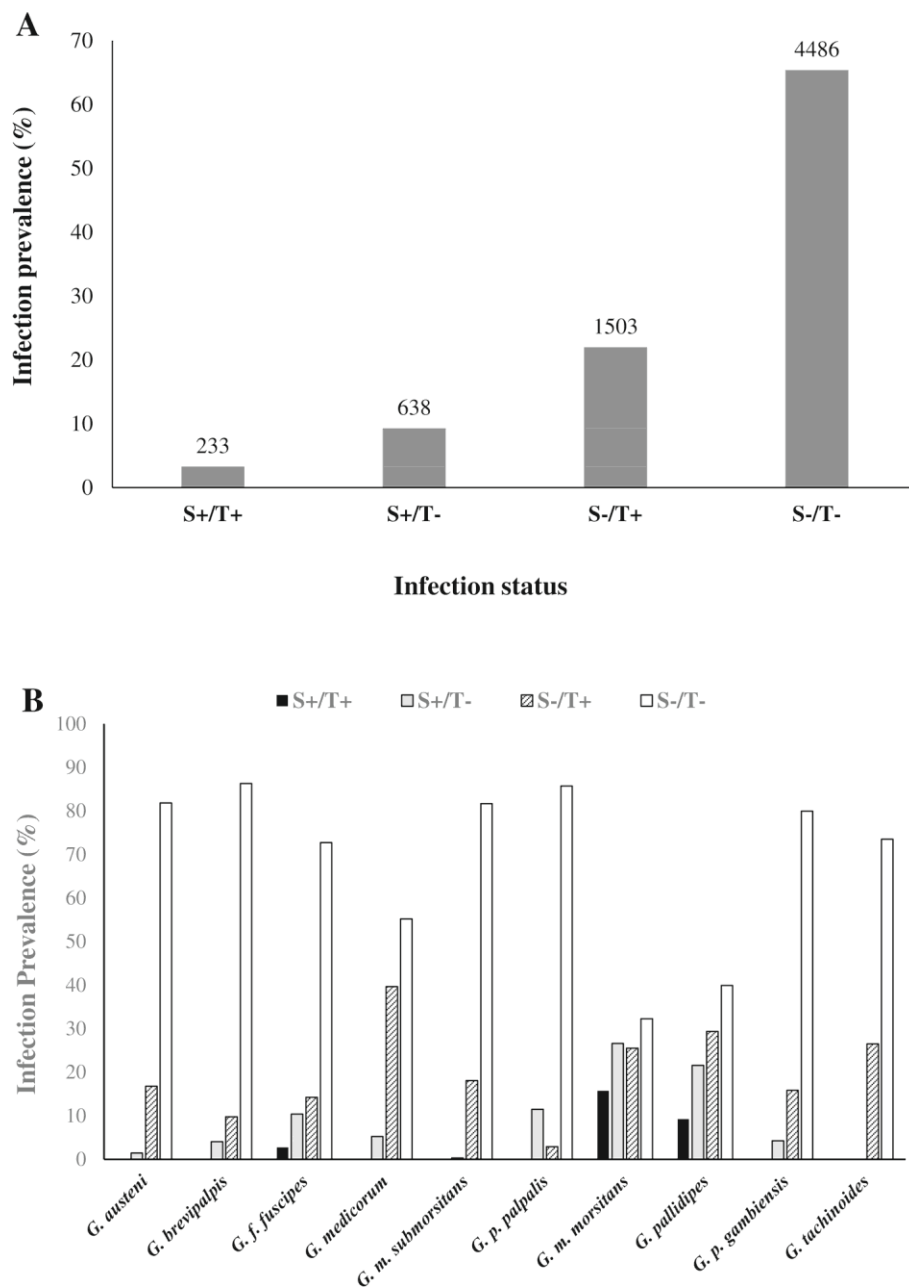
31

Figure 3. Prevalence of coinfection of *Sodalis* and *Trypanosoma* infection in wild tsetse populations. (**A**) Prevalence of coinfection, (**B**) prevalence of coinfection by tsetse taxa.

consideration (Table 4). Based on the *Sodalis* prevalence the tsetse samples can be categorized into four groups: (i) samples with high prevalence (> 50%) (ii) samples with medium prevalence (between < 10% and > 50%) (iii) samples with low prevalence (between > 0% and 10%) and (iv) samples with no *Sodalis* infection as shown in Fig. 2 and Table 4. The samples showing high *Sodalis* prevalence includes *G. m. morsitans* from Kenya (63.5%)and Tanzania (76.5%) and *G. pallidipes* from Tanzania (74.6%) and Uganda (75%), however the samples with no *Sodalis* infection includes *G. austeni* from Eswatini, *G. p. gambiensis* from Mali and Senegal and *G. tachinoides*from Burkina Faso and Ghana indicating that there is 95% confidence that the infection rate is less than or equal to 10%, 0.82%, 0.55%, 1.28% and 0.36%, respectively.

Interactions between *Sodalis* and *Trypanosoma* infections. *Prevalence of co-infections of Sodalis with Trypanosoma.* The screening results indicated that the single infection rate was 9.3% (n = 638) and 21.9%(n = 1503) for *Sodalis* and *Trypanosoma,* respectively, over all taxa (Fig. 3A). No *Sodalis* infection was found in *G. tachinoides*, and therefore was excluded from the analysis. A Cochran–Mantel–Haenszel test for repeated tests of independence showed that infection with *Sodalis* and *Trypanosoma* did deviate from independence across all taxa (χ^2 = 41.73, df = 1, P < 0.001) and individual Chi squared tests for independence for each taxon showed significant deviation from independence at the Bonferroni corrected α = 0.00833 in *G. pallidipes*(P < 0.001) and *G. p. gambiensis* (P < 0.001)

(Supplementary Table 4). The prevalence of coinfection of *Sodalis* and *Trypanosoma* in wild tsetse populations varied with tsetse taxon and location. No coinfection was found in many taxa and many locations. The co-infection was found only in *G. f. fuscipes* (2.73%), *G. m. morsitans* (15.72%) and *G. pallidipes* (9.22%) in east, central and southern Africa (Fig. 3B, Table 5 and Supplementary Table 4).

Impact of co-infection on Trypanosoma and Sodalis density. Attempts were to assess the density of *Trypanosoma* and *Sodalis* under single (S⁻/T⁺) and (S⁺/T⁻) or double infection (S⁺/T⁺) conducted using qPCR with primers mentioned in Supplementary Table 5. The results show that *Sodalis* infections did not have a significant impact on *Trypanosoma* density ($X^2 = 0.648$, $df = 2$, $P = 0.723$), however the median value of (S⁺/T⁺) files were slightly (S⁻/T⁺) lower than (S⁺/T⁻) and (S⁻/T⁺) flies and the number of outlier samples with higher trypanosome density (S⁻/T⁺) flies (Fig. 4A). *Trypanosoma* infections significantly reduced the density of *Sodalis* as indicated by com-paring (S⁺/T⁺) flies with (S⁺/T⁻) flies ($P = 0.014$) although the median values in (S⁺/T⁺) files is higher than the other samples indicating that the increased of *Sodalis* density in (S⁺/T⁻) might be affected with the outlier flies with high *Sodalis* density (Fig. 4B). No significant different was found in the *Trypanosoma* density determined by qPCR in the flies tested negative (S⁺/T⁻) or positive (S⁺/T⁺) and (S⁻/T⁺) with the standard PCR, however, *Sodalis* density showed significant difference between flies with different infection type ($X^2 = 14.54$, $df = 2$, $P < 0.001$) (Fig. 4B). The results showed no correlation between *Sodalis* and *Trypanosoma* density ($r = 0.007$, $t = 0.055$, $df = 69$, $P = 0.9561$) Supplementary Fig. 2, Supplementary File 1).

Discussion

The implementation of the SIT in the context of an AW-IPM strategy to eradicate tsetse flies relies on the release of sterile males in the targeted area. This was successful in eradicating a population of *G. austeni* from Unguja Island of Zanzi-bar[37] and significant progress has been made in the eradication programme implemented against *G. p. gambiensis* in the Niayes area of Senegal[38]. However, as both male and female tsetse flies are vectors of *Trypa- nosoma* species, the release of large numbers of sterile male flies bears a potential risk of temporarily increasing disease transmission during the initial release phase of an SIT programme[39]. Therefore, mitigating measures are required to reduce or eliminate this potential risk, especially in areas where sleeping sickness (HAT) is endemic. To date, to mitigate such risks, sterile males are offered two or three blood meals mixed with the trypanocidal drug isometamidium chloride, before being released which reduces the risk of *Trypanosoma* transmission sig- nificantly but does not eliminate it[40,41]. In addition, other approaches were proposed to minimize such risks such as paratransgenesis[42,43] and combining paratransgenesis with SIT[44].

The vector competence of tsetse flies for different trypanosome species is highly variable and is suggested to be affected by various factors, amongst which bacterial endosymbionts. Here, the interaction of *Sodalis glossinidius* with tsetse tryp-anosome infection is still under debate. Several studies reported a potential positive correlation between *Sodalis* and *Trypanosoma* infections[28,30,32,36,45–48], leading to the hypothesis that *Sodalis* might facilitate the establishment of *Tryp-anosoma* infections in the tsetse midgut[23,26,27]. However, other studies indicated the lack of correlation between *Sodalis* and *Trypanosoma* infection[34–36]. The presence of *Sodalis* infections in tsetse rearing colonies has been well studied and previous studies indicated that *Sodalis* is more frequently present in colonized tsetse flies than in wild tsetse popula-tions[36,49] with a prevalence of 80 and 100% in colonized *G. m. morsitans* and *G. p. gambiensis,* respectively[49,50], which is higher than the symbiont prevalence in wild popula- tions of these tsetse species. This seems to indicate that the rearing process of tsetse flies favours the transmis-sion and spread of *Sodalis* infections within the colonized population. Recently, colonies of *G. pallidipes, G. p. gambiensis, G. f. fuscipes, G. m. morsitans, G. m. centralis* and *G. m. submorsitans* maintained at the FAO/IAEA Insect Pest Control Laboratory were screened for *Sodalis* infections and showed a 100% prevalence of *Sodalis;* only the *G. brevipalpis* colony had a lower prevalence of 95% (data not shown). Taken into consideration that mass-rearing conditions enhances *Sodalis* infections and that *Sodalis* infections might facilitate the establish- ment of a *Trypanosoma* infection in the midgut, sterile male tsetse flies that are derived from colonies might be effective vectors for different *Trypanosoma* species and, therefore, might increase the trypanosome transmission after flies being released. It is therefore important that the managers and planners of SIT programmes are aware which tsetse species show a positive correlation between *Sodalis* and *Trypanosoma* infections to be able to take the necessary mitigating actions.

Various studies have examined the prevalence of *Sodalis* and *Trypanosoma* species in wild tsetse populations[30,32,35,45,51], but our study presents for the first time the prevalence of *Sodalis* and *Trypanosoma* spe-cies on a continent-wide scale. In addition, the DNA extraction and PCR methods we have used were standard- ized and were all carried out in one laboratory to avoid discrepancies in the results due to different handling of tsetse samples or to different methods to discriminate trypanosome species in tsetse tissues. Our results indicate that *Sodalis* and *Trypanosoma* preva-lence varied with tsetse species and geographical location (with an overall trypanosome prevalence of 23.5%), which agrees with many previous studies[52]. A high *Trypanosoma* prevalence (> 30%) was found in *G. m. morsitans* and *G. palli-dipes* from central and east Africa. This finding is in agreement with previous reports on *G. m. morsitans* and *G. m. centralis* from Zambia[36,52] and *G. m. morsitans* sampled in Malawi[53]. Moreover, a high prevalence of *Trypanosoma* infection in *G. pallidipes* was also previously reported in Tanzania[54] and Kenya[33]. However, another study in northern Tanzania indicated a lower prevalence of *Trypa- nosoma* infection (<10%) both in *G. m. morsitans* and *G. pallidipes*[55].

Our study showed that the prevalence of different *Trypanosoma* species and or subspecies can be different in differ-ent tsetse taxa. In *G. tachinoides* in Ghana, the *Trypanosoma vivax* (Tv) infection was high (> 10%) as well as the infections of the *T. brucei* sspp (Tz) and the *T. simiae/T. godfreyi* (Tsg) group and the mixed infections of Tv–Tsg. In contrast, the prevalence of *T. congolense* was very low. These results are in agreement with the

Glossina taxon	Country (Area, Collection Date)	N	S^+/T^+	S^+/T^-	S^-/T^+	S^-/T^-	χ^2	P
G. austeni	Tanzania (Jozani, 1997)	4	0	0	1	3		
G. austeni	Tanzania (Zanzibar, 1995)	6	0	1	0	5		
G. austeni	Tanzania (Uguja Island, 1995)	30	0	2	3	25		
G. austeni	South Africa (North eastern Kwazulu Natal, 1999)	39	0	2	2	35		
G. austeni	South Africa (Lower Mkhuze, 2018)	53	0	0	23	30		
G. austeni	South Africa (Saint Lucia, 2018)	57	0	0	22	35		
G. austeni	South Africa (False Bay Park, 2018)	77	0	0	2	75		
G. austeni	Mozambique (Reserva Especial de Maputo, 2019)	50	0	0	5	45		
G. austeni	Eswatini (Mlawula Nature Reserve, 2019)	30	0	0	0	30		
G. austeni	All locations	346	0	5	58	283	1.02	0.31
G. brevipalpis	South Africa (North eastern Kwazulu Natal, 1995)	50	0	0	2	48		
G. brevipalpis	South Africa (Phinda, 2018)	170	0	7	0	163		
G. brevipalpis	South Africa (Saint Lucia, 2018)	30	0	0	13	17		
G. brevipalpis	South Africa (Hluhluwe, 2018)	50	0	0	16	34		
G. brevipalpis	Mozambique (Reserva Especial de Maputo, 2019)	50	0	7	3	40		
G. brevipalpis	All locations	350	0	14	34	302	1.57	0.21
G. f. fuscipes	Uganda (Buvuma island, 1994)	94	0	4	10	80		
G. f. fuscipes	Kenya (Ikapolock, 2007) [1]	51	5	15	14	17		
G. f. fuscipes	Kenya (Obekai, 2007)	38	0	0	2	36		
G. f. fuscipes	All locations	183	5	19	26	133	0.3	0.59
G. medicorum	Burkina Faso (Comoe, 2008)	94	0	8	32	54		
G. medicorum	Burkina Faso (Folonzo, 2008)	60	0	0	29	31		
G. medicorum	All locations	154	0	8	61	85	5.53	0.02
G. m. submorsitans	Burkina Faso (Comoe, 2007)	206	0	0	20	186		
G. m. submorsitans	Burkina Faso (Folonzo, 2008)	134	0	1	42	91		
G. m. submorsitans	Burkina Faso (Sissili, 2008)	3	0	0	0	3		
G. m. submorsitans	All locations	343	0	1	62	280	0.22	0.64
G. p. palpalis	Democratic Republic of Congo (Zaire, 1995)	35	0	4	1	30		
G. m. morsitans	Tanzania (Kwekivu 2, 2005)	81	35	27	9	10		
G. m. morsitans	Zambia (Mfuwe, Eastern Zambia, 2007)	64	1	7	30	26		
G. m. morsitans	Zimbabwe (Mukondore, 2007)	13	1	2	0	10		
G. m. morsitans	Zimbabwe (M. chiuyi, 2007)	9	0	1	0	8		
G. m. morsitans	Zimbabwe (Rukomeshi, 2006)	15	0	3	0	12		
G. m. morsitans	Zimbabwe (Kemukura, NA)	18	0	4	1	13		
G. m. morsitans	Zimbabwe (Mushumbi, 2006)	6	0	0	2	4		
G. m. morsitans	Zimbabwe (Makuti, 2006)	78	19	2	52	5		
G. m. morsitans	Kenya (Kari, 2006)	85	2	52	0	31		
G. m. morsitans	All locations	369	58	98	94	119	1.8	0.18
Glossina taxon	Country (Area, Collection Date)	N	S^+/T^+	S^+/T^-	S^-/T^+	S^-/T^-	χ^2	P
G. pallidipes	Zambia (Mfuwe, Eastern Zambia, 2007)	146	2	1	64	79		
G. pallidipes	Kenya (Mwea, Katotoi, Emsos, Kari, Kiria, Koibos, Meru and Ruma national park, 2007)	834	88	126	337	283		
G. pallidipes	Ethiopia (Arba Minch, 2007)	459	15	79	77	288		
G. pallidipes	Tanzania (Kwekivu 1, 2005)	217	54	108	27	28		
G. pallidipes	Zimbabwe (Mushumbi 2006)	26	1	0	4	21		
G. pallidipes	Zimbabwe (Gokwe, 2006)	4	0	0	0	4		
G. pallidipes	Zimbabwe (Rukomeshi, 2006)	4	0	0	0	4		
G. pallidipes	Zimbabwe (Makuti, 2006)	38	6	0	27	5		
G. pallidipes	Uganda (Lira, Omogo, Budaka, Moyo, NA)	116	4	83	5	24		
G. pallidipes	All locations	1844	170	397	541	736	25.4	0
G. p. gambiensis	Burkina Faso (Lorepeni)	10	0	0	8	2		
G. p. gambiensis	Burkina Faso (Bouroum bouroum)	18	0	0	16	2		
G. p. gambiensis	Burkina Faso (Kourignon)	24	0	0	10	14		
G. p. gambiensis	Burkina Faso (Kampty)	98	0	0	85	13		
G. p. gambiensis	Burkina Faso (Ouarkoye)	5	0	0	5	0		
G. p. gambiensis	Burkina Faso (Dedougou)	57	0	0	33	24		
G. p. gambiensis	Burkina Faso (Bama)	77	0	0	0	77		
Continued								

Glossina taxon	Country (Area, Collection Date)	N	S⁺/T⁺	S⁺/T⁻	S⁻/T⁺	S⁻/T⁻	χ²	P
G. p. gambiensis	Burkina Faso (Comoe)	123	0	0	3	120		
G. p. gambiensis	Burkina Faso (Folonzo)	212	0	2	25	185		
G. p. gambiensis	Burkina Faso (Kartasso)	136	0	0	0	136		
G. p. gambiensis	Burkina Faso (Kenedougou)	41	0	0	0	41		
G. p. gambiensis	Burkina Faso (Moussodougou)	142	0	0	49	93		
G. p. gambiensis	Guinea (Bafing)	33	0	0	1	32		
G. p. gambiensis	Guinea (Dekonkore)	16	0	0	1	15		
G. p. gambiensis	Guinea (Kangoliya)	126	0	90	0	36		
G. p. gambiensis	Guinea (Kerfala	13	0	0	1	12		
G. p. gambiensis	Guinea (Kifala)	30	0	0	0	30		
G. p. gambiensis	Guinea (Lemonako)	20	0	0	0	20		
G. p. gambiensis	Guinea (Mimi)	45	0	0	1	44		
G. p. gambiensis	Guinea (Tinkisso)	31	0	0	2	29		
G. p. gambiensis	Mali	364	0	0	25	339		
G. p. gambiensis	Senegal	547	0	0	79	469		
G. p. gambiensis	All locations	2168	0	92	343	1733	18.06	0
G. tachinoides	Burkina Faso	834	0	0	140	694		
G. tachinoides	Ghana	234	0	0	143	91		
G. tachinoides	All locations	1068	0	0	283	785		

Table 5. Distribution of the association between the presence of *Trypanosoma* spp and the presence of *Sodalis* according to the tsetse species and the country.

prevalence of *T. brucei* s.l (11%) and *T. congolense* forest type (2.6%) reported in the same tsetse species in Cam- eroon. The same study reported a prevalence of 13.7% of *T. congolense* savannah type[35], which was not observed in our study. Our results of trypanosome infection rates in *G. tachinoides* also agree with former studies[56,57], except for T,c for which a high fly infection rate (31.8%) was previously shown[57]. The Tc infection rates in our study were high in *G. pallidipes* and *G. m. morsitans;* for the latter tsetse fly species, a study in Malawi reported a high prevalence for *T. brucei* (64.4%) but much lower for all other *Trypanosoma* infections (<10%)[58]. The mixed infection of *Trypanosoma* spe- cies/subspecies is in agreement with previous reports[35,52,57,59].

Likewise, the prevalence of *Sodalis* infection varied significantly with tsetse taxon and location and the highest prevalence was found in *G. m. morsitans* and *G. pallidipes*. Our results agree with the high prevalence of *Sodalis* reported in *G. pallidipes* (~ 50%) in one location in Kenya regardless of the fly age[33]; however, the same study reported low *Sodalis* prevalence in another location. In another study in Kenya, Wamwiri et al.,[32] reported moderate *Sodalis* prevalence in *G. pallidipes* (16%) and low prevalence in *G. austeni* (3.7%), which is in agree- ment with our results. On the other hand, our results are different from the low prevalence (<8%) found in *G.m. morsitans* and *G. pallidipes* in Zambia[36]. In another study in Zambia, *Sodalis* prevalence in *G. m. centralis*, was reported to be 15.9% with no significant difference between inter-site prevalence[52]. The prevalence of *Sodalis* in *G. brevipalpis* in our study was found to be low (<2.3%) which contradicts with the high prevalence (93.7%) reported in this species in Zambia[36]. In the DRC, the global prevalence of *Sodalis* in *G. fuscipes quanzensis* midgut averaged 15.5%, but in certain locations the prevalence exceeded 40%[60]. In Nigeria, *Sodalis* prevalence in *G. p. palpalis* and *G. tachinoides* was 35.7%[61] which is higher that the prevalence reported in our study for both species. Our data indicate that the *Trypanosoma* and *Sodalis* infections were very low or absent in some tsetse taxa from certain locations such as *G. austeni* in Eswatini for *Trypanosoma* and *Sodalis* infections and several species in west Africa for *Sodalis*. The lack of *Sodalis* and/or *Trypanosoma* infection in these samples might be due to (i) low number of tested samples (ii) the use of the DNA extracted from the whole body of tsetse adults (iii) the possibility of the collected samples being infected with different strains/genotypes that might not be detected with the primers used and (iv) the infection of *Sodalis* and *Trypanosoma* are under the detection limit of the used PCR. It is important to note that due to the high number of samples tested in our study, the more sensitive nested PCR to detect low infection level was excluded due to logistic reasons.

