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TECHNISCHE UNIVERSITÄT WIEN
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DISSERTATION

Improving Sterile Insect Technique for tsetse flies through research on their symbiont and pathogens

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

Univ. Prof. Dr. Robert Mach

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Table of Contents

Acknowledgment i
Table of Contents iii
List of Abbreviations v
Abstract viii
Zusammenfassung x
Declaration xii
List of Publications and Conference Proceedings xiv
Chapter 1 1
1. General Introduction 2
 1.1. Tsetse flies and African trypanosomiasis 2
 1.2. Distribution of Tsetse species 3
 1.3. Biology of *Glossina* spp. 4
  1.3.1. Taxonomy of tsetse flies 4
  1.3.2. Lifecycle and reproduction of tsetse flies 5
  1.3.3. The feeding process of tsetse flies 6
 1.4. Biology of *Trypanosoma spp.* 7
 1.5. Current treatment strategies 8
 1.6. Control of Tsetse Flies 8
  1.6.1. Sterile Insect Technique 9
 1.7. Insect Symbiosis 11
  1.7.1. Tsetse symbiosis 12
   1.7.1.1. The primary symbiont *Wigglesworthia glossinidius* 12
   1.7.1.2. The secondary symbiont *Sodalis glossinidius* 13
   1.7.1.3. The third symbiont *Wolbachia* 13
   1.7.1.4. The fourth tsetse symbiont *Spiroplasma* 14
 1.8. Tsetse Pathogen-*Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV) 14
 1.9. Tsetse paratransgenesis 15
 1.10. Effects of radiation on symbiont and pathogens 15
Aim of the Thesis 17
Chapter 2 19
A nuclear, mitochondrial and *Wolbachia*-based approach for the rapid and accurate identification of tsetse species 20

Chapter 3 60

Prevalence of trypanosomes, salivary gland hypertrophy virus and *Wolbachia* in wild populations of tsetse flies from West Africa 61

Chapter 4 93

Susceptibility of Tsetse Species to *Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV) 94

Chapter 5 123

Impact of Glossina pallidipes salivary gland hypertrophy virus (GpSGHV) on a heterologous tsetse fly host, *Glossina fuscipes fuscipes* 124

Chapter 6 149

Combining paratransgenesis with SIT: Impact of ionizing radiation on the density of *Sodalis glossinidius* 150

Chapter 7 189

Effect of antibiotic treatment and gamma-irradiation on cuticular hydrocarbon profiles and mate choice in tsetse flies (*Glossina m. morsitans*) 190

Chapter 8 219

General Discussion and Conclusion 220

Reference List 224

Curriculum Vitae xvii

List of Abbreviations

Abbreviation/ Term	Definitions
AAT	Animal African Trypanosomiosis
AICc	Akaike information criterion
Amp	Ampicillin
ANOVA	Analysis of Variance
Apo	Aposymbiotic
AU	African Union
AW-IPM	Area Wide Integrated Pest Management
BSF	Blood Stream Form
CAR	Central African Republic
CHC	Cuticular Hydrocarbon
CI	Cytoplasmic Incompatibility
CIRDES	Centre International de Recherche-Developpement sur l'Elevage en zone Subhumide
COI	Cytochrome oxidase 1
COII	Cytochrome oxidase 2
Ctr	Control
CYTB	Cytochrome b
DAs	Discriminant Analysis
DDT	Dichlordiphenyltrichlorethan
DNA	Deoxyribonucleic Acid
dNTP	Deoxy nucleotide Triphosphate
dsDNA	Double-stranded Deoxyribonucleic acid
El-MS	Electron Ionization Mass Spectra
FAO	Food and Agricultural Organization of the United Nations
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GLM	General Linear Model
GpSGHV	Glossina pallidipes Salivary Gland Hypertrophy Virus
GSS	Genetic sexing strains
Gy	Gray
H	Haplotype
HAT	Human African Trypanosomiasis
HSD	Tukey's Honesty significant difference Test

Abbreviation/ Term	Definitions
HSG	Hypertrophied Salivary Gland
Hytrosaviridae	A virus family name derived from “*Hy*per*tro*phia *s*ialoadenitis”, a Greek word for “salivary gland inflammation.”
IAEA	International Atomic Energy Agency
IPCL	Insect Pest Control Laboratory
ITM	Institute of Tropical Medicine
ITS1	Internal Transcribed Spacer 1
m	meter
MabrNPV	Mamestra brassicae Nuclear Polyhedrosis Virus
MCL	Maximum Composite Likelihood
MdSGHV	*Musca domestica* Salivary Gland Hypertrophy Virus
Me	methyl
ML	Maximum Likelihood
NADH	Nicotinamide adenine dinucleotide dehydrogenase
NAFA	Nuclear Applications in Food and Agriculture
ND2	NADH dehydrogenase 2
NSG	Normal Salivary Gland
NTDs	Neglected Tropical Diseases
ORF	Open Reading Frame
PATTEC	The Pan African Tsetse and Trypanosomiasis Eradication Campaign
PBS	Phosphate Buffered Saline
PCAs	Principal Component Analyses
PCR	Polymerase Chain Reaction
Pf10d	Pupae per female per 10 days
PfNPV	Panolis flammea Nuclear Polyhedrosis Virus
Ph.D.	Doctor of Philosophy
PPIF	Pupae Production Per Initial Female
PVC	Polyvinyl Chloride
qPCR	quantitative Polymerase Chain Reaction
RH	Relative Humidity
RLO	Rickettsia-Like Organism
RNA	Ribonucleic Acid
rRNA	Ribosomal ribonucleic acid
S	Secondary
SAS	Slovak Academy of Science

Abbreviation/ Term	Definitions
SAT	Sequential aerosol technique
SG	Salivary Gland
SGH	Salivary Gland Hypertrophy
SGHV	Salivary Gland Hypertrophy Virus
SGs	Salivary Glands
SIT	Sterile Insect Technique
SPSS	Statistical Package for the Social Sciences
Tbb	*Trypanosoma brucei brucei*
Tet	Tetracycline
USD	United States Dollar
USDA	United State Department of Agriculture
UK	United Kingdom
WHO	World Health Organization
WspecF	*Wolbachia* specific Forward
WspecR	*Wolbachia* specific Reverse

Abstract

African trypanosomoses are major neglected tropical diseases in both humans (sleeping sickness or Human African Trypanosomosis (HAT)) and animals (nagana or Animal African Trypanosomosis (AAT)) in sub-Saharan Africa and are transmitted by cyclical vector tsetse flies (Dipteria: Glossinidae). Due to the lack of efficient vaccines, an increased resistance against anti-trypanosomal drugs, and lack of inexpensive drugs against the diseases, tsetse fly control is currently considered the most powerful, innovative and efficient pest control tactic. The Sterile Insect Technique (SIT) has been proven to be one of the most effective and sustainable methods when it is applied as a part of Area-Wide Integrated Pest Management (AW-IPM) programmes for the suppression and/or eradication of tsetse fly populations. SIT requires the production of a large-number of males, sterilized by exposure to ionizing radiation and their release into the target area where they compete with wild males for wild females. Matings between sterile males and virgin wild females result in no offspring. A repeated release of a higher ratio of sterile males to wild males increases the chances of successful sterile matings and thus results in the suppression and/or eradication of the target insect population over time. Despite the fact that successful implementation of SIT/AW-IPM programmes for eradication tsetse flies in several infested areas has been shown, several constraints need to be addressed to enhance its efficiency. One of the important challenges of the SIT for tsetse is the mass production of sufficient sterile males due to their low productivity and infection with a pathogenic virus. Another challenge facing tsetse SIT programs is the ability of sterile males to transmit the disease due to their hematophagous feeding nature, which represents a potential risk of increasing the disease incidence in a SIT treatment site. Some of the constraints could be solved by understanding the interactions of tsetse flies with their symbionts and pathogens. Laboratory colonies, as well as field populations of many tsetse species, are infected by *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV; family hytrosaviridae) and harbor endosymbiotic bacteria *Wigglesworthia glossinidius*, *Sodalis glossinidia, Wolbachia,* and *Spiroplasma*. GpSGHV infected flies of *Glossina pallidipes* show ovarian abnormalities and testicular degeneration and salivary gland hypertrophy (SGH) symptoms. In the absence of an effective virus management strategy, the SGHV can reduce the colony productivity and may even result in the collapse of the infected colony. Therefore, it is important to assess the virus' impact on several tsetse species. In addition, it is important to assess the impact of irradiation on tsetse symbionts as well as on its vectorial capacity. The aim of this research was to investigate (i)