Our results indicate significant deviation from independence (correlation) of *Sodalis* and *Trypanosoma* infec- tions in *G. medicorum, G. p. gambiensis* and *G. pallidipes*. However, the lack of detection of any tsetse adult with co-infection of *Sodalis* and *Trypanosoma* in *G. medicorum,* and *G. p. gambiensis* might indicate a negative cor- relation. Such neg- ative trend might be supported by the lower density of *Sodalis* in the flies with co-infection (S⁺/T⁺) compared to these with *Sodalis* infection only (S⁺/T⁻). On other hand the lack of impact of *Sodalis* infection on *Trypanosoma* density does not support the negative trend and agreed with the results of Trappe- niers et al.,[24] reported on colonized flies. This results also agreed with previous results reporting the absence of direct correlation between the presence of *Sodalis* and the acquisition of a *Trypanosoma* infection[63]. However, an inverse correlation was reported between *So- dalis* and the vector competence where the presence of *Sodalis* in both midgut and proboscis of *G. p. gambiensis* was associated with its status as a poor vector, whereas it is not found in the proboscis of *G. m. morsitans* (major vector). It is worth noting that all previous studies of *Sodalis* infection in *G. p. gambiensis* and its interaction with *Trypanosoma* infection was carried out with flies reared under laboratory conditions[28,29,64]. The correlation between *Sodalis* and *Tryp- anosoma* infection in *G. pallidipes* is
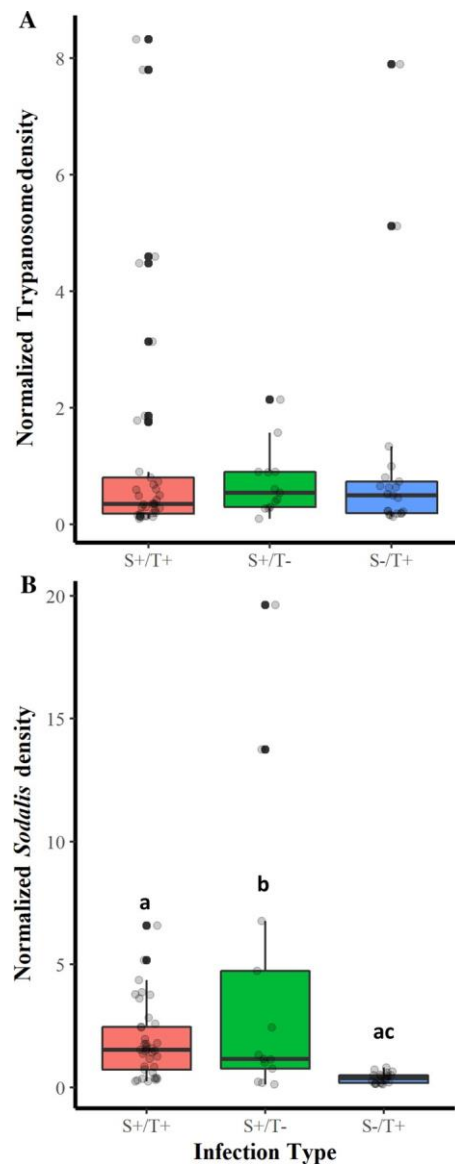
Figure 4. Impact of co-infection with *Trypanosoma* and *Sodalis* on *Trypanosoma* (**A**) and *Sodalis* (**B**) density in *Glossina pallidipes* and *G. m. morsitans*. Bars marked with the same lower-case letter do not differ significantly at the 0.05 level.

positive, evidenced with the relative high number (n = 170) of tsetse with co-infection. This positive correlation was also found in *G. pallidipes* from Kenya although with too few flies with co-infection to enable us to draw a definite conclusion[32]. Although co-infections were found in *G. m. morsitans* and *G. f. fuscipes* in some locations, the global correlation was missing. This is in agreement with the positive correlation found between *Sodalis* and *Trypanosoma* infection in *G. m. centralis* in Zambia, in which there was a 6.2 fold increase in the likelihood of a fly being infected with *Trypanosoma* if *Sodalis* was present[52]. More studies are needed to enhance the potential control interventions mediated by endosymbionts to reduce parasitic infections[61].

The results of this study clearly indicate that the interaction between *Sodalis* and *Trypanosoma* infection is complex, species-specific and requires further investigation. The prevalence results indicate that *Sodalis* and *Trypanosoma* infections are not independent in some species, such as *G. p. gambiensis* and *G. medicorum* in west Africa and *G. pallidipes* in central and east Africa, In case of a positive correlation between *Sodalis* and *Trypanosoma* infection in these species, additional measures could be suggested when implementing the SIT to reduce the *Sodalis* density in the sterile males released in the targeted area to maximize the safe implementation of the SIT. These measures might include the mixing of *Sodalis* phage(s)[29,65] with the blood meals to feed the mass-reared flies to reduce the *Sodalis* density in these flies. In addition, the blood meal offered to the males before release can be supplemented with one or more of the following antimicrobial products to reduce *Sodalis* density, i.e. streptozotocin[23], indolicidin and OaBAC 5 mini[66]. The use of the *Sodalis* phage as well as these antimi- crobial agents requires further studies to (1) develop methods to isolate the phage, (2) determine the conditions (e.g. suitable concentration) for its use, and (3) determine the impact on *Sodalis* density, tsetse productivity and

survival. For *G. m morsitans* and *G. pallidipes*, our results suggest that *Sodalis* infection does not have an impact on *Trypanosoma* infection so here no additional measures need to be taken during the implementation of SIT against these species.

Conclusion

Sodalis and *Trypanosoma* infection varied with tsetse taxon and location. There is a significant positive correla- tion between *Sodalis* and *Trypanosoma* infection in *G. medicorum*, *G. p. gambiensis* and *G. pallidipes*; however, no signifi- cant correlation was found in other tsetse taxa and locations. The results of this study will enable the decision makers of SIT projects to better plan and take the necessary measures to fine-tune and optimize SIT efficiency and safety.

Methods

Tsetse collection and DNA extractions. Tsetse flies were collected in 1995 and between 2005 and 2018 from 95 different geographical locations in fifteen countries in east, central, southern, and western Africa(Table 6, Supplementary Table 6). The tsetse flies were collected with species-specific traps which included thebiconical trap[67], the monoconical trap[68], the Vavoua trap[69], the Ngu trap[70,71], the odour-baited Epsilon trap[72], theNZI trap[73], and the odour baited H trap[74]. A total of 6860 tsetse flies, belonging to ten tsetse species, were col- lected for this study (Table 6). The majority of the samples were collected in Burkina Faso (2274), Kenya (1008), Senegal (547) and South Africa (526). As the distribution of most tsetse species is allopatric (only few species are sympatric), not all tsetse species were collected from each country. Following collection, fly samples were pre-served in 95% ethanol or propylene glycol and shipped to the FAO/IAEA Insect Pest Control Laboratory (IPCL)in Seibersdorf, Austria and stored at − 20 °C until analysis. Total DNA was extracted from individual whole fly bodies using the DNeasy tissue kit (QIAGEN Inc., Valencia, CA) following the supplier's instructions. TheDNA quality and concentration were measured by spectrophotometry (Synergy H1 Multi-Mode Reader, BioTek,Instruments, Inc., USA) and subsequently kept at 4 °C until screened for *Sodalis* and *Trypanosoma* infections.To verify the quality of the extracted DNA, a set of specific primers amplifying the *Glossina* spp. microsatelliteGpCAG133 sequence (Supplementary Table 5) and only the successful samples were included in the analysis[21,75].

***Trypanosoma* prevalence and genotyping.** Polymerase chain reaction (PCR), following the method of Njiru et al.[76] that used the primers ITS1-CF and ITS1-BR (Supplementary Table 5) previously designed to amplify the internal transcribed spacer (ITS1) of the ribosomal DNA, was used to detect *Trypanosoma* infec- tion and *Trypa- nosoma* species in the fly samples. The PCR was carried out in 25 µl reaction mixtures contain-ing 22.5 µl of 1.1 × Pre-Aliquoted PCR Master Mix (0.625 units Thermoprime Plus DNA Polymerase, 75 mM Tris–HCl (pH 8.8 at 25 °C), 20 mM $(NH_4)2SO_4$, 2.0 mM $MgCl_2$, 0.01% (v/v) Tween-20 and 0.2 mM each of thedNTPs (ABgene, UK), 1 µl primers (at 200 nM final concentration of forward and reverse primer) and 1.5 µl oftemplate DNA. PCR cycles were: 94 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and final extension 72 °C for 5 min. Interpre- tation of the results after resolving the amplification productsin a 2% agarose gel (Fisher Biotech) stained with SafeGreen or ethidium bromide, was based on the characteristic band size of *Trypanosoma* taxa: all members of the subgenus *T. brucei* sspp (*T. b. brucei*, *T. b. gambiense*, *T. b.rhodesiense*: 480 bp); *T. congolense* savannah (700 bp); *T. congolense* Kilifi (620 bp); *T. congolense* forest (710 bp); *T. simiae* (400 bp); *T. simiae* Tsavo (370 bp); *T. godfreyi* (300 bp) and *T. vivax* (250 bp). The positive control DNA was from *T. congolense* savannah, *T. congolense* forest, *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. evansi*, and *T. vivax*. DNA samples validated with GpCAG133 primer amplification were screened for trypanosome infec-tion. A tsetse sample was recorded as positive if one or more of the indicated band sizes was de- tected. *Trypano- soma* infection status and species were recorded for each fly.

Prevalence of *Sodalis* infection. The detection of *Sodalis* in natural tsetse samples was based on the *Sodalis fliC* (flagellin) gene which results in an amplicon length of about 508 base pairs with the *Sodalis* specificprimers Sod-fliC-F and Sod-fliC-R (Supplementary Table 5)[77]. These primers were used in single pairs or in multiplex PCR with GpCAG133 primers. For all PCR reactions, 22.5 µl of 1.1 × Pre-Aliquoted PCR Master Mix (ABgene, UK) was used. In a final volume of 25 µl, 1.5 µl of template DNA plus forward and reverse primerswere added to a final concentration of 0.2 mM per primer in a volume of 1 µl. Samples were considered *Sodalis*-infected if the expected symbiont PCR product amplicon was detected. Data were accepted only if the control gene GpCAG133 sequence was amplified. The PCR cycling conditions were: 95 °C for 5 min followed by 34 cycles of 95 °C for 30 s, 52.5 °C for 30 s, 72 °C for 30 s and lastly at 72 °C for 10 min; PCR products were separated by agarose (2%) gel electrophoresis and SafeGreen or eth- idium bromide staining.

Analysis of the *Trypanosoma* and *Sodalis* infection in wild tsetse populations. *Co-infection of tsetse adults with Sodalis and Trypanosoma infection.* The co-infection of *Sodalis* and *Trypanosoma* infectionwas evaluated based on the PCR prevalence. The infection status was divided into four categories *Sodalis* posi-tive and *Trypanosoma* positive (S^+/T^+), *Sodalis* positive and *Trypanosoma* negative (S^+/T^-), *Sodalis* negative and*Trypanosoma* positive (S^-/T^+) and *Sodalis* negative and *Trypanosoma* negative (S^-/T^-).

Analysis of the Trypanosoma and Sodalis density. Samples showing *Trypanosoma* infection (not *T. vivax*) with*Sodalis* (S^+/T^+) and samples not infected with *Trypanosoma* but infected with *Sodalis* (S^+/T^-) were evaluated with quantitative PCR (qPCR) to assess the impact of *Trypanosoma* infection (regardless the *Trypanosoma* type) on*Sodalis* density. The qPCR was performed using a CFX96 Real Time PCR Detection System (Bio-Rad). The *fliC*

Country	No. of locations	No. of collection flies with valid DNA	Collection year
Ethiopia	1	459	2007
Kenya	11	1008	2007, 2008, 2009
Uganda	5	210	2007
Tanzania	5	338	2005, 2009
Democratic R. of Congo	1	35	1995
Zambia	1	210	2007
Zimbabwe	7	211	2006
South Africa	7	526	1995, 2018, 2019
Mozambique	1	100	2019
Eswatini	1	30	2018, 2019
Burkina Faso	14	2274	2008, 2010, 2013, 2015, 2018, 2019
Ghana[a]	11	234	2008
Guinea[a]	8	314	2008, 2009
Mali[a]	10	364	2008, 2010, 2011, 2012, 2013
Senegal	12	547	2008, 2009
Total	95	6860	

Table 6. List of collections of tsetse adults with valid DNA screened for *Sodalis* and Trypanosome[a] infection in wild tsetse population in east, central, southern and west Africa. [a]Part of the trypanosome infection in west Africa was screened by Ouedraogo et al. 2018.

gene was amplified with the following primers: sodqPCR-FliCF and sodqPCR-FliCR[78] (Supplementary Table 5) to assess the density of the symbiont present within *Trypanosoma* infected and noninfected, additional criteria for the selection of the samples was the presence of the two groups (S^+/T^+) and (S^+/T^-) in a given population. Based on the preceding criteria 96 individual flies (52 and 44 flies with infection status of (S^+/T^+) and (S^+/T^-), respectively, were selected from the *G. pallidipes* and *G. m. morsitans* collected in Kenya, Tanzania and Zimba-bwe. In addition, samples with (S^+/T^+) and (S^-/T^+) were used to assess the impact of *Sodalis* infection on *Trypa-nosoma* density. *Trypanoso-matidae*18S specific primers (18S_Typ_F and18S_Typ_R) (Supplementary Table 5)were used to assess the *Trypano-soma* density in the tested samples. The DNA from all selected samples was diluted to a final concentration of 4 ng/μl and 5 μl of the diluted DNA was used for qPCR to determine *Sodalis*and *Trypanosoma* DNA density normalized to the housekeeping β-tubulin gene. The amplification mixture con-tained 5 μl of DNA template, 200 nM of each primer, and 7.5 μl iQ™ SYBER Green Supermix (Bio-Rad). qPCRcycling conditions for *Sodalis* were as follows: initial denatura-tion at 95 °C for 2 min; 39 cycles of 95 °C for 5 s,55 °C for 30 s, one step at 95 °C for 5 s and a melting curve constructed from 65 °C to 95 °C in increments of

0.5 °C for 5 s. The same conditions were used for *Trypanosoma* except the annealing temperature was at 60 °C.The analysis of the *Sodalis, Trypanosoma* and Tubulin densities was based only on qPCR data with the expectedmelting curve at 81.5 °C, 85.5 °C and 86 °C, respectively.

Data analysis. The prevalence data were recorded and analyzed with the general linear model (GLM)[79]. The prevalence of *Sodalis*, *Trypanosoma* species and each *Trypanosoma* species and co-infection were tested for differ-ences between the tsetse taxa and between countries. For each country, the prevalence was assessed again for differ-ences between the localities where the flies were collected and between the tsetse species presentin each country. In the absence of PCR detected *Sodalis* or *Trypanosoma* infection, the upper 95% confidence interval for the true rate of infection was calculated following the method of Couey and Chew[62]. *Trypanosoma*prevalence between taxa was com-pared between species by a pairwise comparison of proportions with a Bonfer-roni correction and Benjamini–Hochberg correction. The analyses were executed in R v 4.0.5[79] using RStudio V 1.4.1106[80,81] with the packages ggplot2 v3.3.2.1[82], lattice v0.20-41[83], car[84], ggthems[85] and MASS v7.3-51.6[86] except for the Chi squared tests for independ-ence, Spearman correlation coefficient and Cochran–Mantel– Haenszel test for repeated tests of independence, which were performed using Excel 2013 The R Markdown fileis available in Supplementary File 1.

To analyse the qPCR data, normalized density of *Trypanosoma* and *Sodalis* against the house keeping gene(tubu-lin) was extracted from the CFX Maestro software. Samples giving a valid density (not N/A) for both *Trypa-nosoma* and *Sodalis* were retained for further statistical analysis in R. Similarities in the structure of *Sodalis* and *Trypanosoma* (sin-gle and multiple) infection and the role of different factors such as countries and tsetse taxa, were assessed using the matrix display and metric multidimensional scaling (mMDS) plot with bootstrap aver-ages in PRIMER version 7+. The bootstrap averages plots were displayed with a Bray and Curtis matrix basedon the square-root transformation of the *Sodalis* and *Trypanosoma* (single and multiple) infection abundance data[87]. The tests were based on the multivari-ate null hypothesis via the use of the non-parametric statistical method PERMANOVA[88]. The Permanova test was conducted on the average of the abundance data based on the country-species after excluding the data of Eswatini (low number of tested samples).

Data availability

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Author contributions

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Chapter 3

Prevalence of *Spiroplasma* and interaction with wild *Glossina tachinoides* microbiota

Prevalence of *Spiroplasma* and interaction with wild *Glossina tachinoides* microbiota

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Abstract

Tsetse flies (Diptera: *Glossinidae*) are vectors of the tropical neglected diseases sleeping sickness in humans and nagana in animals. The elimination of these diseases is linked to the control of the vector. The Sterile Insect Technique (SIT) is an environment-friendly method that has been shown to be effective when applied in an area-wide integrated pest management (AW-IPM) approach. However, as irradiated males conserve their vectorial competence, there is the potential risk of trypanosome transmission with the release of these males. Analyzing the interaction between the tsetse fly and its microbiota, and between different microbiota and the trypanosome, might provide important information to enhance the fly's resistance to trypanosome infection. This study on the prevalence of *Spiroplasma* in wild populations of seven tsetse species from East, West, Central and Southern Africa showed that *Spiroplasma* presence was confirmed only in *Glossina fuscipes fucipes* and *Glossina tachinoides*. In *G. tachinoides,* a significant deviation from independence in co-infection with *Spiroplasma* and *Trypanosoma* spp was observed. Moreover, *Spiroplasma* infections seem to significantly reduce the density of the trypanosomes, suggesting that *Spiroplasma* might enhance tsetse fly's refractoriness to the trypanosome infections. This finding might be useful to reduce risk associated with the release of sterile males during SIT implementation in trypanosome endemic areas.

Keywords

Glossina spp, *Trypanosoma* spp, *Spiroplasma*, microbe infection rate, interactions, paratransgenesis

Résumé

Les mouches tsé-tsé (Diptera : *Glossinidae*) sont des vecteurs de maladies tropicales négligées, telles que la maladie du sommeil chez l'homme et le nagana chez les animaux. L'élimination de ces maladies est liée à la lutte contre le vecteur. La technique de l'insecte stérile (TIS) est une méthode respectueuse de l'environnement qui s'est avérée efficace lorsqu'elle est appliquée dans le cadre d'une approche de gestion intégrée des parasites à l'échelle d'une zone (AW-IPM). Cependant, comme les mâles irradiés conservent leur compétence vectorielle, le risque d'infection par le trypanosome augmente avec la libération de ces mâles. L'analyse de l'interaction entre la mouche et son microbiote, et entre différents microbiotes et le trypanosome, pourrait fournir des informations importantes pour améliorer la résistance de la mouche à l'infection par le trypanosome. Cette étude sur *Spiroplasma* dans les populations

sauvages de sept espèces de tsé-tsé d'Afrique de l'Est, du Sud et de l'Ouest a montré que l'infection par *Spiroplasma* n'a été détectée que chez *Glossina fuscipes fucipes* et *Glossina tachinoides*. Chez *G. tachinoides* on a observé un écart significatif par rapport à l'indépendance dans la co-infection avec *Spiroplasma* et *Trypanosoma* spp. De plus, les infections par *Spiroplasma* semblent réduire de manière significative la densité des trypanosomes, ce qui suggère que *Spiroplasma* pourrait renforcer la réfractariat de la mouche tsé-tsé à l'infection par les trypanosomes. Cette découverte pourrait être utile pour réduire le risque associé à la libération de mâles stériles lors de la mise en œuvre de la TIS dans les zones où les trypanosomoses sont endémiques.

Introduction

Tsetse flies (Diptera: *Glossinidae*) transmit trypanosomes the causative agent of one of the most neglected vector borne diseases in sub-Saharan Africa, i.e., African animal trypanosomosis or AAT (also called nagana) and human African trypanosomosis or HAT (sleeping sickness) which affect animals and humans, respectively [9,37]. Tsetse flies are principally hematophagous and exclusively feed on vertebrate blood [2,57]. During a blood meal on an infected host, the fly can ingest the trypanosomes which are established in the midgut. After several series of proliferation and differentiation, they mature in the salivary gland or the mouth parts depending on the trypanosome species. The parasite can then be transmitted to a mammalian host during a subsequent blood meal [60,61].

The lack of effective prophylactic drugs or a vaccine [9] and the development of resistance to trypanocidal drugs [17], makes tsetse control the most efficient alternative for sustainable management of these diseases. One effective method for tsetse control is the sterile insect technique (SIT) that needs to be implemented as part of an area-wide integrated pest management (AW-IPM) approach. The sterile insect technique requires the mass production of the target insect, sterilization with irradiation and the release of these sterile insects to mate with wild females to reduce fertility of the targeted population. However, the irradiation does not affect the tsetse fly's susceptibility to develop mature trypanosome infections [18], and hence, the desirability to enhance refractoriness of tsetse flies for trypanosome infections that would be used for release in an SIT program [59]. The use of paratransgenesis has been suggested as an approach that could confer resistance against pathogens by genetic engineering of symbionts of the vector [14]. This approach has been implemented successfully in triatomes bugs [22] and mosquitoes [62], but still under evaluation for tsetse flies.

Symbiotic associations have been described in insects and typically involve bacteria that are vertically transmitted through progeny and may influence several functions of their hosts [51]. Tsetse flies harbor tree major endosymbiotic bacteria, i.e., the obligate mutualist *Wigglesworthia glossinidia*, the mutualist *Sodalis glossinidius* and the parasitic *Wolbachia pipientis* [42]. Recently, a fourth endosymbiont, i.e., *Spiroplasma* was discovered in some natural tsetse populations and some laboratory colonies of *Glossina palpalis palpalis*, *Glossina fuscipes fuscipes* and *Glossina tachinoides*, both belonging to the palpalis group [21,28]. In addition, the Multi locus sequencing typing (MLST) analysis performed by Doudoumis et al., [21] identified two different strains of *Spiroplasma* in *G. f. fuscipes* and *G. tachinoides*.