tsetse species identification using molecular approaches, (ii) prevalence of virus, *Wolbachia* and trypanosome in natural populations, (iii) the susceptibility of virus to different laboratory tsetse species, (iv) the impact of GpSGHV infection on heterogenous tsetse hosts, (v) the impact of irradiation on the tsetse symbiont Sodalis and the potential of using a combined approach of paratransgenesis and SIT, and lastly (vi) the impact of symbiont depletion and radiation treatment on *G. m. morsitans* cuticular hydrocarbon profiles and mate choice. The results indicate the susceptibility of different tsetse species to GpSGHV infection, however only *G. pallidipes* permit virus trans-generation transmission. Irradiation did not affect the CHC profile or male vectorial capacity, however, it reduced the density of symbionts. Exposing treatment on 22-day old puparia to radiation allows a significant recovery of the Sodalis density which enables a combination of paratransgenesis and SIT to eliminate the risk of increasing the disease incidence in areas using SIT. Finally, novel molecular tools provide easy, cheap and precise species identification methods where morphological identification methods formerly lacked accuracy.

Key Words: Tsetse flies, SIT, Trypanosomiosis, Symbionts, Irradiation

Zusammenfassung

Afrikanische Trypanosomiasen sind bedeutende, jedoch wenig beachtete Tropenkrankheiten der Menschen (Afrikanische Schlafkrankheit oder Human African Trypanosomiasis (HAT)), und Tieren (Nagana, oder Animal African Trypanosomiasis (AAT)), die in den subsaharischen Regionen Afrikas vorkommen und durch Tsetsefliegen übertragen werden (Dipteria: Glossinidae). Aufgrund fehlender effektiver Impfungen, der zunehmenden Resistenz gegen anti-trypanosomale Medikamente, und des Mangels an verfügbaren und leistbaren Therapien gegen diese Erkrankungen ist die Kontrolle der Tsetsefliegen Populationen momentan die effektivste Lösung um diese Krankheitsvorkommen zu reduzieren. Die Sterile Insekten Technik (SIT) ist eine erwiesene, effektive und nachhaltige Methode, wenn sie im Rahmen einer flächendeckenden, integrierten Schädlingsbekämpfungsstrategie ("Area-Wide Integrated Pest Management (AW-IPM)") für die Dezimierung oder Ausrottung der Tsetsefliegen Populationen angewendet wird. Die SIT benötigt die künstliche Erzeugung einer großen Anzahl des Schadeninsekts, die Sterilisierung der Männchen durch ionisierende Strahlung, und deren Aussetzung in das Zielgebiet. Diese sterilen Männchen konkurrieren mit den in der Natur lebenden fertilen Männchen um die Weibchen, und erfolgreiche Paarungen der sterilen Männchen erzeugen kein Nachkommen. Bei einer verhältnismäßig höheren Anzahl der freigelassenen sterilen Männchen im Vergleich zu den natürlich vorhandenen fertilen Männchen steigen die Chancen der gewollten Paarungen und dadurch kann die Populationen des Zielinsekts stark verringert und sogar komplett ausgerottet werden. Trotz erfolgreiches Einsetzen der SIT/AW-IPM gegen Tsetsefliegen sind einige Beschränkungen vorhanden, die überwunden werden können um die Effizienz der Technik zu verbessern. Eine dieser Beschränkungen ist die niedrige Produktivität dieser Insekten in der Zucht, und deren Infektion mit einem schädlichen Virus. Eine zusätzliche Problematik ist die Fähigkeit der männlichen Tsetsefliegen, wegen deren haematophagen Ernährungsverhalten die erwähnten Krankheiten zu übertragen. Dadurch entsteht das Risiko einer erhöhten Übertragungsrate in der Region. Diese Probleme könnten gelöst werden, indem man die Interaktionen zwischen der Tsetsefliege und deren Symbionten und Pathogenen besser zu verstehen lernt. Sowohl Laborkolonien, als auch in der Natur existierenden Tsetsefliegenpopulationen sind zu hohem Anteil mit der *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV; Familie hytrosaviridae) infiziert und sind gleichzeitig Träger der endosymbiotischen Bakterien *Wigglesworthia glossinidius*, *Sodalis glossinidia, Wolbachia,* und *Spiroplasma*. GpSGHV infizierte *Glossina pallidipes* Fliegen