Bacteria belonging to the genus *Spiroplasma* are Gram-positive, wall-less and described in arthropods and plants and are classified into three major monophyletic group based on the 16S ribosomal RNA gene (rDNA) sequence: Ixodetis, Citri-Chrysopicola-Mirum (CCM) and Apis [24,27]. They belong to the class of Mollicutes and are characterized by an helical shape and the lack of a cell wall and are enveloped by only a cholesterol containing cell membrane [26]. The *Spiroplasmas* are unique in having a well-defined, dynamic, helical cell geometry and a flat, monolayered, membrane-bound cytoskeleton, which follows, intracellularly, the shortest helical line on the cellular coil. They have a cytoskeleton which controls both the dynamic helical shape and the consequent motility of the cell [47,58]. Their cell size varies between 100 to 240 nanometers [1]. The genome size ranges from 780 to 2,220 kbp and the DNA is rich of AT (GC represent 24 - 31 mol%) [1,47]. The role of *Spiroplasma* in the tsetse fly host is currently unclear, but it is known to have an impact on the insects' fitness (assumption of negative effect on the viability of *Harmonia axyridis*, male killing on *Drosophila melanogaster* and *Drosophila neotestacea*) [5,24]. Many studies revealed that *Spiroplasma* might cause disease in arthropods and plants [5,39]. Conversely, some *Spiroplasma* might have a positive effect in their hosts conferring resistance against pathogens [34,41,46]. The *Spiroplasma-Wolbachia* association is not well understood, and both symbionts can be found in the ovaries. However, *Spiroplasma* resides primary in the hemolymph but can invade fat body and salivary glands. In tsetse flies, *Spiroplasma* seems to interact with trypanosomes as reported by Schneider et al [50], who used a laboratory colony of *G. f. fuscipes* in their experiments. They found that flies that harbored *Spiroplasma* showed a lower prevalence of trypanosome infection in the midgut, indicating a potential negative correlation between *Spiroplasma* presence and trypanosome infection. They also indicated a vertical transmission and the possibility of horizontal transmission of *Spiroplasma* through the environment was not excluded. These findings supported the use of *Spiroplasma* to use the novel symbiont-based paratransgenesis approach to develop trypanosome refractoriness. Paratransgenesis is a genetic method that consists of modifying symbiotic organism of insect vectors to enhance their expression of effector molecules, especially ones that can potentially block pathogen development [7]. In this respect, the use of the endosymbiont *Sodalis* was recommended previously [6,15,16]. In addition, irradiating 22 day-old pupae did not impact the copy number of *Sodalis* in *G. morsitans morsitans* as compared with non-irradiated flies also support this idea [18]. However, it has been reported that this bacterium has a negative impact on the metabolic and reproductive fitness of *G. f. fuscipes* [56].

In this study, the prevalence of *Spiroplasma* infection was assessed in natural tsetse populations collected from different countries in Africa. The potential interaction between *Spiroplasma* with the trypanosomes and *Wigglesworthia* was studied using a *G. tachinoides* population from Burkina Faso and Ghana. In this paper, we also report on the genotyping and assessing the different *Spiroplasma* strains that are circulating in wild *G. tachinoides* populations.

Materials and Methods

Tsetse taxon collection and DNA purification

Wild populations of tsetse flies were collected in 40 locations in 10 different countries in West Africa (Burkina Faso, Ghana, Guinea, Mali and Senegal), Central Africa (Democratic Republic of Congo), East Africa (Ethiopia and Uganda) and southern Africa (South Africa and Zimbabwe). Eight tsetse taxa were analyzed including *G. brevipalpis*, *G. f. fuscipes*, *G. m. morsitans*, *Glossina morsitans submorsitans*, *Glossina pallidipes*, *Glossina palpalis gambiense*, *Glossina palpalis palpalis* and *G. tachinoides* **(Figure 1, Supplementary table 1)**.

Adult flies were collected in 1995 and between 2005 and 2018 using the biconical Challier-Laveissière trap, the monoconical Vavoua trap [11,13], the Ngu trap and the Epsilon trap baited with acetone [25] and the odour baited H trap [32]. The collected flies were stored in 95% absolute ethanol or propylene glycol and shipped to the Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria. At the IPCL, the samples were stored at -20 °C until further use. The total DNA was extracted from the whole body of each individual fly using the DNeasy tissue kit (QIAGEN Inc., Valencia, CA) following the supplier's instructions.
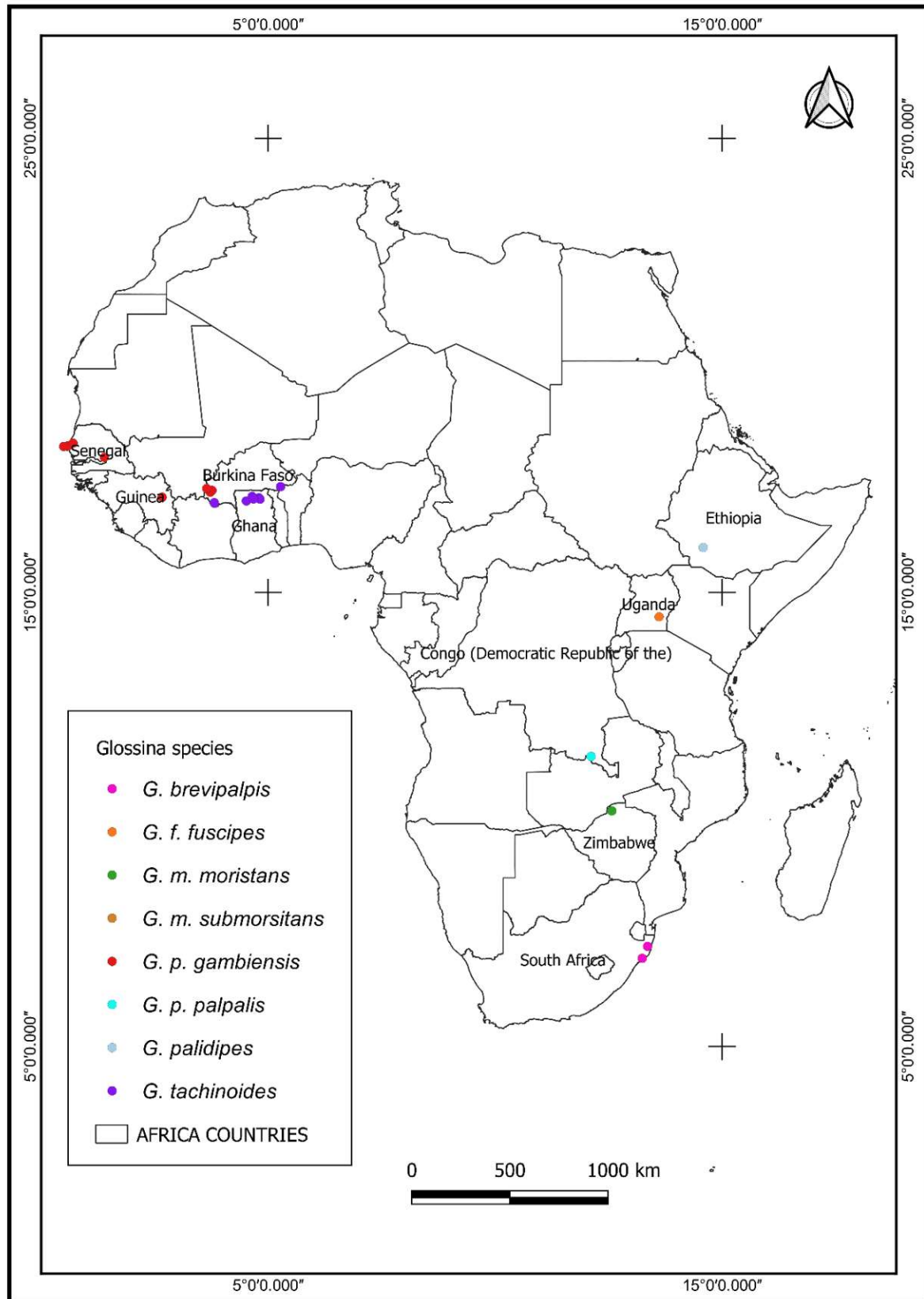
Figure 1: The geographical locations of tsetse samples in Africa

Prevalence of *Spiroplasma* and *Trypanosoma*

To detect *Spiroplasma* infection, PCR amplification of a sequence of approximately 455 bp in the 16S rRNA was performed. The PCR was carried out in 25 µl reaction mixtures containing 22.5 µl of 1.1x Pre-Aliquoted PCR Master Mix (0.625 units Thermoprime Plus DNA Polymerase, 75 mM Tris–HCl (pH 8.8 at 25°C), 20 mM (NH4)2SO4, 2.0 mM MgCl2, 0.01% (v/v) Tween-20 and 0.2 mM each of the dNTPs (ABgene, UK), and 1.5µl of template DNA plus 1µl of *Spiroplasma* 16sRNA primers (63F and TKSS) **(Supplementary table 2)** to a final concentration of 0.2 mM per primer. PCR conditions were 95°C for 5 min, followed by 34 cycles of 95°C for 30 s, 58°C for 30s, 72°C for 30s, and final extension 72°C for 10 min. PCR products were electrophoresed in 2% molecular grade agarose (Fisher Biotech) stained with SafeGreen. The DNA of *G. f. fuscipes* from the IPCL colony that was known to be infected with *Spiroplasma* and sterilized distilled water were included in each PCR run as positive and negative controls, respectively. As described in Dieng et al [19], the GpCAG primer was used to validate the DNA and only sample positive for this primer was considered for *Spiroplasma* or *Trypanosoma* infection status. To confirm that the amplified PCR product obtained with *G. pallidipes, G. m. morsitans, and G. p. gambiensis*, were *Spiroplasma* specific sequences, two approaches were used: the first is run PCR with MLST primers indicated in Supplementary table 2 to confirm the amplification. The second is to sequence the PCR product obtained by the 16sRNA primers. To conduct the sequence, PCR products were purified using the High Pure PCR Clean-up Micro Kit (Roche, Basel, Switzerland) and ligated to the pGEM-T vector (Promega, Madison, Wisconsin, USA), following the supplier's instructions. The recombinant plasmids were transformed into DH5α competent bacteria (Invitrogen, Carlsbad, California, USA) following the supplier's instructions. The recombinant plasmids and the inserted sequences were confirmed by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany) with the universal vector primers M13F_uni (-21) (5′-TGT AAA ACG ACG GCC AGT-3′) and M13R_rev (-29) (5′-CAG GAA ACA GCT ATG ACC-3′). For other tsetse species including *G. f. fuscipes, G. brevipalpis and G. tachinoides* amplified PCR products were purified with the ZR-96 DNA Clean & Concentrator®-5 (California, USA) following the manufacturer's protocol and submit directly to sequencing without cloning using 63F and TKSS (Eurofins Genomics, Ebersberg, Germany). The resulted sequences were blasted against non-redundant protein sequence (nr) data base in NCBI server using the BLAST tool https://blast.ncbi.nlm.nih.gov/Blast.cgi to identify and annotate the sequence. The sequence

was considered a *Spiroplasma* sequence if it matched with *Spiroplasma* sequence in the data base. The prevalence of *Trypanosoma* was assessed and reported in Ouedraogo et al [43].

Analysis of the interaction between *Spiroplasma* and *Trypanosoma* in *G. tachinoides*

The co-infection of *Spiroplasma* and *Trypanosoma* spp was evaluated with PCR prevalence. The trypanosome prevalence was determined as previously described and reported in Ouedraogo et al [43]. The infection status was divided into four categories: 1. *Spiroplasma* positive and *Trypanosoma* positive (Sp+/T+), 2. *Spiroplasma* positive and *Trypanosoma* negative (Sp+/T-), 3. *Spiroplasma* negative and *Trypanosoma* positive (Sp-/T+) and 4. *Spiroplasma* negative and *Trypanosoma* negative (Sp-/T-).

Analysis of the density of *Spiroplasma*, *Trypanosoma* and *Wigglesworthia* infection density

Samples showing the following infection status (Sp+/T+), (Sp+/T-) and (Sp+/T-) were used to assess the density of *Spiroplasma*, *Trypanosoma* spp and *Wigglesworthia* using quantitative PCR (qPCR). The qPCR was performed using a CFX96 Real Time PCR Detection System (Bio-Rad). *The Spiroplasma* density was assessed by the amplification of 16sRNA gene with the qPCR *Spiroplasma* primers **(Supplementary table 2)** In addition, the density of *Wigglesworthia* was evaluated as previously described [18] using the thiC (thiamine biosynthesis gene) **(Supplementary table 2)**. Based on the above-mentioned criteria 212 individual G. tachinoides (76, 65 and 71 flies with infection status of (Sp+/T+), (Sp+/T-), and (Sp-/T+), respectively, were selected from Burkina Faso and Ghana samples. In addition, samples with (Sp+/Tryp+), (Sp+/T-) and (Sp-/T+) were used to assess the impact of *Spiroplasma* infection on *Trypanosoma* density. Trypanosomatidae 18S specific primers (18S_Typ_F and 18S_Typ_R) **(Supplementary table 2)** were used to assess the *Trypanosoma* density in the tested samples. The DNA from all selected samples was diluted to a final concentration of 4 ng/μl and 5 μl of the diluted DNA was used for qPCR to determine *Spiroplasma*, *Wigglesworthia* and *Trypanosoma* DNA density normalized to the housekeeping β-tubulin gene. The amplification mixture contained 5 μl of DNA template, 200 nM of each primer, and 7.5 μl iQTM SYBER Green Supermix (Bio-Rad). qPCR cycling conditions for *Spiroplasma* and *Wigglesworthia* were as follows: initial denaturation at 95 °C for 2 min; 39 cycles of 95 °C for 5 s, 55 °C for 30 s, one step at 95 °C for 5 s and a melting curve constructed from 65°C to 95 °C in increments of 0.5 °C for 5 s. Same conditions were used for *Trypanosoma* except the annealing temperature, which was at 60 °C. The analysis of the

Spiroplasma, *Wigglesworthia*, *Trypanosoma* and Tubulin densities was based only on qPCR data with the expected melting curve at 81.5°C, 85.5°C and 86°C, respectively.

Genetic variation and phylogenetic analysis of *Spiroplasma* in *G. tachinoides*

To assess the genetic variation of *Spiroplasma* in wild *G. tachinoides*, DNA of positives samples from Burkina Faso and Ghana were amplified by PCR on 4 genes 16sRNA, *Spiroplasma* fructose repressor (fruR), *Spiroplasma* DNA Topoisomerase 4 subunit B (parE), and RNA polymerase subunit beta (rpoB) with a multi-locus sequence typing (MLST) approach. Primer sets used for each reaction, product sizes, and PCR conditions, are shown in the **(Supplementary table 2)**.

All amplified PCR products were purified using The High Pure PCR Cleanup Micro Kit (Roche Diagnostics, Indianapolis, IN, USA) and the ZR-96 DNA Clean-up Kit™ (Zymmo research, California, USA). Sequencing was performed as described previously. The results of the sequence were analysed using Geneious Prime® 2023.0.2. The sequences were analysed based on the quality of the sequencing chromatograms. Sequences were first cleaned manually to avoid any ambiguity and those who showed a lot of ambiguities were removed from the analysis. For each gene, sequences were aligned and to have the same length, the smallest sequence was kept as the standard size and the extra nucleotides to the left and right of the longest sequences were removed. Sequences were then blasted on the "Blast" resource of NCBI to confirm that it was a *Spiroplasma* sequence.

Phylogenetic trees were built for each gene (16sRNA, fruR, pare and rpoB) and for a concatenated sequence of the 4 genes. Multiple alignments were then performed using MUSCLE alignment with the default parameters on Geneious Prime® 2023.0.2. and the Neighbor-joining tree was built using the Tamura-Nei genetic distance model.

Data analysis

The data of the prevalence were analysed in R using Rstudio version V 1.4.1106 [4]. For all the samples, the prevalence of *Spiroplasma* and *Trypanosoma* spp was analysed for differences between countries and between localities in each country. The significant differences between the different prevalence was analysed with general linear model (glm) [45] combined with the ANOVA respectively provided by the package ggplot [63] and car. The data of the density of *Spiroplasma*, *Trypanosoma* and *Wigglesworthia* was normalised with the tsetse house-keeping tubulin gene before analysis and only the samples which shown quantification for the 3 microorganisms were used for the analysis. Like the prevalence, the glm was used to analyse

the significant differences between the density of *Spiroplasma*, *Trypanosoma* and *Wigglesworthia* according to the different *Spiroplasma* and *Trypanosoma* co-infection status **(Supplementary file 1)**. To evaluate the association between *Spiroplasma* and *Trypanosoma*, the Cochran-Manthel-Haenzel (CMH) test and the chi-square test were performed on the excel table as described in Dieng et al [19].

Results

Prevalence of *Spiroplasma*

The presence of *Spiroplasma* in wild populations of tsetse flies was assessed using a PCR-based method to amplify the 16sRNA sequence. Positive samples were identified based on the observed amplicon band size in the electrophoresis gel for all tsetse species. The sequencing work revealed that *Spiroplasma* infection was only confirmed in *G. tachinoides (N=41)* and *G. f. fuscipes (N=6)*, both belonging to the *palpalis* subgenus **(Table 1)**. In the case of *G. brevipalpis*, *G. m. morsitans*, *G. m. submorsitans, G. palidipes, G. p. gambiensis*, and *G. p. palpalis*, the amplified sequence belonged to different microbiota primarily consisting of *Bacillus cereus*, *Bacillus thuringiensis*, *Enterococcus cecorum*, and some uncultured bacteria **(Data not shown)**.

The PCR results indicated an overall *Spiroplasma* prevalence of 39.27% in *G. tachinoides.* The prevalence did not differ significantly between Burkina Faso, Ghana and the samples of the colony ($\chi2=2.12$, df=2, and $p=0.34$), with Burkina Faso and Ghana showing a prevalence rate of 46.56% and 52.94%, respectively **(Table 2).** However, a significant variation in *Spiroplasma* prevalence was found across the various sampling locations ($\chi2=22.61$, df=8, and $p=0.003$) **(Table 2 and Figure 2 and 3).** Specifically, there was a significant difference in prevalence between the two sampling locations in Burkina Faso ($\chi2=6.459$, df=1, and $p=0.01$), with a higher prevalence observed in Folonzo. Similarly, a significant difference was found between the prevalence rates of different locations in Ghana ($\chi2=11.955$, df=5, and $p=0.03$), with the highest prevalence observed in the Mortani region (98.44%), where 100% of the female flies were infected. Conversely, the lowest prevalence of *Spiroplasma* was recorded in Kumpole, Ghana (25%), with no male flies showing any signs of an infection **(Table 2, Figures 2 and 3)**.

Table 1: *Spiroplasma* identification in 8 *Glossina* species from 10 different countries using 16sRNA, Multi locus sequence typing (MLST) and sanger sequencing

Species	Country	Location	Total number of analysed flies	Number of flies with valid DNA	[a]Nb of *Spiroplasma*-positive using 16sRNA	[b]Samples positives with MLST/ Samples tested with MLST	[c]Samples successfuly sequenced	Confirmation after sequencing
G. brevipalpis	South Africa	Zululand	50	0	0			
		Colony	**94**	**94**	**37**	**0/16**	**4**	No
		Phinda	180	180	0			
G. f. fuscipes	**Uganda**	**Buvuma Island**	**147**	**94**	**6**	**6/6**	**6**	Yes
G. m. moristans	**Zimbabwe**	**Makuti**	**94**	**94**	**17**	**0/6**	**4**	No
G. m. submorsitans	Burkina Faso	Singou	3	3	0			
		Comoe	32	31	4	0/4		
		Folonzo	**152**	**135**	**24**	**0/12**	**2**	No
G. p. gambiensis	Senegal	**Kedougou**	**62**	**60**	**57**	**0/22**	**4**	No
		Pout	207	199	169	0/18		
		Sebikotane	41	39	23	0/12		
		Diaka Madia	80	79	44	0/41		
		Diacksao peulh	70	65	62			
		Hann	31	28	24	0/21		
	Guinea	Kansaba	32	31	29	0/9		
		Minipontda	32	29	15	0/6		
		Kindoya	87	83	80	0/66		
		Ghanda Oundou	27	20	14	0/10		
		Fefe	10	10	7	0/2		
		Togoue	21	21	21	0/21		
		Alahine	13	12	12	0/12		
		Boureya Kolonko	60	46	46	0/38		
	Mali	Fijira	14	14	11	0/11		
		Astan	138	126	85	0/32		

Species	Country	Location	Total number of analysed flies	Number of flies with valid DNA	[a]Nb of *Spiroplasma*-positive using 16sRNA	[b]Samples positives with MLST/ Samples tested with MLST	[c]Samples successfully sequenced	Confirmation after sequencing
	Burkina Faso	Comoe	116	82	69	0/26		
		Kenedougou	12	12	5			
		Folonzo	153	123	70	0/23		
		Moussodougou	54	49	1			
		Kartasso	136	118	107	0/20		
G. p. palpalis	**Republic Democratic of Congo**	**Katanga**	**44**	**23**	**4**	**0/4**	**4**	No
G. palidipes	**Ethiopia**	**Arba mich**	**94**	**94**	**24**	**0/8**	**8**	No
G. tachinoides	Burkina Faso	Comoe	119	119	29	24/24	24	Yes
		Folonzo	347	347	188	17		
		Colony (CIRDES)*	**25**	**19**	**2**	**2/2**	**2**	Yes
	Ghana	**Walewale**	**108**	**108**	**47**	**15/38**	**15**	Yes
		Mortani	41	41	40			
		Fumbissi	14	14	4	4/4		Yes
		Sissili Bridge	6	6	3			
		Grogro	11	11	4			
		Kumpole	7	7	1			