zeigen Abnormitäten in den Ovarien und Testikel und Speicheldrüsen-Hypertrophie (salivary gland hypertrophy (SGH)). Ohne einer Strategie zur Virenkontrolle kann eine SGH Virus-Infektion in der Tsetsefliegenkolonie deren Produktivität stark verringern und sogar zum völligen Kollaps bringen. Es ist deswegen wichtig, die Auswirkungen dieser Viren auf die verschiedenen Tsetsefliegenspezien zu eruieren. Zusätzlich ist sowohl die Auswertung der Wirkung radioaktiver Strahlen auf Tsetse Symbiontenals auch auf die Krankheitsübertragungskapazität der Fliegen entscheidend. Das Ziel dieser wissenschaftlicher Arbeit war: (i) die Identifizierung der Tsetsefliegen Spezies durch molekulare Methoden, (ii) die Prävalenz von Viren, *Wolbachia*, und Trypanosomen in natürlichen Populationen der Tsetsefliegen, (iii) die Suszeptibilität der verschiedenen Tsetse Spezies zu Viren, (iv) die Eruierung der Auswirkungen von GpSGHV Infektionen auf deren Wirten, (v) die Eruierung der Wirkung ionisierender Strahlen auf die Tsetse Symbionten *Sodalis* und die Möglichkeiten einer kombinierten Paratransgenese und SIT Methode, und (vi) die Eruierung der Auswirkung einer Depletion der Symbionten und radioaktiver Strahlung auf das kutikuläre Hydrokarbonprofil (KHD Profil) der Tsetsefliegen *G. m. morsitans*. Die Ergebnisse weisen darauf hin, dass verschiedene Tsetse Spezies anfällig auf GpSGHV sind, jedoch nur *G. pallidipes* die Fähigkeit einer transgenerationalen Übertragung der Viren aufweist. Ionisierende Strahlen haben weder Auswirkung auf das KHD Profil, noch auf die Krankheitsübertragungskapazität der Fliegen, jedoch konnte eine Auswirkung auf die Dichte der Tsetse Symbionten nachgewiesen werden. Die Bestrahlung von 22 Tage alten Puppen ermöglicht die Wiederherstellung der *Sordalis* Dichte und somit die Kombination von Paratransgenese und SIT, um das Risiko der Krankheitsübertragung in SIT Anwendungsgebieten zu verringern. Letztlich ermöglichen verschiedene molekulare Methoden eine kosteneffektive, einfache und präzise Speziesidentifikation.

Stichworte: Tsetsefliegen, SIT,Trypmanosomiasis, Symbionten, Ionisierende Strahlung

Declaration

I hereby declare that this doctoral thesis entitled "**Improving Sterile Insect Technique for tsetse flies through research on their symbiont and pathogens**" was carried out and written by me for the degree of Doctor of Philosophy in English in Doctoral programme in Natural Sciences Diploma programme: Technical Chemistry under the guidance and supervision of Prof. Robert L. Mach from the Institute of Chemical, Environmental and Biological Engineering and Prof. Adly Abdalla from the Insect Pest Control Laboratory, Joint FAO/IAEA Divisions of Nuclear Techniques in Food and Agriculture. I also confirm that this thesis is the product of original research work obtained at the Insect Pest Control Laboratory, Joint FAO/IAEA Divisions of Nuclear Techniques in Food and Agriculture, Seibersforf, Austria. Furthermore, I took reasonable care to ensure that the work is original and it does not breach copyright law from other sources and such work has been cited and acknowledged. I have not submitted the doctoral thesis for any other degree or professional qualification. The experimental work is almost entirely my work; the collaborative contributions have been indicated clearly and acknowledged. The data herein presented in this thesis was obtained in an experiment carried out in close collaboration Dr. Jan Van Den Abbeele and Dr. Linda De Vooght at the Department of Biomedical Sciences, Unit of Veterinary Protozoology, Institute of Tropical Medicine Antwerp (ITM), Antwerp, Belgium. Mrs. Gisele Ouedraogo at Ecole National de l'Elevage et de la Santé Animale, in Ouagadougou , Burkina Faso and with Dr. Martin Kaltenpoth at the Max Plank Institute for Chemical Ecology in Jena, Germany.

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2. Ouedraogo G.M.S., **Güler Demirbas-Uzel,** Traore A, Rayaisse J.B, Avgoustnos, A, Parker, Sidibe, I, Ouedraogo, A.G, Vreysen MJB, Bourtzis K and Abd-Alla M.M.A, "Prevalence of salivary gland hypertrophy virus *Wolbachia* and trypanosome in wild population of tsetse flies from West Africa*".* (BMC Microbiology, under revision).

3. **Güler Demirbas-Uzel**, Henry M. Kariithi, Andrew G. Parker, Marc J. B. Vreysen, Robert L. Mach and Adly M. M. Abd-Alla. "Comparative Susceptibilities of Tsetse

Species to *Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV)" (Frontier Microbiology, under revision).

4. **Gü**ler **Demirbas-Uzel**, Andrew G. Parker, Marc J. B. Vreysen, Robert L. Mach, Jeremy Bouyer, Peter Takac , and Adly M. M. Abd-Alla. "Impact of *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) on a heterologous host, tsetse fly *G. f. fuscipes*". (BMC Microbiology, Accepted 2018).

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1. **Güler Demirbas Uzel, Henry M. Kariithi, Andrew G. Parker, Marc J. B. Vreysen, Robert L. Mach and Adly M. M. Abd-Alla. "**Comparative Susceptibilities of Tsetse Species to *Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV)", (**Frontier Microbiology. 2018, under revision)**

2. **Güler Demirbas Uzel, Henry M. Kariithi, Andrew G. Parker, Marc J. B. Vreysen, Jeremy Bouyer, Robert L. Mach and Adly M. M. Abd-Alla. "**Impact of *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) on a heterologous host, tsetse fly *G. f. fuscipes*"(**Accepted to BMC Microbiology, 2018***)*

3. **Güler Demirbas Uzel, Linda De Vooght, Andrew Parker, Marc Vreysen, Robert L Mach, Jan Van Den Abbeele Adly Abd-Alla, "**Combining paratransgenesis with SIT; Impact of ionizing radiation on the prevalence of *Sodalis glossinidius",* **(Submitted to BMC Microbiology, 2018***).*

4. **Tobias Engl, Veronika Michalkova, Brian L. Weiss, Güler Demirbas Uzel ,Peter Takac, Wolfgang J. Miller, Adly M. M. Abd-Alla**, **Serap Aksoy**, **Martin Kaltenpoth,** "Effect of antibiotic treatment and gamma-irradiation on cuticular hydrocarbon profiles and mate choice in tsetse flies (*Glossina m. morsitans*)" **(Accepted to BMC Microbiology, 2018***)*

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6. **Augustinos AA†, Meki I†, Güler Demirbas Uzel, Saridaki A, Tsiamis G, van Oers, MM, Vreysen M, Parker A, Abd-Alla A, Bourtzis K**, "Nuclear, mitochondrial and *Wolbachia*-based approaches for the quick and accurate identification of tsetse species, (**Submitted to BMC Microbiology, 2018)**