First, *Spiroplasma* positive flies were identified using primers designed for the 16s rRNA sequence[a]. Samples that were found to be positive then were tested using the Multi locus sequence typing (MLST) gene to confirm the presents of *Spiroplasma*[b]. *Spiroplasma* positive samples confirmed by both primers (16s rRNA, MLST) or at least the 16sRNA sequence for each species were sequenced[c]. Samples sequences that didn't match with the *Spiroplasma* sequences and matched with other bacteria were not considered as positive for *Spiroplasma* infection. *samples from colony maintained in CIRDES,
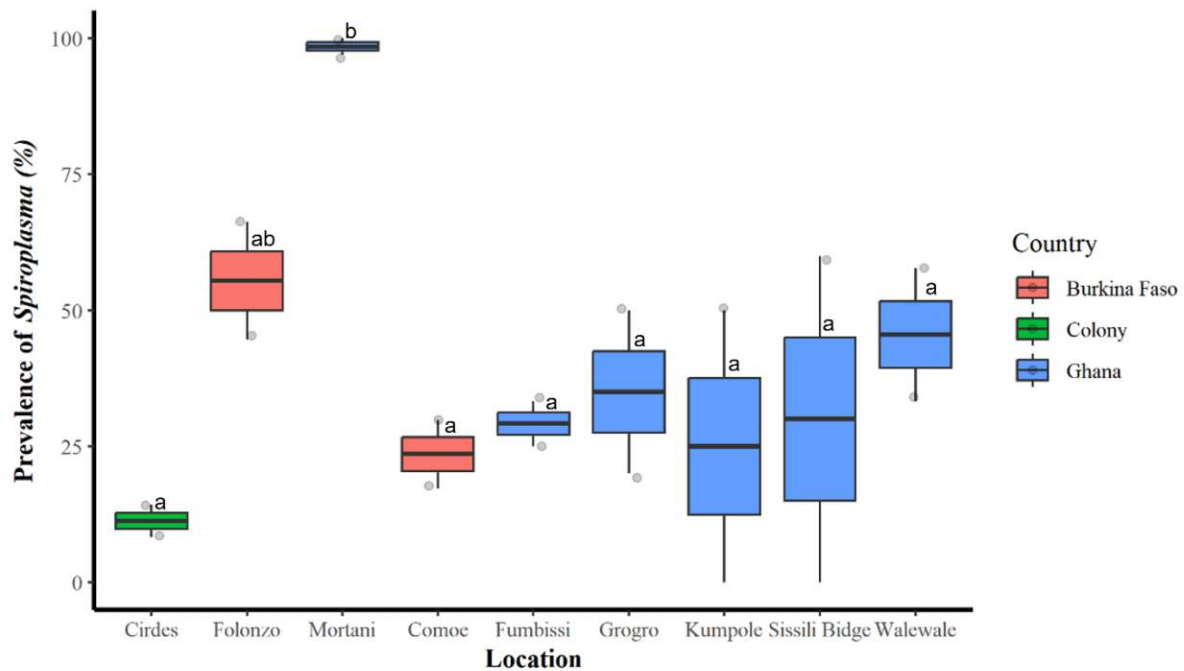
Figure 2: Prevalence of *Spiroplasma* according to the location, Bars marked with the same lower-case letter do not differ significantly at the 0.05 level.
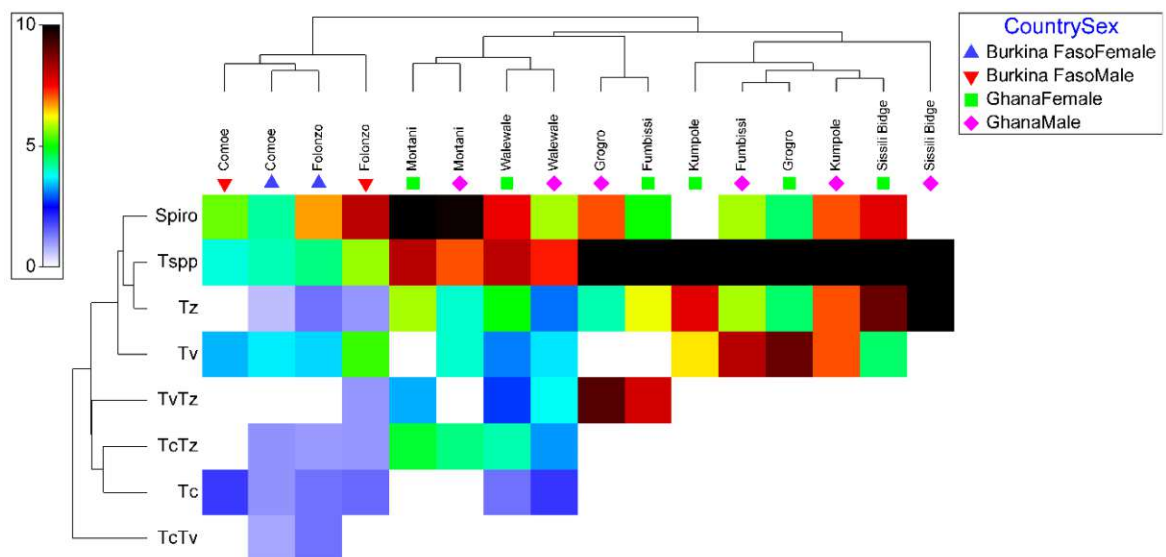
Figure 3: Prevalence of the *Spiroplasma* and *Trypanosoma* (single and multiple) infections per country, location and per sex. Prevalence data were square root transformed and averaged based on location-sex and the matrix display was conducted in PRIMER version 7 + software. Tree on the left of the matrix is the similarity dendrogram based on the similarity index of the square root of the prevalence values. The colour index is the square root of the prevalence values ranged 0–9 which is the square root of 0–81% prevalence.

Table 2: Global prevalence of *Spiroplasma* in *Glossina tachinoides* according to the locations and

Country	Location	Sex	*Spiroplasma* prevalence (%)	*Trypanosoma* Prevalence (%)*
Burkina Faso	Comoe	F	17.31	16.54
	Comoe	M	29.85	15
	Folonzo	F	44.56	18.87
	Folonzo	M	66.23	32.63
	Subtotal		**46.56**	**20.76**
Colony	Cirdes	F	14.29	-
	Cirdes	M	8.33	-
	Subtotal		**11.31**	**-**
Ghana	Walewale	F	57.78	66.04
	Walewale	M	33.33	53.85
	Sissili Bidge	F	60.00	100
	Sissili Bidge	M	0.00	100
	Fumbissi	F	25.00	100
	Fumbissi	M	33.33	100
	Kumpole	F	0.00	100
	Kumpole	M	50.00	100
	Grogro	F	20.00	100
	Grogro	M	50.00	100
	Mortani	F	100.00	66.67
	Mortani	M	96.88	50
	Subtotal		**52.94**	**86.38**
	Total (average)		**39.27**	**69.97**

*Data already published in (Ouedraogo et al., 2018)

Prevalence of single and multiple *Trypanosoma* infections

The screening of the flies indicated the presence of different taxa of *Trypanosoma*, including *Tc* (*Trypanosoma congolense type: Savanah, Kilifi, Forest*), *Tv* (*Trypanosoma vivax*), and *Tz* (*Trypanozoon sp: Trypanosoma brucei brucei, Trypanosoma brucei gambiense, Trypanosoma brucei rhodesiense, Trypanosoma evansi*). The overall prevalence of single or multiple *Trypanosoma* infections among all tested flies was 69.97% (457/653). The prevalence of *Trypanosoma* varied significantly between countries ($\chi2= 37.18$, df=1, and $p < 0.001$) and locations ($\chi2= 452.21$, df=7, and $p < 0.001$). In Ghana, the prevalence was significantly higher than in Burkina Faso, at 86.38% and 20.76%, respectively (**Table 2 and Figure 3**). In Ghana, the prevalence varied significantly with location ($\chi2= 125.43$, df=5, and $p < 0.001$), with a prevalence of 100% in some locations such as Sissili Bridge, Fumbissi, Kumpole, and Grogro (**Figure 3 and Supplementary table 3**).

The most frequently found trypanosomes were *Tz* and *Tv*, with a prevalence of 30.2% and 22.42%, respectively. However, only *Tz* varied significantly with country (χ2= 7.54, df=1, and p = 0.006) and location (χ2= 185.82, df=7, and p < 0.001). *Trypanosoma congolense* was found in the two locations in Burkina Faso (Comoe at 2.37% and Folonzo at 2.00%), and only in one location in Ghana (Walewale (2.87%)). Its prevalence varied significantly with country (χ2= 6.426, df=1, and p = 0.01) and location (χ2= 34.97, df=7, and p < 0.001).

The *TvTz* multiple infection was the most prevalent in the samples with a prevalence of 11.22%. In Ghana, no *TcTv* double infections were found, while in Burkina Faso, no triple infections *TcTvTz* were found. The prevalence of the double infections varied only according with location (χ2= 245.15, df=7, and p < 0.001) (**Figure 3 and** *TcTz* **Supplementary table 3**).

Interaction between *Spiroplasma* and *Trypanosoma*

Prevalence of co-infections

The results of the analysis showed that 12.56% of the flies were infected both with *Spiroplasma* and *Trypanosoma*, regardless of country, location, and sex. However, the prevalence of single infections of *Spiroplasma* (35.83%) was higher than that of *Trypanosoma* (17.46%). (**Figure 4**). The association between *Spiroplasma* and *Trypanosoma* infections was analyzed using the Cochran-Manthel-Haenzel (CMH) test and chi-square test. Across all samples, the CMH test showed a significant deviation from independence between the two infections (χ2MH = 5.19, df = 1, p= 0.02). The chi-square test confirmed that the independence between *Spiroplasma* and *Trypanosoma* infections was significant with a Bonferroni corrected α = 0.006 (χ2 = 9.85, p = 0.03). However, when considering countries, only in Ghana the chi-square test did show a significant deviation from independence between the two microbiotas (χ2 = 13.004, p<0.001) (**Table 3 and Supplementary tables 4**).
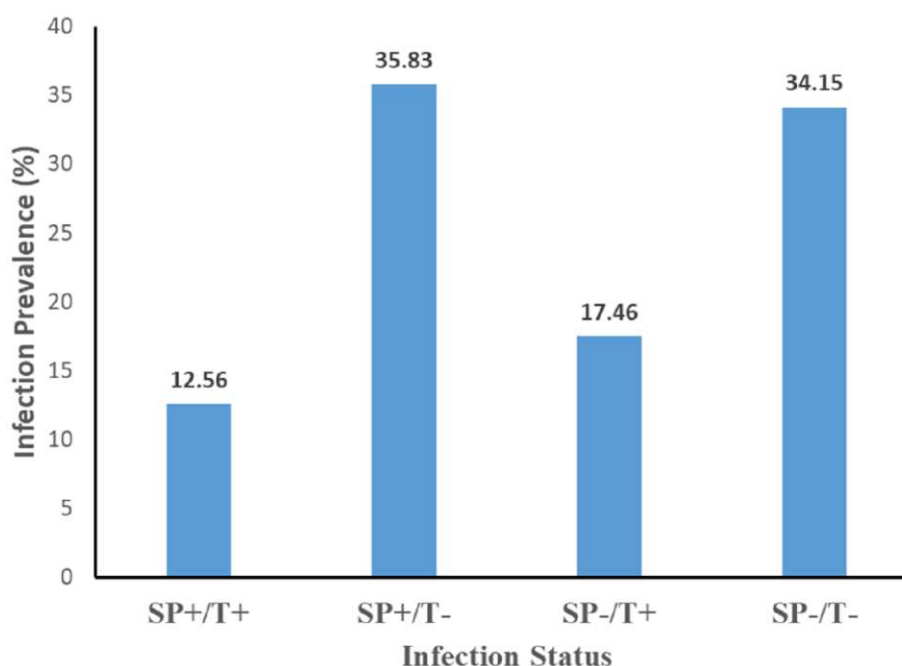
Figure 4: Prevalence of co-infection *Spiroplasma-Trypanosoma* in wild *G. tachinoides*

Table 3: Distribution of the association between the presence of *Trypanosoma* spp and the presence of *Spiroplasma* according to the country and the locations

Glossina species	Country (Area, Collection Date)	N	Sp+/T+	Sp +/T-	Sp-/T+	Sp-/T-	χ^2	*P*
G. tachinoides	Burkina Faso (Comoe,)	119	1	28	15	75		
G. tachinoides	Burkina Faso (Folonzo,)	347	27	161	29	130		
Sub-Total		**466**	**28**	**189**	**44**	**205**	**2.01**	**0.15**
G. tachinoides	Ghana (Walewale,)	108	24	23	43	18		
G. tachinoides	Ghana (Sissili Bidge,)	6	3	0	3	0		
G. tachinoides	Ghana (Fumbissi,)	14	4	0	10	0		
G. tachinoides	Ghana (Kumpole,)	7	1	0	6	0		
G. tachinoides	Ghana (Grogro,)	11	3	1	7	0		
G. tachinoides	Ghana (Mortani,)	41	19	21	1	0		
Sub-Total		**187**	**54**	**45**	**70**	**18**	**13.03**	**0.0003**
Total		**840**	**136**	**279**	**184**	**241**	**9.85**	**0.001**

Co-infection and the density of *Spiroplasma*, *Trypanosoma* and *Wigglesworthia*

The density of *Spiroplasma*, *Trypanosoma*, and *Wigglesworthia* was evaluated using relative qPCR based on the single (Sp+/T-; Sp-/T+) and double co-infection (Sp+/T+) status. As expected, the results showed that flies infected with *Spiroplasma* (Sp+/T- and Sp+/T+) had a significantly higher density of *Spiroplasma* compared to those not infected (Sp-/T+) which indicate that flies classified as uninfected with PCR showed lower infection rate with qPCR. However, there was no significant difference in the density of *Spiroplasma* between flies

infected with *Spiroplasma* and not infected with *Trypanosoma* (Sp+/T-) and those infected with both (Sp+/T+) (**Figure 5A**). Furthermore, obviously flies with double co-infection (Sp+/T+) had a significantly higher density of the trypanosome than those with single co-infection (Sp+/T- and Sp-/T+) (**Figure 5B**). However, no significant difference was found in the density of *Wigglesworthia* in the three categories of co-infection (**Figure 5C**).
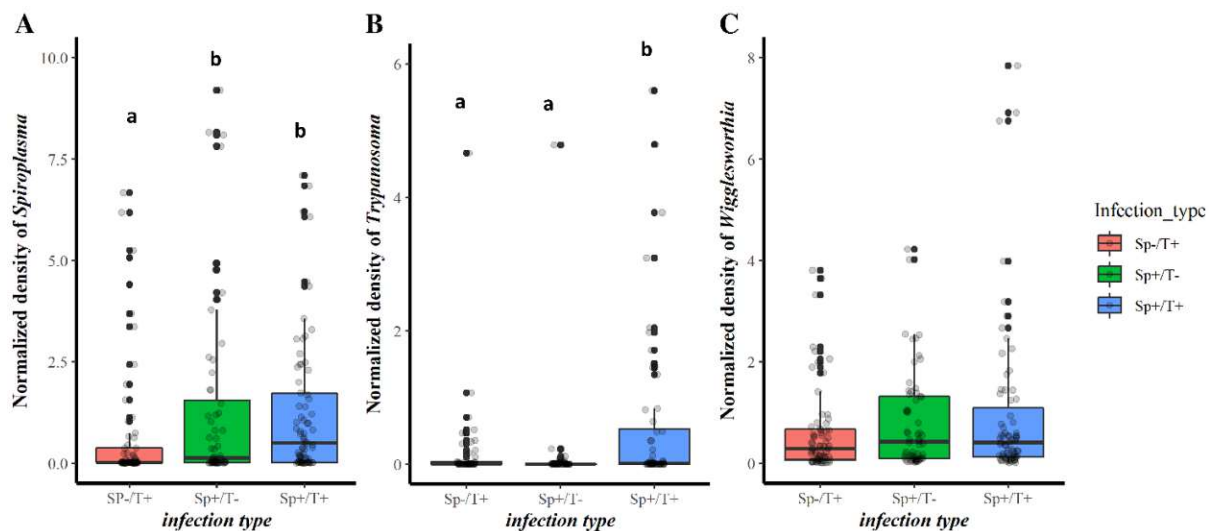

Figure 5: Normalized density of *Spiroplasma* (A), *Trypanosoma* (B) and *Wigglesworthia* (C) according to the co-infection *Spiroplasma-Trypanosoma* in wild *G. tachinoides*. Bars marked with the same lower-case letter do not differ significantly at the 0.05 level.

Genetic variation and phylogenetic analysis of *Spiroplasma* in wild *G. tachinoides*

Among the 35 samples sequenced, a total of 14 sequences from Comoe in Burkina Faso, 2 from the CIRDES colony and 2 from Wale Wale in Ghana was used for the analysis. For the 4 genes used for the sequencing, 2885 base pairs were generated. The comparison of the sequence showed a global nucleotide mutation rate of 0.06% with 2SNPs. (**Table 4**). This 2 SNPs were found on the *parE* gene (1SNP/745bp) and *rpoB* (1SNP/1455). None of these substitutions was non-synonymous and the percentage of amino-acid mutations was 0.40% (1/248) for the *parE* gene and 0.20% (1/485) for the *rpoB* gene. For the *parE* gene, the mutation was between Isoleucine and Valine, but for the *rpoB* gene it was between Phenylalanine and Serine. All the samples from all the locations have shown the same profile for 16sRNA and *fruR* genes. In Burkina Faso and Ghana, 2 genotypes profiles were found where only one was found for CIRDES (**Table 5 and 6).** Three different haplotypes were found in the sampling areas with a specific haplotype for the cirdes colony and Burkina Faso and Ghana sharing the same haplotypes (**Table 6, Fig 6**).

Table 4: Summary of information for the nucleotide polymorphisms detected in the partial sequences of *Spiroplasma* in *G. tachinoides*

Genes	Length (bp)	No. of SNP/total number of nucleotides (%)	No. of nucleotides substitutions/total no. of nucleotides (%)		No. of amino acid mutations (%)
			No. of silent nucleotides substitution	No. of non-silent nucleotides substitutions	
16sRNA	352	0/352 (0.00)	-	-	-
fruR	333	0/333 (0.00)	-	-	-
parE	745	1/745 (0.13)	0/1 (0.00)	1/1 (100)	1/248 (0.40)
rpoB	1455	1/1455 (0.06)	0/1 (0.00)	1/1 (100)	1/485 (0.20)
Total	2885	2/2885 (0.06)	0/2 (0.00)	2/2 (100)	2/733 (0.27)

Table 5: Alleles of *Spiroplasma* in different locations of tested countries. Numbers between brackets indicate the number of sequences tested per each allele.

Country	Location	No of flies tested per sequence	16sRNA	*fruR*	*parE*	*rpoB*
Burkina Faso	Comoe	14	1 (14)	1 (14)	1 (6) 2 (8)	1 (14)
	Cirdes*	2	1 (2)	1 (2)	1 (2)	3 (2)
Ghana	Wale wale	2	1 (2)	1 (2)	1 (1) 2 (1)	1 (2)

Cirdes*: colony of *G. tachinoides* established in CIRDES insectary

Table 6: The *Spiroplasma* haplotypes found in the same individuals collected in eastern and southern African countries. The frequency of occurrence of the haplotypes is shown in the last column. The number in parentheses indicates the total number of flies in which the haplotype was detected

Country	Location	No of flies tested	No of gene profiles	16sRNA	*fruR*	*parE*	*rpoB*	Haplotype No	Frequency
Burkina Faso	Comoe	14	2	1	1	1	1	**H1**	6 (14)
				1	1	2	1	**H2**	8(14)
	Cirdes*	2	1	1	1	1	2	**H3**	2 (2)
Ghana	Wale wale	2	2	1	1	1	1	**H1**	1 (2)
				1	1	2	1	**H2**	1 (2)

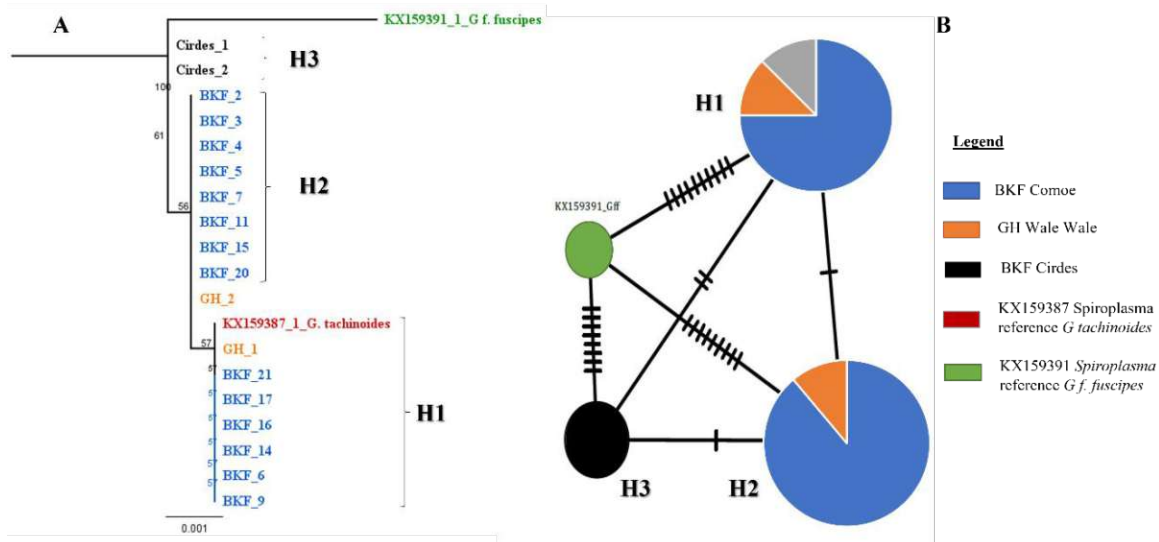Cirdes*: colony of *G. tachinoides* established in CIRDES insectary

Figure 6: Neighbor-Joining consensus tree (**A**) and Haplotype network analysis (**B**) of the *Spiroplasma* in *G tachinoides* in Burkina Faso and Ghana. **(A)**The Neighbor-Joining consensus tree was built after alignment of all the concatenated sequences. The method used to calculate the distance was Tamura-Nei. **(B)** The haplotype network generated based on the ML tree generated based on *Spiroplasma* sequences. The black lineaments on the lines represent mutations events between the haplotypes. The different colours represent the locations. The reference sequence of *Spiroplasma* in *G. fuscipes fuscipes* species with the reference KX159391 was used as outgroup for both phylogenetic tree and haplotype network.

Discussion

In this study, we evaluated the prevalence of the endosymbiont *Spiroplasma* and the *Trypanosma* parasite in wild *G. tachinoides* in Burkina Faso and Ghana, and the interaction between these two microbiotas and *Wigglesworthia*. The discovery of the presence of *Spiroplasma* in tsetse flies is quite recent although its presence in other insects and plants, has been shown a long time ago. Doudoumis et al [21] showed the presence of *Spiroplasma* in both laboratory colonies and field populations of *G. tachinoides*, *G f. fuscipes* and *G. p palpalis*, all belonging to the palpalis group.

The present study has confirmed the presence of *Spiroplasma* in *G. tachinoides* in both wild populations and colonised insectary flies. Using the 16sRNA gene sequencing, we observed amplification of a bacterial community in the tsetse species that did not belong to the palpalis group that was different from *Spiroplasma*. Since the 16sRNA gene is shared with all bacterial species and one of the most conserved bacterial genes [64], primers designed targeting this region could have a broad range of bacterial detection. It is therefore necessary to carry out taxonomical confirmation by sequencing amplicons of the positive PCRs [30]. The prevalence in the field was found to be similar to that observed by El Khamlichi et al [23], but higher than that reported by Doudoumis et al [21]. However, the prevalence of *Spiroplasma* in the colony

was lower than that observed by Doudoumis et al [21]. The prevalence of the infection varied significantly with location. Furthermore, although the prevalence rates did not differ significantly between Burkina Faso and Ghana, the observed differences in prevalence rates between individual sampling locations suggest that regional variations may impact the infection and the distribution of *Spiroplasma*.