7. **Kariithi, HM; Boucias, DG; Murungi, EK ; Meki, IK; Uzel, Demirbas Guler; van Oers, MM; Vreysen, MJB; Abd-Alla, AMM and Vlak, JM "**Coevolution of Hytrosaviruses and Host Immune Responses***",* (**Accepted to BMC Microbiology, 2018)**

8. **Kariithi, HM,†; Meki, IK†; Schneider, DI†; De Vooght, L†; Khamis, FM†; Geiger, A†; Demirbas Uzel, Guler†; Vlak, JM; iNCE, ikbal Agah; Kelm, S, and Abd-Alla, AMM.** "Enhancing Vector Refractoriness to Trypanosome Infection: Achievements, Challenges and Perspectives" (**Accepted - BMC Microbiology, 2018**).

Conference Proceedings

1. **Güler Demirbas Uzel and Adly M. M. Abd-Alla. "**Current research activities in at IPCL; Irradiation impact on tsetse symbiont*"* (**Oral Presentation**), "Enhancing vector refractoriness to trypanosome infection", Final Reserach Coordinating Meeting of the Joint FAO/IAEA Division of nuclear tecniques in food and agriculture. Tanga, Tanzania, December, 2017.

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4. **Guler Demirbas Uzel, Andrew Parker, Marc Vreysen, Robert L Mach, Kostas Bourtzis, and Adly Abd-Alla,** "Analysis of the impact of irradiation treatment on the establishment of *Sodalis* in ***Glossina morsistans morsitans*** Species", (**Poster Presentation**), Third FAO–IAEA International Conference on Area-wide Management of Insect Pests: Integrating the Sterile Insect and Related Nuclear and Other Techniques, 22-26 May 2017, Vienna, Austria.

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7. **Guler Demirbas Uzel, Andrew parker, Robert L Mach, Adly Abd-Alla**, "Host range of *Glossina palidipes* salivary gland hypertrophy virus (GpSGHV)", (**Oral Presentation**),

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9. **Güler Demirbas Uzel, Vangelis Doudoumis, AntoniosAugustinos, Gisele Ouedroogo, Andrew Parker, Drion Boucias, Kostas Bourtzis, AdlyAbd-Alla,** "Interactions between salivary gland hypertrophy virus and tsetse microbiota", **(Oral Presentation)**, 47th Annual Meeting of the Society for Invertebrate Pathology and International Congress on Invertebrate Pathology, No 174, 2014, Julius Kühn-Institute |JKI Journal

Chapter 1

General Introduction

1. General Introduction

This Ph.D. thesis introduction aims to provide necessary information on tsetse flies, their pathogens, symbionts, and Trypanosomosis disease. In addition, further details on tsetse mating behaviors, control methods and disease management strategies with new directions, guidelines, and recommendations are briefly described. There are several challenges facing the implementation of tsetse SIT programs such as the lack of accurate species identification tools, challenges in large-scale mass production of tsetse species, and the ability of tsetse sterile males to transmit trypanosomiosis. In the following section the molecular identification tools to verify accurate species identification, the impact of salivary gland virus infection and the interaction with tsetse symbiont and the impact of irradiation on tsetse symbionts are discussed.

1.1. Tsetse flies and African trypanosomiasis

Tsetse flies are the vectors of a debilitating, fatal zoonotic disease of domestic livestock Animal African Trypanosomiosis (AAT) and Human African Trypanosomiosis (HAT) in sub-Saharan Africa. The disease is one of the important restrictive factors for improvement of the animal and agriculture sector, and around 50 million cattle are at risk of infection [1]**.** Trypanosomes are parasitic protozoa that circulate in the blood and tissues of an extensive variety of vertebrate hosts and are transmitted cyclically by tsetse fly (*Glossina spp.*) [2]. The link between tsetse flies, parasite trypanosomes, and Trypanosomosis was revealed over 100 years ago [3].

The Food and Agricultural Organisation of the United Nations (FAO) estimates that tsetse infested lands suffer US$ 4750 million economic losses per year and that AAT causes about 3 million deaths in cattle with approximate losses of USD 600-1200 million in the production of cattle annually [4]. Around 35 million doses of trypanocidal drugs are administered annually. HAT disease prevalence reported by World Health Organisation (WHO) is 300,000-500,000 cases/year which represents 10-15 % of the real number of infected individuals[5]. However, due to increased surveillance and available drugs, the increased number of educated technicians and the commitment of the international community in the last decades, the prevalence of the disease has reduced to less than 3000 case/year, and the situation has become more encouraging .(http://www.who.int/trypanosomiasis_african/country/en/) [6, 7].

In general, people at highest risk of tsetse bites and HAT largely depend on agriculture, fishing, animal husbandry and hunting in rural populations. Resurgence and epidemics of HAT are mainly associated with economic decline, civil disturbance/wars, population and refugee movements [8]. HAT is one of the most critical and severe neglected tropical diseases (NTDs) [9], which are affecting low-income populations in Asia, Africa and the Americas [10].

1.2. Distribution of Tsetse species

Tsetse (the word for 'fly' in the South African Tswana language) or tik tik flies are enormous biting flies that are endemic to mid-continental sub-Saharan Africa. To date, tsetse flies are restricted to Africa and small areas of the Arabian Peninsula [11]. The first tsetse fly fossils were discovered in 26 million-year-old shales in Florissant, Colorado, USA [12]. Their dissemination is limited to a 'belt' region between the Sahara and Somali deserts in the north and the Kalahari and Namibian Desert in the south of the African continent and the eastern part [13] (**Figure 1**). Tsetse fly distribution covers approximately 10 km^2 of the sub–Saharan African continent [14].

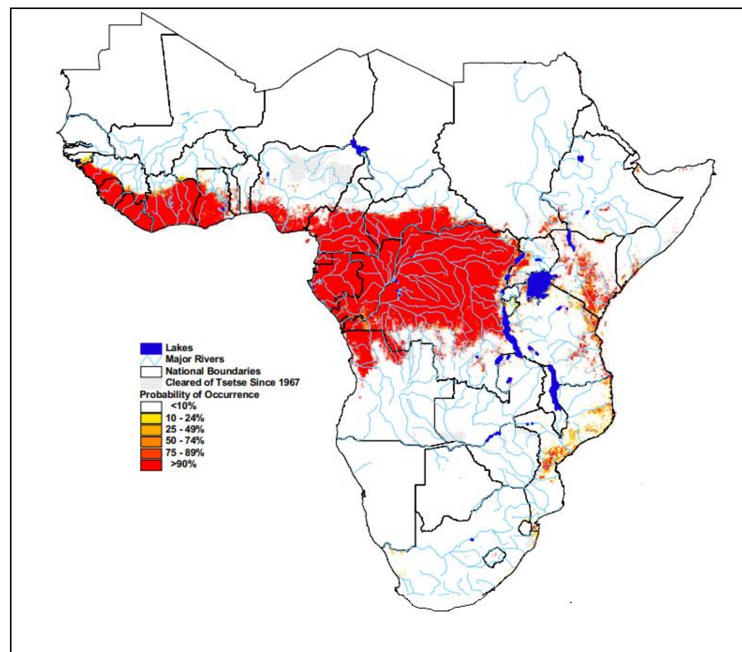

Figure 1. Distribution of Tsetse flies in Africa. (Images were taken from http://www.fao.org/ag/againfo/programmes/en/paat/documents/maps/pdf/tserep.pdf)

Tsetse flies are widely distributed and have been shown to transmit trypanosomes to animals and/or humans [15]. The most important tsetse vectors are the riverine species in Western and Central Africa and the savannah species in Eastern and Southern Africa [8]. The distribution

of each species and their habitats vary due to the different requirements, i.e., humidity, temperature, vegetation cover, surrounding habitats, and available food. Tsetse flies feed on available host animals [16]. Temperature and humidity affect the flies reproductive cycle and development and therefore are the primary factors determining tsetse distribution [17]. Temperature increases due to global climate change are thus expected to have a significant influence on tsetse population dynamics [18].