In the study area, three major trypanosomes of human and animals were found, *T. brucei* s.l. (Tz), *T. congolense* (Tc) and T. vivax (Tv), with a relatively high prevalence, particularly in Ghana. This high prevalence of *Trypanosoma* in the flies explains the presence of AAT in the sampling site, highlighting the significant risk of infection in this area. A human infection risk is not to exclude as Tz was identified. Indeed, they are the main cause of HAT [10,37]. The presence of Tv in Burkina Faso was already shown previously [55]. The prevalence of *Trypanosoma* was almost similar to the result of Djohan et al [20] in Cote d'Ivoire (61.4%) but significantly higher (69.97%) than the prevalence obtained by Kame-Ngasse et al [31] in the north of Cameroon (34.81%) and Meharenet and Alemu [38] in Ethiopia. The difference of the prevalence with the work of Meharenet and Alemu [38] could be due to the diagnostic method used. In their study, the authors used dissection to identify the presence or absence of the parasite, which has some disadvantages, including low sensitivity and susceptibility to the examiner's technicity [12]. Female flies appeared to have higher infection rates than males, which is in line with the results of Meharenet and Alemu [38] and Lefrancois et al [33], and may be due to their longer lifespan. *Trypanozoon* spp and *T. vivax* were the most predominant *Trypanosoma*. Djohan et al [20] also found the same predominant species of trypanosome with Tv present at 27.2%. Conversely, Kame-Ngasse et al [31] observed that in *G. tachinoides* in Cameroon, Tc was dominant. Since the active foci of the HAT are different, the distribution of parasites will depend on the working area. Previous studies have shown the predominance of Tc in the "Faro and Deo" region in Cameron [35,36].

Mixed infections were predominantly TvTz (11.22%) and TcTz (4.45%). Previous studies have shown that *G. tachinoides* is commonly infected with various types of trypanosomes. However, the composition of the mixed infections may depend on the distribution of the parasite and the identification method used. For instance, in Côte d'Ivoire, TvTcs (9.4%) and TcTcs (12.5%) were the predominant mixed infections [20], whereas in Cameroon TcsTcf (4.8%) was predominant [31].

Our analyses with RT-qPCR showed that a *Spiroplasma-Trypanosoma* coinfection had no significant effect on the density of *Wigglesworthia*. This bacteria is an obligate tsetse fly endosymbiont that provides essential nutrients that are absent in blood meals [8]. It is maternally transmitted, making it difficult for other microbiota to invade its niche [3]. Although *Spiroplasma* can be maternally transmitted [56], its presence or absence did not affect the density of *Wigglesworthia* or *Sodalis* in laboratory *G. f. fuscipes* flies.

The analysis of the prevalence of *Spiroplasma* and *Trypanosoma* coinfections suggests a significant deviation from independence, as most of the flies infected with *Spiroplasma* were not infected with *Trypanosoma*, and vice versa. This may indicate that the presence of *Spiroplasma* could confer a certain level of refractoriness to *Trypanosoma* infection. This hypothesis was confirmed by the Cochran-Mantel-Haenzel (CMH) and chi-square tests, that showed a significant deviation from independence between the two microorganisms across all samples. Our results align with those of Schneider et al [50] who reported that only 2% of *Spiroplasma* infected flies in *G. f. fuscipes* species were also infected with trypanosomes. The same study also found that, under laboratory conditions, trypanosomes were less likely to colonize the midgut of *G. f. fuscipes* infected with *Spiroplasma*. However, our results did not agree with the higher prevalence of *Spiroplasma* found in *Trypanosoma* infected *Glossina palpalis palpalis* flies than uninfected one [40]. The mechanism by which this bacterium enhances refractoriness to trypanosomes in flies remains unclear. It could be related to competition for proliferation niches, given that *Spiroplasma* is found in both the midgut and haemolymph, or to the induction of an immune response in the fly or specific gene regulation. It might also be due to competition for specific nutrients that both microbiota need for their development. This has been observed with the endosymbiont *Sodalis*, which competes with the host and parasite for host nutrients [52–54]. Our study indicates that the possible refractory effect of *Spiroplasma* on trypanosome infection is not species-dependent, as it was observed in both *G. tachinoides* and *G. f. fuscipes*. However, these two species belong to the palpalis subgroup, within which *Spiroplasma* was exclusively found in our study. Our genotyping analysis showed that the strains of *Spiroplasma* found in *G. tachinoides* in Burkina Faso and Ghana are most closely related to the citri group, as previously reported. This clade is composed of various taxa that are pathogens for plants, such as *S. phoeniceum* [49], and *S. citri* [48], as well as protecting Drosophila, against nematodes infection and parasitic wasps such as *S. poulsonii* [29,44,65,66]. Despite belonging to the same citri group, three haplotypes were identified, with Burkina Faso and Ghana sharing two haplotypes and one specific haplotype

for the CIRDES colony samples. These information sheds light on the genetic diversity of *Spiroplasma* in the field and in colonies, which could help understanding its evolution. The colonization process may induce several mutations that could lead to the development of new haplotypes. Insect microbiota can be influenced by a variety of factors, including environmental conditions, host genetics, and interactions with other organisms. When an insect is colonized, its microbiota may be exposed to different environmental conditions or may interact with new microbial communities, resulting in changes in the composition or function of the microbiota.

The SIT for tsetse flies relies on the release of sterile males within the context of area-wide insect pest management (AW-IPM). To prevent or reduce the transmission of trypanosomes by the released sterile males, they receive at least 2 bloodmeals with trypanocidal drugs before being released. However, this is cumbersome and costly, so the discovery that *Spiroplasma* infection could confer refractoriness to the trypanosome infection in flies presents an elegant way to mitigate the transmission risk. Releasing of sterile males infected with *Spiroplasma*, that are to a certain degree refractory to the trypanosome parasite, would reduce the risk of transmission. Moreover, since paternal transmission of *Spiroplasma* occurs, albeit imperfectly, the offspring from the residual fertility of the sterile males released could also be infected with *Spiroplasma* and be relatively refractory to the parasite. *Spiroplasma* is an endosymbiont that can significantly improve the effectiveness of SIT, making the study and management of this microbe crucial.

Conclusion

This study reinforced the hypothesis that *Spiroplasma* could enhance refractoriness to trypanosome infections in certain species of tsetse flies, and this would make this symbiont a good candidate for paratransgenesis in addition to *Sodalis* as previously described. More investigations are required with field samples to understand better the interaction of *Spiroplasma* and the trypanosome, and to evaluate the impact of ionization radiation on the dynamism of *Spiroplasma*.

Author Contributions: A.M.M.A. and M.J.B.V. conceived and designed research. P.M., S.P., F.N., F.S.N.F., C.D.B., collected biological materials from the field. K.M.D., M.M.D., P.M. and A.M.M.A. collected data and conducted research. K.M.D., M.M.D., and A.M.M.A. analyzed and interpreted data. K.M.D., M.M.D., and A.M.M.A. wrote the initial paper; K.M.D., M.M.D., P.M., G.M.S.O., S.P., F.N., F.S.N.F., C.J.D, R.L.M., M.J.B.V., and A.M.M.A. revised the paper; A.M.M.A. had primary responsibility for final content. All authors read and approved the final manuscript.

Data Availability Statement: Materials described in the paper, including all relevant raw data, are available in this link https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/ZKIR8I

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Ethics approval and consent to participate

Not applicable.

Conflict of interests

The authors declare that they have no conflict interests.

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Supplementary file 1: R markdown

Supplementary tables

<u>Supplementary table 1</u>: Country of origin and location

Country of origin and location	Species	Country	Location	Latitude	Longitude
South Africa (Zululand)	*G. brevipalpis*	South Africa	Zululand	-28.85	31.833333
South Africa (Phinda)	*G. brevipalpis*	South Africa	Phinda	-27.82598	32.2863
Uganda (Buvuma Island)	*G. f. fuscipes*	Uganda	Buvuma Island	0.224444	33.273333
Burkina Faso (Singou)	*G. m. submorsitans*	Burkina Faso	Singou	11.3	1.033333
Burkina Faso (Comoe)	*G. m. submorsitans*	Burkina Faso	Comoe	9.89106718	-4.58976269
Bukina Faso (Folonzo)	*G. m. submorsitans*	Burkina Faso	Folonzo	9.92967851	-4.60801757
Senegal (Kedougou)	*G. p. gambiensis*	Senegal	Kedougou	12.62932251	-12.09137828
Senegal (Diacksao peulh)	*G. p. gambiensis*	Senegal	Diacksao peulh	15.005758	-16.639484
Senegal (Pout)	*G. p. gambiensis*	Senegal	Pout	14.7818	-17.062551
Senegal (Sebikotane)	*G. p. gambiensis*	Senegal	Sebikotane	14.7450749	-17.1336827
Senegal (Diaka Madia)	*G. p. gambiensis*	Senegal	Diaka Madia	13.8	-13.967
Senegal (Diacksao peulh)	*G. p. gambiensis*	Senegal	Diacksao peulh	15.005758	-16.639484
Senegal (Hann)	*G. p. gambiensis*	Senegal	Hann	14.725682	-17.43591
Guinea (Kansaba)	*G. p. gambiensis*	Guinea	Kansaba	1246788	253124
Guinea (Minipontda)	*G. p. gambiensis*	Guinea	Minipontda	1219003	278716
Guinea (Kindoya)	*G. p. gambiensis*	Guinea	Kindoya	1349672	215596
Guinea (Ghanda Oundou)	*G. p. gambiensis*	Guinea	Ghanda Oundou	1303042	220377
Burkina Faso (Comoe)	*G. p. gambiensis*	Burkina Faso	Comoe	9.89106718	-4.58976269
Burkina Faso (Kenedougou)	*G. p. gambiensis*	Burkina Faso	Kenedougou	10.98166737	-4.80305222
Burkina Faso (Folonzo)	*G. p. gambiensis*	Burkina Faso	Folonzo	9.92967851	-4.60801757
Burkina Faso (Moussodougou)	*G. p. gambiensis*	Burkina Faso	Moussodougou	10.833333	-4.95
Burkina Faso (Kartasso)	*G. p. gambiensis*	Burkina Faso	Kartasso	11.141786	-5.253033
Guinea (Fefe)	*G. p. gambiensis*	Guinea	Fefe	1286931	434802
Guinea (Togoue)	*G. p. gambiensis*	Guinea	Togoue	1257379	437447
Guinea (Alahine)	*G. p. gambiensis*	Guinea	Alahine	1286684	509017
Guinea (Boureya Kolonko)	*G. p. gambiensis*	Guinea	Boureya Kolonko	1297685	307815
Guinea (Boureya Kolonko)	*G. p. gambiensis*	Guinea	Boureya Kolonko	1297685	307815
Guinea (Kansaba)	*G. p. gambiensis*	Guinea	Kansaba	1246788	253124
Guinea (Kindoya)	*G. p. gambiensis*	Guinea	Kindoya	1350069	215087
Mali (Fijira)	*G. p. gambiensis*	Mali	Fijira	542170	1397893
Mali(Astan)	*G. p. gambiensis*	Mali	Astan	10.400434	-9.053083
Republic Democratic of Congo	*G. p. palpalis*	Republic Democratic of Congo	Katanga	-11.666667	27.483334
Burkina Faso (Comoe)	*G. tachinoides*	Burkina Faso	Comoe	9.89106718	-4.58976269
Burkina Faso (Folonzo)	*G. tachinoides*	Burkina Faso	Folonzo	9.92967851	-4.60801757
Ghana (Walewole)	*G. tachinoides*	Ghana	Walewale	10.351613	-0.79846
Ghana (Fumbissi)	*G. tachinoides*	Ghana	Mortani	10.23479058	-0.714119074
Ghana (Funsi)	*G. tachinoides*	Ghana	Fumbissi	10.47282856	-1.386834989
Ghana (Sissili Bridge)	*G. tachinoides*	Ghana	Sissili Bridge	10.33035865	-1.319208122
Ghana (Grogro)	*G. tachinoides*	Ghana	Grogro	10.08224767	-1.883133222
Ghana (Kumpole)	*G. tachinoides*	Ghana	Kumpole	10.25432141	-1.270183374
Ethiopia (Arba mich)	*G. palidipes*	Ethiopia	Arba mich	6.11667	37.03333
Zimbabwe (Makuti)	*G. m. moristans*	Zimbabwe	Makuti	-16.3	29.25

Supplementary Table 2: List of Primers used for PCR and quantitative PCR (qPCR) analyses of microbiome in *Glossina tachinoides*

Target Gene	Primer Name	Primer Sequence (Listed 5- to -3)	Annealing Temperature (˚C)	Amplicon Size (bp)	References
Spiroplasma 16sRNA	63F	GCCTAATACATGCAAGTCGAAC	59 ºC	455	(Doudoumis et al. 2017)
	TKSS	TAGCCGTGGCTTTCTGGTAA			
Spiroplasma fructose repressor (*fruR*)	*fruR-F*	GTCATAATTGCAATTGCTGG	56 ºC /	398	
	FruR-R	CAATGATTAAAGCGGAGGT			
Spiroplasma DNA Topoisomerase 4 subunit B (*parE*)	*ParE-F*	GGAAAATTTGGTGGTGATGG	57 ºC	1126	
	ParE-R	TGGCATTAATCATTACATTAATTTCT			
RNA polymerase subunit beta (*rpoB*)	*rpoB*	ATGGATCAAACAAATCCATTAGCAGA	60 ºC	1703	
	rpoB	GCATGTAATTTATCATCAACCATGTGTG			
qPCR *Spiroplasma*	qPCR *Spiroplasma F*	TGAAAAAAACAAACAAATTGTTATTACTTC	56 ◦C	138	
	qPCR *Spiroplasma R*	TTAAGAGCAGTTTCAAAATCAGG			
GpCAG133	GpCAG133-F	ATT TTT GCG TCA ACG TGA	52.5	185-205	(Baker and Krafsur 2001)
	GpCAG133-R	ATG AGG ATG TTG TCC AGT TT			
thiC (thiamine biosynthesis gene)	WiggqPCRthiCF	GACATCAAATCGCGTTACTGG	60	645	(Boucias et al. 2013)
	WiggqPCRthiCR	GACTTGTACGTGATATTTCCAAGC'			
ITS 1	ITS 1-CF	CCG GAA GTT CAC CGA TAT TG	60	250-710	(Njiru et al. 2005)
	ITS 1-BR	TTG CTG CGT TCT TCA ACG AA			
18S	18S_Typ_F	CGC CAA GCT AAT ACA TGA ACC AA	60		Kindly provide by Jan Van Den Abbeele
	18S_Tryp_R	TAA TTT CAT TCA TTC GCT GGA CG			

Supplementary table 3: Prevalence in percentage of *Spiroplasma*, *Trypanosoma spp* and the different *Trypanosoma* species, single or multiple infection in Burkina Faso and Ghana, according to the sampling location and the sex. Spiro = *Spiroplasma*, T. spp = *Trypanosoma spp*, Tc = *T. congolense*, Tv = *T. vivax*, Tz = *Trypanosoma brucei spp*, TcTv = Coinfection *T. congolense T. vivax*, TcTz = Coinfection *T. congolense T. brucei spp*, TvTz = Coinfection *T. vivax T. brucei spp*, TcTvTz = Coinfection *T. congolense, T. vivax and T. brucei spp*

Country	Location	Sex	Spiro	T. spp	Tc	Tv	Tz	TcTv	TcTz	TvTz	TcTvTz
Burkina Faso	Comoe	F	17.31	16.54	1.1	13.24	0.37	0.74	1.1	0	0
Burkina Faso	Comoe	M	29.85	15	3.64	11.36	0	0	0	0	0
Burkina Faso	Folonzo	F	44.56	18.87	1.89	12.26	1.89	1.89	0.94	0	0
Burkina Faso	Folonzo	M	66.23	32.63	2.11	27.37	1.05	0	1.05	1.05	0
Ghana	Walewale	F	57.78	66.04	1.89	9.43	24.53	0	16.98	7.55	5.66
Ghana	Walewale	M	33.33	53.85	3.85	12.82	8.97	0	10.26	14.1	1.28
Ghana	Sissili Bidge	F	60	100	0	20	80	0	0	0	0
Ghana	Sissili Bidge	M	0	100	0	0	100	0	0	0	0
Ghana	Fumbissi	F	25	100	0	0	37.5	0	0	62.5	0
Ghana	Fumbissi	M	33.33	100	0	66.67	33.33	0	0	0	0
Ghana	Kumpole	F	0	100	0	40	60	0	0	0	0
Ghana	Kumpole	M	50	100	0	50	50	0	0	0	0
Ghana	Grogro	F	20	100	0	80	20	0	0	0	0
Ghana	Grogro	M	50	100	0	0	16.67	0	0	83.33	0
Ghana	Mortani	F	100	66.67	0	0	33.33	0	22.22	11.11	0
Ghana	Mortani	M	96.88	50	0	15.63	15.63	0	18.75	0	0

Supplementary table 4: Chi squared analysis of coinfection of *Spiroplasma* and Trypanosome in *Glossina tachinoides* natural population in Burkina Faso

Supplementary table : Chi squared analysis of coinfection of *Spiroplasma* and Trypanosome in *Glossina tachinoides* natural population in Burkina Faso

| Glossina species | Country (Area, Collection Date) | N | Observed | | | | Expected | | | | χ^2 | P |
			SPIRO+/T+	SPIRO+/T-	SPIRO-/T+	SPIRO-/T-	SPIRO+/T+	SPIRO+/T-	SPIRO-/T+	SPIRO-/T-		
G. tachinoides	Burkina Faso (Comoe, …)	119	1	28	15	75	3.90	25.10	12.10	77.90		
G. tachinoides	Burkina Faso (Folonzo, …)	347	27	161	29	130	30.34	157.66	25.66	133.34		
Total		**466**	**28**	**189**	**44**	**205**	**33.53**	**183.47**	**38.47**	**210.53**	**2.02**	**0.16**

Taxa with χ2 test 6
Bonferroni corrected α 0.05/6 = 0.00625

Supplementary table 5a: Chi squared analysis of coinfection of *Spiroplasma* and Trypanosome in *Glossina tachinoides* natural population in Ghana

Glossina species	Country (Area, Collection Date)	N	SPIRO+/T+	SPIRO+/T-	SPIRO-/T+	SPIRO-/T-	SPIRO+/T+	SPIRO+/T-	SPIRO-/T+	SPIRO-/T-	χ²	P
G. tachinoides	Ghana (Walewale,...)	108	24	23	43	18	29.16	17.84	37.84	23.16		
G. tachinoides	Ghana (Sissili Bidge,...)	6	3	0	3	0	3.00	0.00	3.00	0.00		
G. tachinoides	Ghana (Fumbissi,...)	14	4	0	10	0	4.00	0.00	10.00	0.00		
G. tachinoides	Ghana (Kumpole,...)	7	1	0	6	0	1.00	0.00	6.00	0.00		
G. tachinoides	Ghana (Grogro,...)	11	3	1	7	0	3.64	0.36	6.36	0.64		
G. tachinoides	Ghana (Mortani,...)	41	19	21	1	0	19.51	20.49	0.49	0.51		
Total		**187**	**54**	**45**	**70**	**18**	**65.65**	**33.35**	**58.35**	**29.65**	**13.03**	**0.00**

Taxa with χ2 test   6

Bonferroni corrected α 0.05/6 = 0.00625

Supplementary table 5b: Cochran–Mantel–Haenszel test for repeated tests of independence with continuity correction on the coinfection of *Spiroplasma* and Trypanosome in *Glossina tachinoides* natural population

Glossina taxon	No. of Locations (Samples)	Total N	SPIRO+ /T+	SPIRO+ /T-	SPIRO- /T+	SPIRO- /T-	Proportion S+	Proportion T+		
G. tachinoides	8	653	82	234	114	223	0.484	0.300	-12.848	34.310
Σ	8	653	82	234	114	223	0.484	0.300	-12.848	34.31
									178.180	
								χ^2_{MH}	**5.193**	
								df	**1**	
								P	**0.02**	**n.s.**

Glossina species	Country (Area, Collection Date)	N	Observed				χ^2	P
			SPIRO+/T+	SPIRO+/T-	SPIRO-/T+	SPIRO-/T-		
G. tachinoides	Burkina Faso (Comoe, …)	119	1	28	15	75		
G. tachinoides	Burkina Faso (Folonzo, …)	347	27	161	29	130		
Sub-Total		**466**	**28**	**189**	**44**	**205**	**2.0174**	**0.155506248**
G. tachinoides	Ghana (Walewale,…)	108	24	23	43	18		
G. tachinoides	Ghana (Sissili Bidge,…)	6	3	0	3	0		
G. tachinoides	Ghana (Fumbissi,…)	14	4	0	10	0		
G. tachinoides	Ghana (Kumpole,…)	7	1	0	6	0		
G. tachinoides	Ghana (Grogro,…)	11	3	1	7	0		
G. tachinoides	Ghana (Mortani,…)	41	19	21	1	0		
Sub-Total		**187**	**54**	**45**	**70**	**18**	**13.034**	**0.000305889**
Total		**840**	**136**	**279**	**184**	**241**	**9.8593**	**0.001689779**

Taxa with χ2 test   6

Bonferroni corrected α 0.05/6 = 0.00625

Dieng *et al. Parasites & Vectors* (2022) 15:447

Chapter 4

Chapter 4

Interactions between *Glossina pallidipes* salivary gland hypertrophy virus and tsetse endosymbionts in wild tsetse populations

Dieng *et al. Parasites & Vectors* (2022) 15:447

Chapter 4

RESEARCH

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Interactions between *Glossina pallidipes* salivary gland hypertrophy virus and tsetse endosymbionts in wild tsetse populations

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Abstract

Background: Tsetse control is considered an effective and sustainable tactic for the control of cyclically transmitted trypanosomosis in the absence of effective vaccines and inexpensive, effective drugs. The sterile insect technique (SIT) is currently used to eliminate tsetse fly populations in an area-wide integrated pest management (AW-IPM) context in Senegal. For SIT, tsetse mass rearing is a major milestone that associated microbes can influence. Tsetse flies can be infected with microorganisms, including the primary and obligate *Wigglesworthia glossinidia*, the commensal *Sodalis glossinidius*, and *Wolbachia pipientis*. In addition, tsetse populations often carry a pathogenic DNA virus, the *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) that hinders tsetse fertility and fecundity. Interactions between symbionts and pathogens might affect the performance of the insect host.

Methods: In the present study, we assessed associations of GpSGHV and tsetse endosymbionts under field conditions to decipher the possible bidirectional interactions in different *Glossina* species. We determined the co-infection pattern of GpSGHV and *Wolbachia* in natural tsetse populations. We further analyzed the interaction of both *Wolbachia* and GpSGHV infections with *Sodalis* and *Wigglesworthia* density using qPCR.

Results: The results indicated that the co-infection of GpSGHV and *Wolbachia* was most prevalent in *Glossina austeni* and *Glossina morsitans morsitans*, with an explicit significant negative correlation between GpSGHV and *Wigglesworthia* density. GpSGHV infection levels > $10^{3.31}$ seem to be absent when *Wolbachia* infection is present at high density (> $10^{7.36}$), suggesting a potential protective role of *Wolbachia* against GpSGHV.

Conclusion: The result indicates that *Wolbachia* infection might interact (with an undefined mechanism) antagonistically with SGHV infection protecting tsetse fly against GpSGHV, and the interactions between the tsetse host and its associated microbes are dynamic and likely species specific; significant differences may exist between laboratory and field conditions.

Keywords: *Hytrosaviridae*, Tsetse microbiota, Virus transmission, *Wigglesworthia*, *Wolbachia*, *Sodalis*

Introduction

Mutualistic bacteria are functionally essential to the physiological well-being of their animal hosts. They benefit their hosts by providing essential nutrients, aiding in digestion and maintaining intestinal equilibrium. Furthermore, mutualistic symbionts foster the development, differentiation, and proper function of their host's

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Dieng *et al. Parasites & Vectors* (2022) 15:447

Chapter 4

immune system [1–5]. Insects provide a useful model for studying host-microbe interactions because they areassociated with bacterial communities that can be easilymanipulated during their host's development [6]. Tsetseflies (*Glossina* spp.) accommodate various types of bacte-ria, including two gut-associated bacterial symbionts, the obligate *Wigglesworthia glossinidia* and the commensal *Sodalis glossinidius*, the widespread symbiont *Wolbachia pipientis*, and a recently discovered *Spiroplasma* endo-symbiont [7–13]. In addition, tsetse flies can house differ-ent types of viral infection, including the salivary glandhypertrophy virus (GpSGHV), iflavirus, and negevirus,besides trypanosome parasites [14–17]. Symbiotic associations between insect disease vectors, gut and endosymbiotic bacteria have been particularly well studiedto determine how these microbes influence their host's ability to be infected and transmit disease [18–22]. For example, in tsetse flies, the obligate bacteria *W. glossinidia* are essential for maintaining female fecundity andthe host immune system by providing important nutritional components (vitamin B6) and folate (vitamin B9) [22–24]. In addition, *Sodalis* may modulate tsetse susceptibility to infection with trypanosomes, and several studies using field-captured tsetse have noted that the prevalence of trypanosome infections positively cor-relates with increased *Sodalis* density in the fly's gut [25–29]. In contrast, the exogenous bacterium *Kosakonia cowanii* inhibits trypanosome infection by creating an unfavorable environment for trypanosome establishmentin the mid-gut [30].

Flies in the genus *Glossina* (tsetse flies) are unique toAfrica and are of great medical and economic importance as they serve as a vector for the trypanosomes responsible for sleeping sickness in humans (human African trypanosomosis or HAT) and nagana in animals (African animal trypanosomosis or AAT) [31, 32]. The presence oftsetse and trypanosomes is considered one of the major challenges to sustainable development in Africa [33, 34].The lack of adequate and affordable vaccines coupled with pathogen resistance to drug treatments severely limits AAT control, leaving vector control as the most feasible option for sustainable management of the disease [31, 32]. In addition to various pesticide- and trapping-based methods for tsetse control, the sterile insect technique (SIT) is considered an efficient, sustainable and environmentally friendly method when implemented in the frame of area-wide integrated pest management (AW- IPM) [35, 36]. However, the SIT requires the mass rearing of many males to be sterilized with ionizing radiationbefore release into the targeted area [33, 37].

Tsetse fly biology is characterized by its viviparous reproduction rendering tsetse mass rearing a real challenge. Tsetse flies nourish their intrauterine larvae

from glandular secretions and give birth to fully developed larvae (obligate adenotrophic viviparity) [38, 39]. They also live considerably longer than other vector insects, which somewhat compensates for their slow reproduc- tion rate [40]. The ability to nourish larvae on the milk gland secretion, although limiting the number of lar-vae produced per female lifetime (8–12), facilitates the transmission of endosymbitic bacteria and pathogens from females to lar-vae such as *Wigglesworthia*, *Sodalis*, *Wolbachia,* *Spiroplasma*, and GpSGHV [8, 10, 13, 41].Moreover, as strictly hematophagous, tsetse rely on the associated endosymbionts to obtain essential nutrients for female reproduction. Therefore, tsetse well-being inmass rearing for SIT is affected by the status of its endos- ymbionts as well as infection with pathogenic viruses and the interactions between them. Although *Wiggleswor- thia* is an obligate endosymbiont and found in all tsetse species, *Sodalis*, *Wolbachia*, and *Spiroplasma* infectionvaried from one species to another [8, 10, 42–45]. In addition, infection with GpSGHV, although reported in different tsetse species, is mainly symptomatic in *G. pal- lidipes* [46–48]. As GpSGHV is horizontally transmit- ted via the feeding system under laboratory conditions, leading to high infection rates [49–51], and the virus hasa negative effect on the reproductive system of the host causing reduced fecundity and fertility [52, 53], control ofthe virus infection is important in tsetse mass rearing forefficient production of irradiated males for SIT program implementation.

The variable responses of different tsetse species to the GpSGHV infection might indicate a possibility of the tsetse microbiota modulating the molecular dia- logue among the virus, symbiont, and host, shaping the response of each species to the virus infection. It was necessary, therefore, to investigate the infection status ofthe major tsetse endosymbionts (*Wigglesworthia*, *Sodalis*, and *Wolbachia*) in different tsetse species and their potential interactions. We have recently investigated theinteraction between GpSGHV and tsetse symbionts in six tsetse species after virus injection under laboratory conditions [54]. The results indicated that the interac- tion between the GpSGHV and tsetse symbionts is a complicated process that varies from one tsetse species to another. It is worth noting that the study of Demirbas-Uzel et al. [54] was conducted in tsetse flies maintainedunder controlled laboratory conditions (sustainable food availability, constant environmental conditions (tempera- ture and humidity), and high density of the flies), whichfavors the increase of tsetse symbionts [45, 55, 55–57]. In addition, this study was done using adults artificiallyinfected with GpSGHV by injection. Therefore, we investigated the associations of the GpSGHV and tsetsesymbionts in field-collected samples by evaluating the

Dieng *et al. Parasites & Vectors* (2022) 15:447

Chapter 4

prevalence of co-infection of GpSGHV and *Wolbachia* and their potential association with Wigglesworthia and *Sodalis* infection in natural tsetse populations. The results are also discussed in the context of developing an effective and robust mass production system of high-quality sterile tsetse flies for implementing SIT programs.

Methods

Tsetse samples, extraction of total DNA, and PCR amplifications

The field collection of tsetse fly samples, DNA extraction, and the PCR-based prevalence of GpSGHV and *Wol- bachia* infections were reported previously [7, 47, 58, 59]. Based on these publications, and using *G. m. morsitans*, *G. pallidipes*, *G. medicorum*, *G. brevipalpis*, and *G. aus- teni* samples collected from Burkina Faso, South Africa, Tanzania, Zambia, and Zimbabwe, four infection patterns (i.e. presence) were determined: (i) flies PCR posi- tive for both GpSGHV and *Wolbachia* (W^+/V^+), (ii) flies PCR positive for *Wolbachia* alone (W^+/V^-), (iii) flies PCR positive for GpSGHV alone (W^-/V^+), and (iv) flies PCR negative for both GpSGHV and *Wolbachia* (W^-/V^-). It has to be noted that the prevalence of the symbionts was assessed using a conventional PCR assay while their den- sities (see below) were determined using a qPCR assay. Since these two assays were different in several aspects including the size of the amplicons and visualization pro- cess, this resulted in some discrepancies regarding the infections status of some virus samples initially consid- ered virus free by conventional PCR that were found to be positive dur- ing the qPCR analysis.

Analysis of the associations among SGHV and *Wolbachia*, *Sodalis*, and *Wigglesworthia* infection in wild tsetse populations

The associations among GpSGHV and *Wolbachia, Soda- lis*, and *Wigglesworthia* were assessed by qPCR analysis. Tsetse fly samples were selected for qPCR analysis only if a given population of each species was characterized by the presence of two or three of the infection patterns (W^+/V^+), (W^+/V^-), and (W^-/V^+). Based on this criterion, 203 individual flies (78, 103, and 22 flies with infection pattern (W^+/V^+), (W^+/V^-), and (W^-/V^+), respectively) were analyzed (Table 1). The qPCR analysis was performed as pre- viously described [47, 53, 60]. In brief, for the stand- ard curve, total DNA was diluted tenfold before being used for qPCR analysis on a CFX96 real-time PCR de- tection system (Bio-Rad, Hercules, CA) using the pri- mers and conditions presented in Additional file 2: Table S1. The estimated copy number by qPCR for each sample compared with the standard curve was determined in diluted DNA (4 ng/µl) and corrected through the multiplication by the inverse dilution

factor to reflect the GpSGHV, *Wolbachia*, *Wiggles- wor- thia*, or *Sodalis* copy number (hereafter mention as den- sity) per fly. Analysis of the *Wolbachia*, *Wig- glesworthia*, *Sodalis,* and SGHV density levels (ti- ters) was based only on qPCR data with the expected melting curves at 85.5–86 °C, 78.5–80 °C, 81.5–82 °C, and 76.5–77 °C, respectively. Data with a melt- ing curve outside the indi- cated range were excluded from the analysis. The status of *Sodalis* and *Wiggles- worthia* infection of the samples used for the qPCR analysis was not determined by tradi- tional PCR. Based on the estimated copy number per fly for SGHV, *Wolbachia*, *Wigglesworthia*, and *Sodalis*, the average copy number was calculated for all tested flies. Flies with copy number values less than the me- dian were considered infected with low density and flies with copy number value greater than the median were considered infected at a high level. The median copy numbers of the GpSGHV, *Wolbachia*, *Sodalis*, and *Wigglesworthia* in all tested samples were $10^{3.31}$, $10^{7.36}$, $10^{6.07}$, and $10^{6.84}$ per fly, respectively.

Statistical analysis

The proportion of single and double infections (GpSGHV and *Wolbachia*) in wild flies was ana- lyzed by location and species and for all samples together using the Chi- squared test. The Chi- squared tests for independence, Spearman correla- tion coefficient, and Cochran-Mantel-Haenszel test for repeated tests of independence were performed using Excel 2010. *P*-values were calculated from the data with the significance threshold selected as 0.05.

The difference in *Wigglesworthia, Sodalis, Wolbachia*, and GpSGHV density between different locations and tsetse species and the correlation be- tween densities as well as preparing figures were ex- ecuted in R v 4.0.5 [61] using RStudio v 1.4.1106 [62, 63] with packages ggplot2 v3.3.2.1 [64], lattice v0.20-41 [65], car (version 3.1-0) [66], ggthems (version 4.2.4) [67], and MASS v7.3-51.6 [68]. All regression analyses of symbionts and GpSGHV den- sities were conducted using the gener- alized linear model (glm) for different tsetse species and different countries with analysis of deviance table (type II tests). Pearson correlation coefficient be- tween the density of *Wolbachia* and *Wigglesworthia* and the log transformed density of GpSGHV and *So- dalis* was conducted in R. The analysis details are pre- sented in Additional file 1. Overall similari- ties in *Wolbachia*, *Wigglesworthia*, *Sodalis*, and GpSGHV density levels between tsetse species, countries, and infection pattern were shown using the matrix dis- play and metric multidimensional scaling (mMDS) plot with bootstrap averages in PRIMER version 7 + and were dis- played with a Bray and Curtis matrix based on the square root transformation [69]. The tests were based on the

Dieng *et al. Parasites & Vectors* (2022) 15:447

Chapter 4

Table 1 SGHV and *Wolbachia* infection status of tsetse flies in natural populations of different *Glossina* species

	Country (area, collection date)	N	W^+/V^+	W^+/V^-	W^-/V^+	W^-/V^-	χ^2	P
G. austeni	Tanzania (Jozani, 1997)[a]	42	0	22	2	18		
G. austeni	Tanzania (Zanzibar, 1995)[a,c]	78	3	72	0	3		
G. austeni	South Africa (Zululand, 1999)[a,c]	83	51	28	1	3		
G. austeni	Coastal Tanzania (Muhoro, NA)	2	0	2	0	0		
G. austeni	All locations	205	54	124	3	24	4.32	0.04
G. brevipalpis	South Africa (Zululand, 1995)[a]	50	0	1	0	49		
G. brevipalpis	Coastal Tanzania (Muhoro, NA)	1	0	1	0	0		
G. brevipalpis	Coastal Tanzania (Muyuyu, NA)	1	0	1	0	0		
G. brevipalpis	All locations	52	0	3	0	49		
G. f. fuscipes	Uganda (Buvuma Island, 1994)[a,b]	53	0	0	6	47		
G. medicorum	Burkina Faso (Comoe, 2008)[c]	94	2	18	7	67	0.01	0.94
G. m. submorsitans	Burkina Faso (Nazinga, 2009)	3	0	0	0	3		
G. m. submorsitans	Burkina Faso (Comoe Folonzo, 2007)	30	0	2	3	25		
G. m. submorsitans	Burkina Faso (Comoe, 2008)[c]	109	0	4	9	96		
G. m. submorsitans	All locations	142	0	6	12	124	0.58	0.45
G. p. palpalis	Democratic Republic of Congo (Zaire, 1995)[a]	48	0	0	1	47		
G. tachinoides	Burkina Faso (Nazinga, 2009)	15	0	0	0	15		
G. tachinoides	Burkina Faso (Comoe Folonzo, 2007)	112	3	2	26	81		
G. tachinoides	Burkina Faso (Comoe, 2008)	72	0	0	8	64		
G. tachinoides	Ghana (Pong Tamale, Walewale, 2008)	46	0	5	0	41		
G. tachinoides	Ghana (Walewale, 2008)	149	0	27	6	116		
G. tachinoides	Ghana (Fumbissi, 2008)	39	0	0	0	39		
G. tachinoides	All locations	433	3	34	40	356	0.15	0.70
G. m. morsitans	Coastal Tanzania (Utete, NA)	3	0	2	0	1		
G. m. morsitans	Zambia (MFWE, Eastern Zambia, 2007)[a,c]	122	26	96	0	0		
G. m. morsitans	Tanzania (Ruma, 2005)[a,c]	100	29	71	0	0		
G. m. morsitans	Zimbabwe (Gokwe, 2006)[a]	74	0	7	8	59		
G. m. morsitans	Zimbabwe (Kemukura, 2006)[a]	26	0	26	0	0		
G. m. morsitans	Zimbabwe (M.Chiuy, 1994)[a,c]	36	5	28	0	3		
G. m. morsitans	Zimbabwe (Makuti, 2006)[a,c]	99	11	84	1	3		
G. m. morsitans	Zimbabwe (Mukond, 1994)[a]	36	0	35	0	1		
G. m. morsitans	Zimbabwe (Mushumb, 2006)[a]	8	0	3	0	5		
G. m. morsitans	Zimbabwe (Rukomeshi, 2006)[a,c]	100	8	90	0	2		
G. m. morsitans	All locations	604	79	442	9	74	1.07	0.30
G. pallidipes	Zambia (MFWE, Eastern Zambia, 2007)[a,c]	203	1	4	97	101		
G. pallidipes	Kenya (Mewa, Katotoi, Meru national park, 2007)[a]	470	0	0	10	460		
G. pallidipes	Ethiopia (Arba Minch, 2007)[a]	454	0	2	87	365		
G. pallidipes	Tanzania (Ruma, 2005)[a,c]	83	2	1	42	38		
G. pallidipes	Tanzania (Mlembuli and Tunguli, 2009)[a]	94	0	0	0	94		
G. pallidipes	Zimbabwe (Mushumb, 2006)[a]	50	0	0	1	49		
G. pallidipes	Zimbabwe (Gokwe, 2006)[a]	150	0	0	19	131		
G. pallidipes	Zimbabwe (Rukomeshi, 2006)[a]	59	0	5	0	54		
G. pallidipes	Zimbabwe (Makuti, 2006)[a,c]	96	1	3	5	87		
G. pallidipes	Mainland Tanzania (Death Valley, NA)	6	0	4	0	2		
G. pallidipes	Coastal Tanzania (Muhoro, NA)	4	0	3	0	1		
G. pallidipes	Coastal Tanzania (Muyuyu, NA)	3	0	3	0	0		
G. pallidipes	All locations	1672	4	25	261	1382	0.09	0.76
G. p. gambiensis	Senegal (Diacksao Peul and Pout, 2009)[a]	188	0	1	31	156		
G. p. gambiensis	Guinea (Kansaba, Mini Pontda, Kindoya, Ghada Oundou, 2009)[a]	180	0	0	13	167		

Dieng *et al. Parasites & Vectors* (2022) 15:447

Chapter 4

Table 1 (continued)

Glossina taxon	Country (area, collection date)	N	W⁺/V⁺	W⁺/V⁻	W⁻/V⁺	W⁻/V⁻	χ²	P
G. p. gambiensis	Guinea (Alahine, 2009)[a]	29	0	0	3	26		
G. p. gambiensis	Guinea (Boureya Kolonko, 2009)[a]	36	0	0	1	35		
G. p. gambiensis	Guinea (Fefe, 2009)[a]	29	0	0	1	28		
G. p. gambiensis	Guinea (Kansaba, 2009)[a]	19	0	0	4	15		
G. p. gambiensis	Guinea (Kindoya, 2009)[a]	12	0	1	0	11		
G. p. gambiensis	Guinea (Lemonako, 2009)[a]	30	0	0	4	26		
G. p. gambiensis	Guinea (Togoue, 2009)[a]	32	0	0	1	31		
G. p. gambiensis	Guinea (Conakry, 2010)	138	0	5	0	133		
G. p. gambiensis	Burkina Faso (Comoe, 2008)	12	0	0	7	5		
G. p. gambiensis	Burkina Faso (Comoe Folonzo, 2007)	53	0	1	14	38		
G. p. gambiensis	Burkina Faso (Kenedougou, 2007)	37	0	1	0	36		
G. p. gambiensis	Burkina Faso (Houet Bama, 2007)	69	0	1	41	27		
G. p. gambiensis	Guinea (Fefe, Togoue, Alahine, Boureya Kolonko, 2009-2010)	94	0	5	0	89		
G. p. gambiensis	Guinea (Boureya Kolonko, Kansaba, Kindoya, Ghada Oundou, 2009-2010)	94	0	3	0	91		
G. p. gambiensis	Mali (Fijira, 2009)	14	0	0	0	14		
G. p. gambiensis	Senegal (Diaka Madia, 2009)	42	0	0	0	42		
G. p. gambiensis	Senegal (Tambacounda, 2008)	38	0	3	0	35		
G. p. gambiensis	Senegal (Simenti, 2008)	33	0	6	0	27		
G. p. gambiensis	Senegal (Kédougou, 2008)	15	0	1	0	14		
G. p. gambiensis	All locations	1194	0	28	120	1046	3.20	0.07

[a] In these samples, the presence of *Wolbachia* was tested in Doudoumis et al. [7]

[b] The individuals of *G. f. fuscipes* were considered negative for *Wolbachia* based on the results of the initial PCR amplification. The results from the reamplification method were not considered so that the conditions were consistent for all species

[c] Samples used for qPCR analysis to determine the density of *Wigglesworthia*, *Sodalis*, *Wolbachia*, and GpSGHV

multivariate null hypothesis via the non-parametric statistical method PERMANOVA [70]. The PERMANOVA test was conducted on the average of the qPCR density data based on the country-species sample.

Results
Prevalence of co-infection with GpSGHV and *Wolbachia* in wild tsetse flies

Analysis of the *Wolbachia* and GpSGHV infection status for each individual tsetse adult in the previously reported data [7, 47, 58, 59] indicated that the single infection rate was 10.21% ($n = 459$) and 15.12% ($n = 680$) for GpSGHV and *Wolbachia*, respectively, over all taxa and locations combined (Additional file 4: Fig. S1A). No *Wolbachia* infection was found in two taxa, *G. f. fuscipes* and *G. p.palpalis*, and these were excluded from further examination (Table 1). A Cochran-Mantel-Haenszel test for repeated tests of independence showed that infection with GpSGHV and *Wolbachia* did not deviate from independence across all taxa ($\chi2$ MH $= 0.848$, df $= 1$, n.s.), and individual Chi-squared tests for independence for each taxon did not show any significant deviation from independence at the Bonferroni corrected $\alpha = 0.00714$ (Additional file 3: Table S2). The prevalence of co-infection of GpSGHV and *Wolbachia* (W^+/V^+) in wild tsetse populations varied

based on the taxon and the location (Table 1 and Additional file 4: Fig. S1B). No co-infection was found in *G. brevipalpis*, *G. m. submorsitans*, and *G.p. gambiensis*, and co-infection was absent in many loca- tions in the remaining taxa. However, a low prevalence of co-infection was found in *G. medicorum* (2%), *G. tachi- noides* (0.7%), and *G. pallidipes* (0.2%). A relatively highprevalence of co-infection was only observed in *G. aus- teni* (26%) and *G. m. morsitans* (13%) (Additional file 4: Fig. S1B).

Impact of co-infection (W^+/V^+) on GpSGHV, *Wolbachia*,*Sodalis*, and *Wigglesworthia* density
GpSGHV density

The GpSGHV qPCR data showed overall no statistically significant difference between flies with different infection patterns (W^+/V^+), (W^-/V^+), and (W^+/V^-) ($X^2 = 1.4625$, df$=2$, P$=0.481$) regardless of tsetse taxon(Additional file 5: Fig. S2A). Moreover, no significant difference in GpSGHV copy number was observed between tsetse taxa ($X = 0.752$, df$=3$, P$=0.861$) (Additional file 4: Fig. S1A). However, a significant difference in the virus copy number was observed between different countries ($X^2 = 16.234$, df$=4$, P$=0.0027$) where the virus copy number in the flies collected from Zambia

Dieng *et al. Parasites & Vectors* (2022) 15:447

Chapter 4

was significantly lower than those collected from South Africa, Tanzania, and Zimbabwe (Additional file 1 and 6: Fig. S3A).

Wolbachia density

The copy number of *Wolbachia* infection was signifi-cantly different between tsetse taxa ($X^2 = 6.568$, $df = 2$, $P = 0.037$) (Additional file 4: Fig. S1B), between the infec-tion statuses ($X^2 = 23.723$, $df = 2$, $P < 0.001$) (Additional file 5: Fig. S2B), and between the countries ($X^2 = 73.507$, $df = 3$, $P < < 0.001$) (Additional file 6: Fig. S3B). *Wolbachia* density was signifi-cantly higher in *G. m. morsitans* than in *G. austeni* ($t = 2.029$, $df = 1$, $P = 0.0478$). (Additional file 1 and 4: Fig. S1B).

Overall, a significant difference in *Wolbachia* den-sity was observed in the flies with different infection patterns previously determined by conventional PCR, where flies with a (W^+/V^-) infection pattern showed significantly higher *Wolbachia* density than flies with a (W^+/V^+) infection pattern regardless of the tsetse species ($X^2 = 23.723$, $df = 2$, $P < < 0.001$). This trend was observed in *G. m. morsitans* ($t = 3.184$, $P = 0.0022$) (Additional file 1). The *Wolbachia* density was highest in the flies col-lected from Zambia (Additional file 6: Fig. S3B). Analyz-ing only the flies with co-infection (W^+/V^+) indicated that the *Wolbachia* density was statistically significantly higher in *G. m. morsitans* than in *G. austeni* ($t = -2.353$, $df = 1$, $P = 0.024$) (Additional file 1 and 7: Fig. S4B).
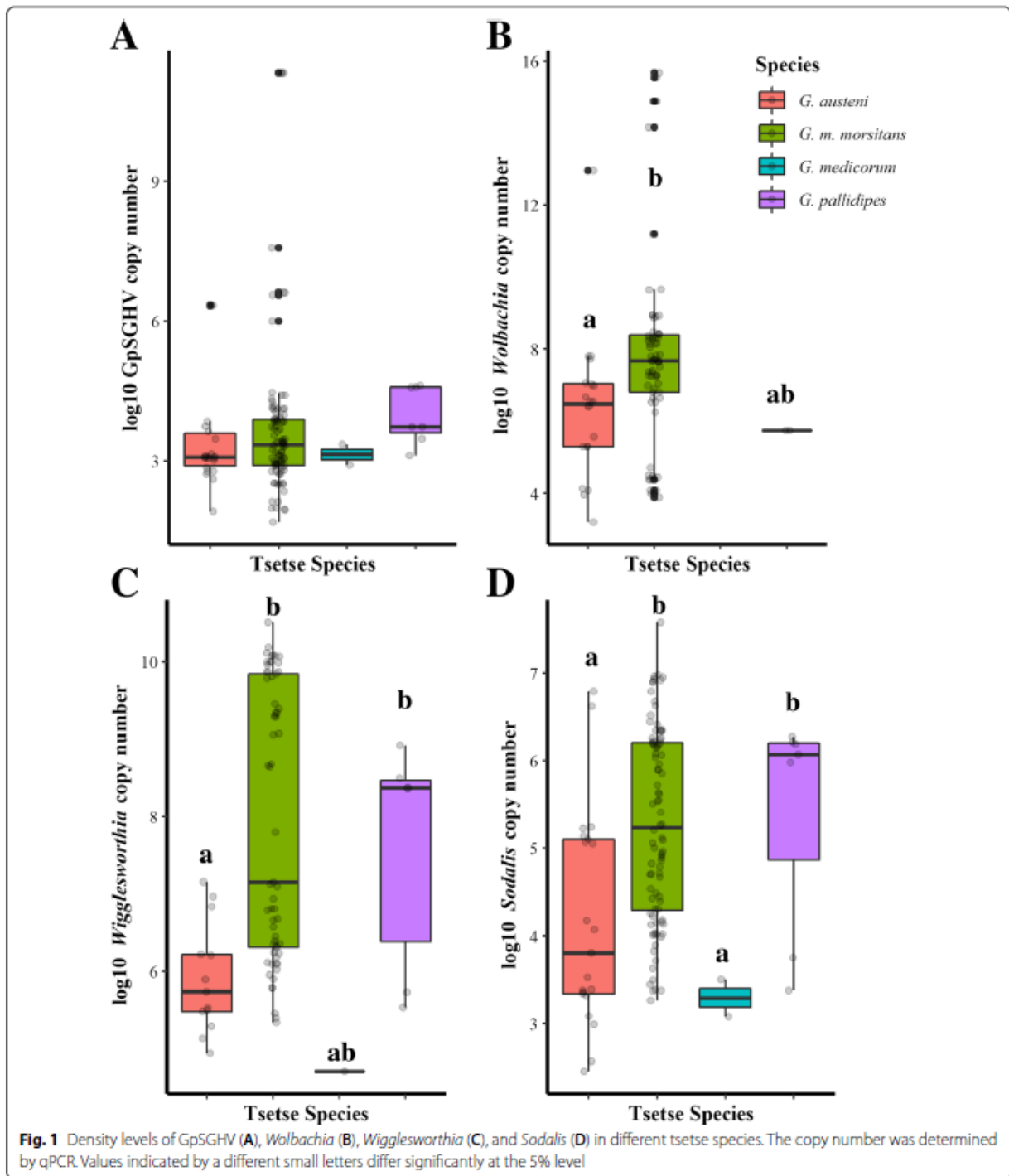
Interaction between GpSGHV infection and Wolbachia, Wigglesworthia, and Sodalis infection

The qPCR results of both *Wigglesworthia* and *Soda-lis* in tsetse adults with different infection patterns (W^+/V^+), (W^-/V^+), and (W^+/V^-) indicated that *Wig-glesworthia* density varies significantly between different infection patterns ($X^2 = 10.706$, $df = 2$, $P = 0.0047$) and its density in flies with co-infection (W^+/V^+) was sig-nificantly lower than in those with *Wolbachia* infection only (W^+/V^-) ($t = 3.137$, $df = 2$, $P = 0.0024$) but did not differ significantly from flies with virus infection only (W^-/V^+) ($t = 1.656$, $P = 0.102$) (Additional file 5: Fig. S2C). *Wiggles-worthia* density varies also between tsetse taxa ($X^2 = 33.479$, $df = 4$, $P < < 0.001$) with higher density in *G. m. morsitans* and *G. pallidipes* than in *G. austeni* (Additional file 4: Fig. S1C) as well as between countries ($X^2 = 19.785$, $df = 3$, $P < 0.001$) (Additional file 1 and 6: Fig. S3C).

Sodalis density also varies between tsetse taxa ($X^2 = 21.612$, $df = 3$, $P < 0.001$) (Fig. 1D) and between countries ($X^2 = 21.179$, $df = 4$, $P < 0.001$) (Additional file 6: Fig. S3D) but there was no significant difference between tsetse flies with different infection patterns ($X^2 = 0.63888$, $df = 2$, $P = 0.727$) (Additional file 1 and 5: Fig. S2D).

Analyzing the pairwise correlation between the GpSGHV and each of the tsetse endosymbionts in *G. aus-teni* and *G. m. morsitans* (species with the highest number of flies with co-infection) indicated different types of cor-relation based on the insect taxa. In *G. m. morsitans*, the GpSGHV density has a significant nega-tive correlation with *Wolbachia* density ($r = -0.558$, $t = -4.150$, $df = 38$, $P < 0.001$). No flies were observed with high virus density ($> 10^{3.3}$ copy number) when *Wolbachia* density was high ($\sim 10^{7.3}$ copy number), although this observation should be considered with caution as it is based on a small sample size. Contrary to *Wolbachia*, GpSGHV has a significant positive cor-relation with *Wigglesworthia* ($r = 0.531$, $t = 3.868$, $df = 38$, $P < 0.001$) but no correlation with *Sodalis* density ($r = 0.203$, $t = 1.276$, $df = 38$, $P = 0.209$). *Wolbachia* density also showed significant negative cor-relation with *Wigglesworthia* density ($r = -0.637$, $t = -5.095$, $df = 38$, $P < 0.001$). No flies with high *Wig-glesworthia* den-sity ($\sim 10^8$ copy number) were detected when *Wolbachia* density was high ($> 10^{7.3}$ copy number). In contrast, *Sodalis* density did not show significant correlation with either *Wolbachia* ($r = 0.193$, $t = 1.214$, $df = 38$, $P = 0.232$) or *Wig-glesworthia* den-sities ($r = 0.072$, $t = 0.443$, $df = 38$, $P = 0.66$) (Fig. 2, Additional file 1). In *G. austeni*, the only significant cor-relation was found to be positive between *Sodalis* and *Wiggles-worthia* density ($r = 0.602$, $t = 2.386$, $df = 10$, $P = 0.038$) (Fig. 2, Additional file 1).
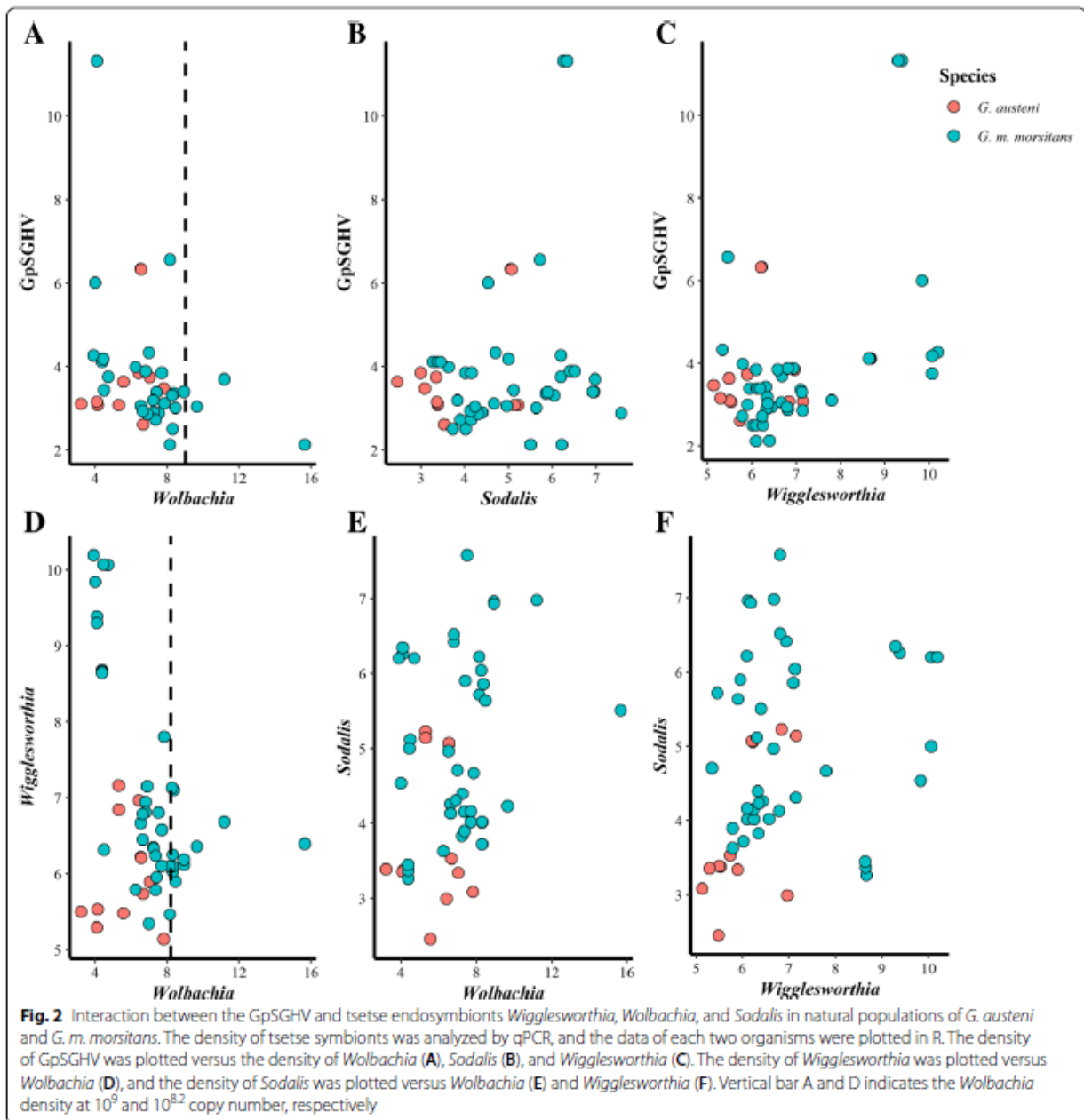
The qPCR results showed that *Wolbachia*-infected flies had relatively high *Wolbachia* density (median $10^{7.3}$ copies/fly) compared to the GpSGHV and other tsetse symbionts (*Wigglesworthia* and *Sodalis*) regard-less of the species, country, or infection pattern (Fig. 3). The heat map analysis of the qPCR data of *G. austeni* and *G. m. morsitans* clearly indicates the contrast between *Wolbachia* copy number and *Wigglesworthia* copy num-ber considering the infection pattern, tsetse taxa, or coun-tries. In addition, it clearly shows the low copy number of GpSGHV in the samples show-ing a high *Wolbachia* copy number (Fig. 3, Additional file 8: Fig. S5). The boot-strap averages of the metric mul-tidimensional scaling (mMDS) produced clusters based on the species, country, and infection pattern (Fig. 4). The PERMANOVA analy-sis of the density of GpSGH, *Wolbachia*, *Wigglesworthia*, and *Sodalis* based on the country, tsetse species, and in-fection pattern indicated that the clusters observed between infection pattern ($P = 0.026$) and country ($P = 0.001$) were statistically significant. The interaction between country and infec-tion pattern was not statistically significant ($P = 0.123$) (Table 2).

Dieng *et al. Parasites & Vectors* (2022) 15:447

Chapter 4

Fig. 1 Density levels of GpSGHV (**A**), *Wolbachia* (**B**), *Wigglesworthia* (**C**), and *Sodalis* (**D**) in different tsetse species. The copy number was determined by qPCR. Values indicated by a different small letters differ significantly at the 5% level

Discussion

The prevalence of GpSGHV and *Wolbachia* in natu-ral tsetse populations clearly indicated that the two infections were independent (not correlated) in most of the tested tsetse species with only *G. m. morsitans* and *G. austeni* presenting a high proportion of co- infections. However, the number of co-infections originally determined by conventional PCR may have been

Dieng *et al. Parasites & Vectors (2022) 15:447*

Chapter 4

Fig. 2 Interaction between the GpSGHV and tsetse endosymbionts *Wigglesworthia*, *Wolbachia*, and *Sodalis* in natural populations of *G. austeni* and *G. m. morsitans*. The density of tsetse symbionts was analyzed by qPCR, and the data of each two organisms were plotted in R. The density of GpSGHV was plotted versus the density of *Wolbachia* (**A**), *Sodalis* (**B**), and *Wigglesworthia* (**C**). The density of *Wigglesworthia* was plotted versus *Wolbachia* (**D**), and the density of *Sodalis* was plotted versus *Wolbachia* (**E**) and *Wigglesworthia* (**F**). Vertical bar A and D indicates the *Wolbachia* density at 10^9 and $10^{8.2}$ copy number, respectively

underestimated with conventional PCR as the qPCR analysis carried out in the frame of the present study clearly indicated that a number of initially considered virus-free samples were found to be positive, albeit at low density. It should also be noted that the *Wolbachia* strains infecting *G. m. morsitans* and *G. austeni* are closely related but different, as has been shown by both MLST analysis and, more recently, genome sequencing[7, 40, 71].

Analysis of *G. morsitans* and *G. austeni* co-infected samples suggested that low density of GpSGHV is asso- ciated with high density of *Wolbachia*. Due to the low number of individuals showing this correlation, further analysis is required. Moreover, the screen of wild tsetse popula- tions for GpSGHV and *Wolbachia* infection indi- cated that not all *Glossina* species harbor *Wolbachia* or GpSGHV. Furthermore, *Wolbachia* and GpSGHV prevalence was found to differ not only between different

Dieng *et al. Parasites & Vectors* (2022) 15:447

Chapter 4
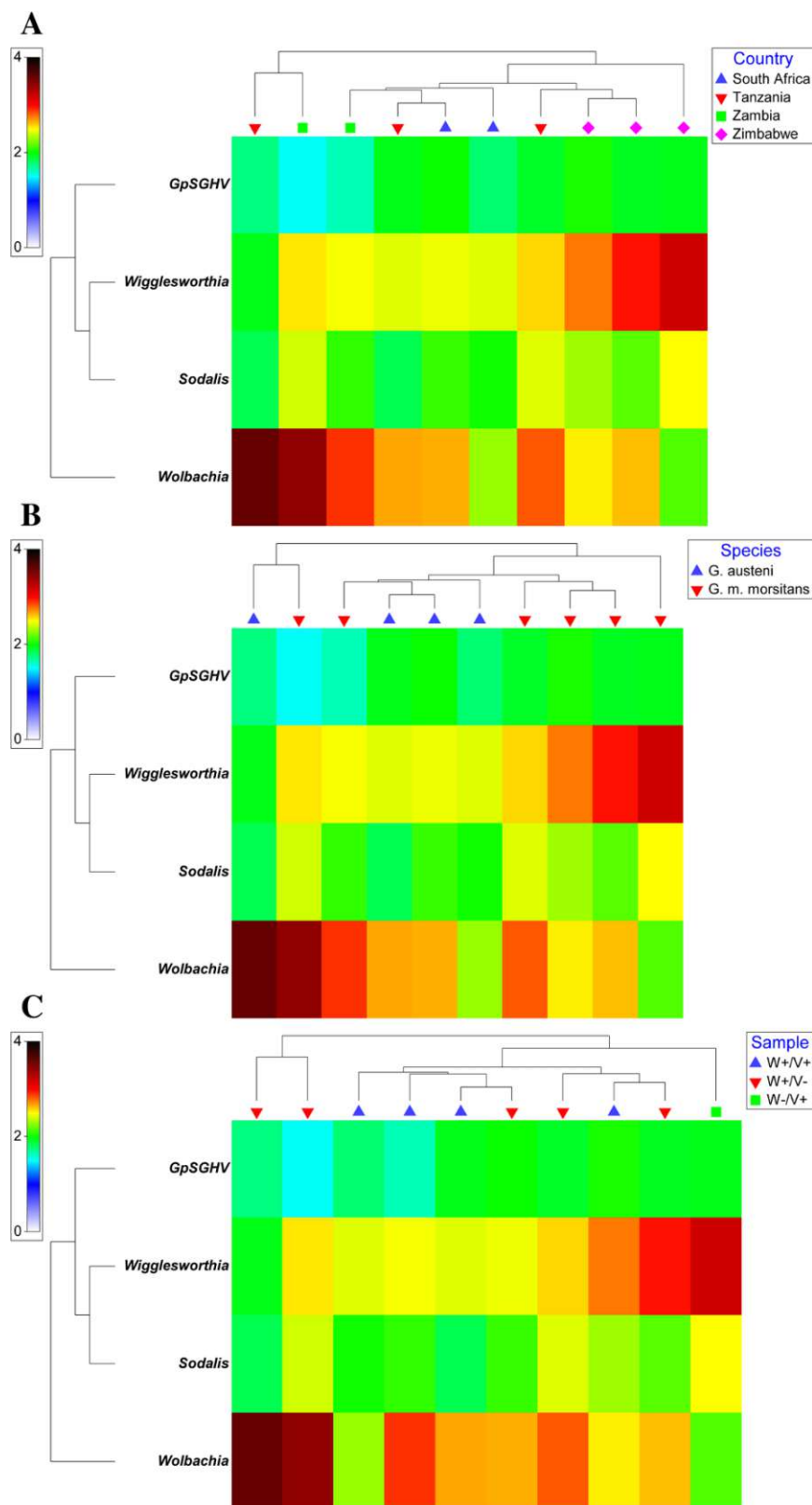
Fig. 3 Relative density of GpSGHV, *Wigglesworthia*, *Sodalis*, and *Wolbachia* in *G. austeni* and *G. m. morsitans* field-collected tsetse flies. The density ofGpSGHV and tsetse symbionts was analyzed by qPCR. Data were transformed to square root and averaged based on country (**A**), tsetse species (**B**), and infection status (Sample) (**C**). The top and the left of the graph indicate the group averaged Bray-Curtis simila
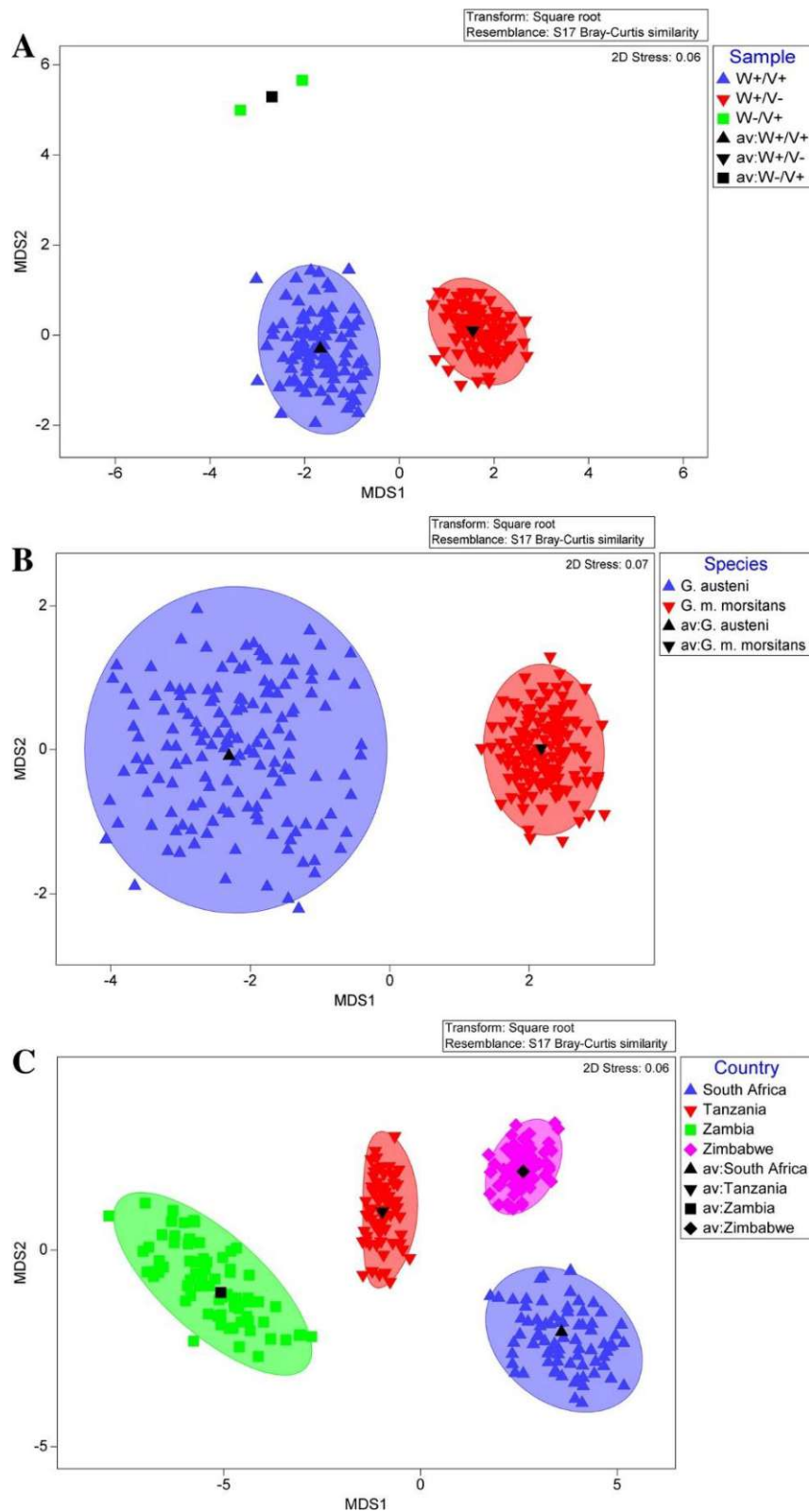
Dieng *et al. Parasites & Vectors (2022) 15:447*

Chapter 4

Fig. 4 Metric multidimensional scaling (mMDS) of GpSGHV, *Wigglesworthia, Sodalis*, and *Wolbachia* relative density in field-collected tsetse flies. The mMDS of GpSGHV, *Wigglesworthia, Sodalis*, and *Wolbachia* relative density was performed in respect to infection status (Sample) (**A**), tsetse species (**B**), or country (**C**). *av* average

Dieng *et al. Parasites & Vectors (2022) 15:447*

Chapter 4

Table 2 PERMANOVA table of results for country and infection status factors and their combinations

Source	df	SS	MS	Pseudo-F	P(perm)	Uniqueperms
Country	2	657.53	328.77	13.003	**0.001**	999
Species	0	0		No test		
Infection status	2	188.97	94.487	3.7369	**0.026**	998
Country × species Country × infection status	0	0		No test		
	2	100.59	50.296	1.9892	0.123	999
Species × infection status	0	0		No test		
Country × species × infection status	0	0		No test		
Res	104	2629.6	25.285			
Total	113	4681.3				

Within the table, statistically significant differences (*P* < 0.05) are shown in bold

Perm(s) permutations

tsetse host species but also between different populations within the same tsetse species [7, 11, 47, 58, 59, 72].

The potential negative impact (antagonistic effect) of *Wolbachia* density on the GpSGHV density in natural tsetse populations is in agreement with the recent report on the interaction of *Wolbachia* and GpSGHV infection in colonized tsetse populations [54]. However, the num-ber of tested flies was not equally distributed between the tsetse taxa and locations, which might explain the lack of detected co-infections in some taxa and, therefore, the low number of taxa (*G. austeni* and *G. m. morsitans*) used for investigating the interactions between the GpSGHV and tsetse symbionts. The negative correlation between *Wolbachia* and GpSGHV infections was also reported in wild-caught *G. f. fuscipes* collected from Uganda [72]. This conflicts with our findings as no *G. f. fuscipes* flies with GpSGHV were reported, which might be due to the low number of tested flies used in our study (*n* = 53).

Several reports have discussed and well documented the negative effect of *Wolbachia* on RNA viruses in different insect models such as mosquitoes and *Drosophila* [73–75], although there have also been reports about *Wolbachia* enhancement of both RNA and DNA viruses [76–78]. It is worth mentioning that the negative correlation of *Wolbachia* with GpSGHV was observed only when *Wolbachia* density was high as the results show the absence of high density (>$10^{3.7}$) GpSGHV infection with high-density *Wolbachia* infection (>$10^{7.5}$). However, at low *Wolbachia* density co-infection occurs with a prevalence of > 10%. Although our study indicated a correlation between high-density *Wolbachia* and low-density GpSGHV, previous reports suggested that the negative impact of *Wolbachia* on insect viruses is density dependent [76, 79].

The assessment of the infection density (copy number per fly) of all four microbes (GpSGHV, *Wolbachia*, *Wigglesworthia*, and *Sodalis*) in the same tsetse flies indicated that *Wolbachia* infection at high density has a significant negative correlation

with *Wigglesworthia* infection in *G. m. morsitans* but not in *G. austeni*. However, the latter might be due to the low number of analyzed *G. austeni* flies (*n* = 21) compared to *G. m.morsitans* (*n* = 91). On the other hand, *Wolbachia* density levels do not correlate with *Sodalis*. The nature of the negative interaction between *Wolbachia* and *Wigglesworthia* is unclear. Whether this negative correlation between *Wolbachia* and *Wigglesworthia* is present in other tsetse species beyond *G. m. morsitans* remains to be seen.

The positive correlation between GpSGHV infection and *Wigglesworthia* infection observed in *G. m. mor- sitans* conflicts with the negative correlation observed in the same species of colonized flies [54]. This result might reflect a specific adaptation between a specific strain of *Wigglesworthia*, which reacts in a specific way to increase its density in the presence of GpSGHV as a manner to restore and enhance the host immune sys- tem against the virus infection [80]. The difference in the interaction between the GpSGHV and *Wigglesworthia* between the results of this study and the results of Demirbas-Uzel et al. [54] might be due to: (i) difference in the host strain/genotype as the *G. m. morsitans* individuals were collected from several countries in east Africa (Tanzania, Zambia, and Zimbabwe) while the colonized flies originated from Zimbabwe and have been maintained in the colony since 1997; (ii) different strain(s) of *Wigglesworthia* circulating in the field samples compared to the ones present in colo- nized flies [60]; (iii) different strain(s) of the GpSGHV in the field samples [58]; (iv) difference between field and laboratory conditions where the stress from han- dling the large number of flies in high density in the laboratory might negatively affect *Wigglesworthia* density levels and/or performance. The same reasons may also explain the difference observed between field and

Dieng *et al. Parasites & Vectors* (2022) 15:447

Chapter 4

laboratory samples regarding the interactions between GpSGHV and *Sodalis*.

Conclusions

The present study, despite its limitations regarding the size of samples and the lack of knowledge about the age, nutritional and trypanosome infection status, and environmental conditions at the time of collection of field specimens, shows a snapshot image of the density levels of tsetse symbionts and SGHV under field conditions and clearly indicates that the interactions/association between the tsetse host and its associated microbes are dynamic and likely species specific, and significant differences may exist between laboratory and field conditions. Further studies are needed to clarify the interaction between tsetse symbionts and GpSGHV under field conditions.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05536-9.

Additional file 1: Interactions between tsetse endosymbionts and *Gloss-ina pallidipes* salivary gland hypertrophy virus in wild tsetse populations.

Additional file 2: **Table S1.** List of primers used for quantitative PCR(qPCR) analyses in *Glossina* species.

Additional file 3: **Table S2.** Cochran-Mantel-Haenszel test for repeated tests of independence with continuity correction on the coingection of GpSGHV and *Wolbachia* in wild tsetse species.

Additional file 4: **Figure S1.** Prevalence of GpSGHV and *Wolbachia* co-infection in natural tsetse populations. A: In all tsetse species; B: in each tsetse species. GpSGHV and *Wolbachia* prevalence was determined by PCR as described previously [7,47].

Additional file 5: **Figure S2.** Density levels of GpSGHV (A), *Wolbachia* (B), *Wigglesworthia* (C), and *Sodalis* (D) determined by qPCR in tsetse flies with different GpSGHV and *Wolbachia* infection statuses. The copy number was determined by qPCR. Values indicated by a different small letter differ significantly at the 5% level. W+/V+: flies infected with both *Wolbachia* and GpSGHV; W+/V-: flies infected only with *Wolbachia*; W-/V-+: flies infected only with GpSGHV. GpSGHV and *Wolbachia* infection status was determined by conventional PCR as described previously [7,47].

Additional file 6: **Figure S3.** Density levels of GpSGHV (A), *Wolbachia* (B), *Wigglesworthia* (C), and *Sodalis* (D) in tsetse flies collected from different countries. The copy number was determined by qPCR. Values indicated by a different small letter differ significantly at the 5% level.

Additional file 7: Figure S4. Impact of GpSGHV and *Wolbachia* co- infection (W+/V+) on the density levels of GpSGHV (A), *Wolbachia* (B), *Wigglesworthia* (C), and *Sodalis* (D) in different tsetse species. The copy number was determined by qPCR. Values indicated by the same lowercase letter do not differ significantly at the 5% level.

Additional file 8: Figure S5. Relative density of GpSGHV, *Wigglesworthia*, *Sodalis*, and *Wolbachia* in *G. austeni* and *G. m. morsitans* field-collected tsetse flies. The density of GpSGHV and tsetse symbionts was analyzed by qPCR. Data were transformed to square root and averaged based on countries and species (A), countries and infection status (sample) (B), and countries, species, and infection status (sample) (C). The top and the left ofthe graph indicate the group averaged Bray-Curtis similarity.

Declarations

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential competing interests.

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Publisher's Note

Chapter 5

Conclusion

Conclusion

The SIT is an important strategy for the sustainable management of Human African Trypanosomiasis and African Animal Trypanosomosis. It involves the mass production of and subsequently release of sterile male tsetse flies to reduce the overall tsetse fly population. The objective of this strategy is to reduce or eliminate the transmission of *Trypanosoma* spp. parasites, which cause these diseases. It is important to note that the success of SIT relies on careful planning, monitoring, and quality control in mass-rearing facilities. It also depends on the accurate release of sufficient numbers of sterile males to outcompete the wild males and reduce the tsetse fly population effectively. Additionally, SIT is typically used as part of an integrated approach to disease control, which may include other methods like insecticide-treated targets or traps and chemotherapy or chemoprophylaxis for animal and human. While the SIT is a valuable tactic for the management of trypanosomiasis, it requires precise execution and consideration of the potential risks associated with early implementation and the vector competency of sterile males. Indeed, the fact that sterile males maintaining their vector competence intended for release in area where sleeping sickness occurs might increase the disease transmission risk. To mitigate the risk of disease transmission during SIT programs, sterile males are offered blood meals mixed with trypanocidal drugs, like isometamidium chloride. This reduces the risk but does not eliminate it. Other approaches, such as paratransgenesis, have been proposed to minimize these risks.

Further, tsetse symbionts and viruses like GpSGHV are integral components of the tsetse fly and can have implications for the success of the SIT. The presence of nutritional symbionts as *Wigglesworthia* as previously describes in **chapter 1** has been important for rearing healthy sterile males, and the interactions between symbionts, viruses, tsetse flies, and *Trypanosoma* spp. have implications for disease transmission and control strategies. Understanding these complex ecological relationships is essential for effective management of Trypanosomiasis.

This thesis dissertation is raising questions of significant relevance in the context of field samples in order to have an overview on the tripartite interrelation between symbionts-pathogen-tsetse fly on trypanosome infection for improving the effectiveness of SIT pro-grammes. Some of the important steps for efficient execution of SIT for tsetse flies include (i) assessing the impact of the interaction between tsetse fly's symbionts and *G. pallidipes* salivary gland hypertrophy virus (GpSGHV) on trypanosome infections and (ii) investigating potential interactions between the new discovery tsetse symbiont *Spiroplasma* with the trypanosome and

the primary symbiont in *Glossina tachinoides* relevant vector of HAT in West and Central Africa.

Within the aforementioned goals, this thesis was conducted on the following themes: (i) evaluation of the prevalence of *Sodalis* and *Trypanosoma* spp. infection in wild population of tsetse flies and exploring their interactions; (ii) determination of the potential association between *Wolbachia* and GpSGHV co-infection with *Wigglesworthia* and *Sodalis* in field samples (iii) assessment of the prevalence of *Spiroplasma* in natural tsetse population and investigating the different strains of the bacterium circulating in *G. tachinoides* wild population. Finally, evaluating of the interactions between *Spiroplasma* with the trypanosome and *Wigglesworthia* in *G. tachinoides*.

The results obtained reveal a snapshot view on the interactions between tsetse fly's symbionts as well as pathogens (GpSGHV) on trypanosome infection. These findings collectively contribute to our understanding of the dynamics of trypanosome infections associated with tsetse fly bacterial symbionts and pathogens and the potential for more effective control strategies.

Chapter 2 highlights the importance of geographic variation in *Trypanosoma* and *Sodalis* prevalence among tsetse fly populations. This geographic variation can inform targeted control measures and strategies specific to affected areas in the context of the disease transmission risk areas. In case of SIT implementation, it is crucial to determine the infection rate of the trypanosome species responsible of HAT in such area. Our findings show that *Trypanosoma brucei* spp. has the lowest prevalence of 2,29 % among all the trypanosome taxa, but higher in West (3,16 %) than Central and South Africa (1,25 %). However, this percentage combines all the trypanosome belonging to the subgenus *Trypanozoon* (*T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*). Therefore, the characterisation of each species is needed to identify the one responsible of HAT. On the other hand, the prevalence of human and animal disease caused by *T. brucei* spp. is controversial while the mature infection rate found in the salivary glands is very low in the range of 0,1% [52,98].

Additionally, the secondary facultative symbiont *Sodalis glossinodius* was previously reported to be present in only some wild population of tsetse fly [125]. Our findings show that the prevalence of the symbiont varied among tsetse fly species and locations. Indeed, the symbiont is more prevalent in East, Central and South Africa than in West Africa. Regardless the

countries, the highest prevalence of *Sodalis* is recorded in *G. m. morsitans* and *G. pallidipes*. In contrast the prevalence of the symbiont is very low in the following species *G. austeni*, *G. p. palpalis*, *G. brevipalpis*, *G. p. gambiense*, *G. medicorum*, *G. m. submorsitans* and absent in *G. tachinoides*. This provides critical insights into the complexity of tsetse fly microbiota. It implies that the composition of microbial communities can be influenced by factors such as geography and tsetse fly species.

The vector competence of tsetse flies for *Trypanosoma* species varies, and might be influenced by various factors, including bacterial endosymbionts like *Sodalis glossinidius*, *Spiroplasma* and *Wolbachia*. Some studies suggested a potential positive correlation between *Sodalis* and trypanosome infections [133,134,207,208], which may facilitate the parasite establishment in the tsetse midgut [9,91,209]. However, other studies indicated a lack of correlation [208,210]. *Spiroplasma* and *Wolbachia* also may protect specifics tsetse fly species as *G. f. fuscipes* and *G. tachinoides* belonging both to the *palpalis* group against trypanosome infection [93,150] while *Wolbachia* has reported to have no influence on the establishment of trypanosomes in some tsetse species [151,152]. Our results suggest a correlation between *Sodalis* and trypanosome in *G. p. gambiense*, *G. pallidipes* and *G. medicorum*. Taking into account that the symbiont is more frequently found in colonized tsetse flies than in tsetse wild population [208], the low prevalence of the symbiont found in some natural tsetse species is not to be minimized. Because e.g. species like *G. p. gambiense* use for SIT programmes can show high *Sodalis* prevalence (100 %) in reared condition [211,212]. This higher prevalence of *Sodalis* in colonized tsetse flies compared to wild populations could impact their role as disease vectors.

Chapter 3 gives as well some outlines in the vector competence and interaction between symbionts and trypanosome co-infection in wild population of tsetse fly. Our research confirms the presence of *Spiroplasma* in *G. tachinoides* in both colonized and wild tsetse and indicate that the presence of the symbiont could have a refractory effect on *Trypanosoma* spp. infection. The mechanism behind this effect is not fully understood but may be related to competition, immune responses, or nutrient availability. Although, the *Spiroplasma*-trypanosome co-infection has no significant effect on the density of *Wigglesworthia*. This may be by the incapacity by others microbiota to invade the niche of the primary symbiont [129]. The *Spiroplasma* strains found in *G. tachinoides* collected in West Africa countries (Burkina Faso and Ghana) are most close to the citri group and exhibits genetic diversity, with different

haplotypes. This genetic diversity may be influenced by factors such as colonization and environmental conditions.

Chapter 4 gives some outputs in the association between GpSGHV, *Wolbachia*, *Wigglesworthia*, and *Sodalis* in several tsetse fly's species. These interactions are complex and can vary among tsetse species and locations. The reported single infection rate with *Wolbachia* and GpSGHV in wild tsetse flies is low [213–216]. However, it was necessary to analyse the potential association between the co-infection *Wolbachia*-GpSGHV and others tsetse symbiotic microbiota. The results emphasize the dynamic nature of interactions between tsetse flies and their associated microbes, suggesting that interactions may not be uniform but species-specific, highlighting the complexity of host-microbe relationships in tsetse flies. Specifically, our results indicate that the *Wolbachia*-GpSGHV co-infection occur only in *G. austeni* and *G. m. morsitans*. In *G. m. morsitans* a negative correlation was observed between *Wolbachia* and *Wigglesworthia* with no correlation with *Sodalis*. It is worth noting that there is a positive correlation between GpSGHV and *Wigglesworthia* in *G. m. morsitans*. This positive correlation conflicts with the negative correlation found in laboratory flies [173]. Finally, our findings suggest an antagonistic relationship between *Wolbachia* and GpSGHV, which protects tsetse flies against GpSGHV. However, the exact mechanism of this interaction remains undefined.

The results of the study collectively underscore the complex interactions among various microorganisms in tsetse flies and their potential implications for controlling tsetse populations and the diseases they transmit. The antagonistic interaction between *Wolbachia* and GpSGHV, along with the potential protective role of *Spiroplasma* against trypanosome infections, present promising avenues for the development of innovative control strategies.

The discovery that *Spiroplasma* infection can reduce trypanosome infection in *G. tachinoides* holds significant implications for SIT programs. Releasing sterile males infected with *Spiroplasma* could mitigate disease transmission risks, enhancing the effectiveness of SIT in tsetse population control. Investigating the specific mechanisms underlying the impact of *Spiroplasma* on trypanosome infection and the undefined mechanisms of *Wolbachia*'s protective effect on GpSGHV are exciting areas for further research. The observed discrepancy between laboratory and field conditions remains a crucial consideration. While these interactions are noted in controlled settings, their practical application in the field may differ.

Therefore, additional research and field trials are essential to assess the viability of these strategies in real-world scenarios.

In conclusion the amalgamated results unveil the multifaceted nature of host-microbe interactions in tsetse flies and underscore the potential for these interactions to guide innovative control measures. A comprehensive understanding the dynamics of tsetse fly microbiota and their relationships with pathogens such as *Trypanosoma* and GpSGHV is crucial for the development of more effective and targeted strategies to control tsetse fly populations and reduce disease transmission in Africa.

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Curriculum vitae

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Personal Data

Date and place of birth: 1st December 1978 in Diourbel/ Senegal

Nationality: Senegalese

EDUCATION

Inter State School of Sciences And Veterinary Medicine Cheikh Anta Diop University	Diploma in Veterinary Medicine- Dakar, Senegal 1999-2004
Malick SY High school	Scientific Baccalaureate- Thies, Senegal 1996-1999

TRAINING FOR RESEARCH BY RESEARCH

Vienna University of Technology	Ph.D. in Natural Science in Technical Chemistry at the Institute of Chemical, Environmental and Bioscience Engineering-Vienna, Austria 2020-2023
Institute of Tropical Medicine	Master of Science in Animal Tropical Health Antwerpen, Belgium 2012-2013

WORK EXPERIENCE

Assistant-Professor, Animal Health and Feeding Department of Animal Husbandry and livestock, Gaston Berger University	Saint Louis- Senegal 2017-Present
Head of the veterinary service of National Gendarmerie	Dakar- Senegal 2022- Present
Consultant Joint FAO/IAEA Agriculture and Biotechnology Laboratories United Nations	2018-2021 Seibersdorf, Austria

Regional department Head of livestock   Dakar, Senegal 2013-2016

Regional department Head of livestock   Kedougou, Senegal 2010-2012

Head of the Veterinary Service
of the Port and Airport   Dakar, Senegal 2007-2010

Departmental Inspector
of Veterinary Services   Thies, Senegal 2006-2007

Deputy Inspector of Veterinary Services   Thies, Senegal 2006-2007

Veterinarian-Hygienist at the United Nations
Mission in Liberia (UNMIL)   Harper, Liberia 2005-2006

RESEARCH SKILLS AND EXPERTISE

- ❖ Entomological experiments and maintaining tsetse colonies

- ❖ Molecular Techniques including DNA isolation, PCR, qPCR, molecular cloning

- ❖ DNA analysis by gel electrophoresis and spectrophotometry

- ❖ Basic Microbiological Techniques including aerobic and anaerobic bacterial growth

- ❖ Culturing *Sodalis* bacteria and attempt to produce *Sodalis*-GFP bacteria

- ❖ DNA cloning and sequencing and analysis the sequence results

- ❖ Attempts to isolate *Sodalis* phages

- ❖ Assess the impact of pupal sex sorting machine of the tsetse performance

TECHNICAL TRAINING

- ❖ Training in the Veterinary Protozoology unit of Institute of Tropical Medicine, Antwerp, Belgium from 15-19 October 2018 to develop a protocol to culture *Sodalis* bacteria (tsetse-endosymbiont).

- ❖ Informatics Bio-Linux and QIIME2 training course held at the IAEA Seibersdorf laboratories, Vienna on Mon-Tue 10-11 December 2018.

❖ Training in Strategic management of eradication projects tsetse fly held at the ELAT (Ecole de Lutte anti tsetse) Bobo Dioulasso, Burkina Faso on 1-14 June 2014.

❖ Training in Implementation of a quality approach according to the Standard -17020 :2012- Dakar, Senegal December 2014.

❖ Training in Milk and meat technology held at the EICA (Egyptian International Center for Agriculture) - Cairo, Egypt March 2012.

❖ Training in Recycling in rural poultry farming -Kedougou-Senegal November 2011.

❖ Training in Zoosanitary inspection -Inter State School of Sciences and Veterinary Medicine- Dakar February 2008.