1.3. Biology of *Glossina* spp.

1.3.1. Taxonomy of tsetse flies

Kingdom:	:	Animalia
Phylum:	:	Arthropoda
Class:	:	Insecta
Superfamily:	:	Hippoboscoidae
Family:	:	Glossinidae
Genus:	;	Glossina

Tsetse flies are classified into one genus, *Glossina*, of the family *Glossinidae*, Order *Diptera.* The two-winged flies have different sizes ranging between 6–13 mm [19]. The hematophagous insects can be recognized by their “hatchet-cell” on their wings, and therefore they are easily distinguished from other blood-sucking insects. Both female and male flies act as vectors, are blood feeders and transmit trypanosomes [20]. There are 31 species, and 8 sub-species of tsetse fly that transmit trypanosomosis. However, only 8–10 species of tsetse fly are important with regard to economic (agricultural-veterinary) or human health [14]. Tsetse flies are divided into three different groups by their habitats: *Morsitans* in savannah regions, *Palpalis* in high humidity areas and *Fusca* in forests [21]. *Glossina* species and subspecies are additionally characterized by their geographic distribution, distinguishing genetics, physiology, morphology, and pathogenicity [18].

All tsetse species belonging to the *Musca* group are limited to savannah woodlands [22]. *G. m. morsitans* is the most important species in Africa and a major vector of AAT. Other species such as *G. swynnertoni, G. longipalpis, G. pallidipes* mainly live in the edge vegetation of forest and thickets. The *Palpalis* group spread mainly in lowland rain forests. However, the habitants live throughout the river system including wet savannah areas [22].

The *Fusca* group species has mostly little or no economic importance, and their habitat is lowland rain forests, the border areas of forests and isolated relic forests [22]. Tsetse flies live best at a temperature of 25° C. However, interspecific differences can be observed [23]. Sufficient moisture conditions are required mainly for larvae to complete pupal development.

1.3.2. Lifecycle and reproduction of tsetse flies

The life cycle of tsetse flies is different from other insects [24]. Females have a pair of ovaries, a uterus, and spermathecae to store spermatozoa received from copulation with males. Males can mate approximately ten times if mating opportunity are offered. The sperm migrates towards the spermathecae where it is stored. A single mating event is enough for the female to produce larvae for several months; spermatozoids can survive for nearly 200 days in the spermathecae. Sometimes a female can accept multiple matings to fill the spermathecae. They mate on the first or second day after emergence [25] and most likely mate only once in nature, although, polyandry has been recorded in small laboratory cages [26] and recently in wild *G. f. fuscipes* populations [27].

Tsetse flies demonstrate adenotrophic viviparity, embryo grows in the eggs and develop to third instar larvae in the uterus of the female where it is provided with nutrients [28]. During intra-uterine development, the larva feeds on a highly nourishing secretion from the milk gland. After development and moulting, third instar larvae are deposited onto soil, where it burrows into the ground and begins pupation (**Figure 2A**). The female has two ovaries, each of them comprising of two polytrophic ovarioles, which are continually at different development stages [25, 29] (**Figure 2B**). Females revive their reproductive cycle and produce full-grown larvae every 9-10 days, which then pupates. The adult fly emergence after a puparium period that varies according to temperature and ranges from 30 and 40 days at 24° C. Therefore, tsetse flies have a very slow reproduction and are closer to small mammals than most of the insects in their reproductive strategy. An insect with such a reproductive strategy are called *k* strategists [24], which differs from most insects which produce a large number of eggs and are termed *r* strategists. In *k* strategists like tsetse flies, the maternal care given to each larva enables better survival of each offspring than would be the case for *r* strategists. In laboratory conditions, a female tsetse can produce 10 offspring in its reproductive life. However, in nature, less than 10 offspring are estimated [25, 29].

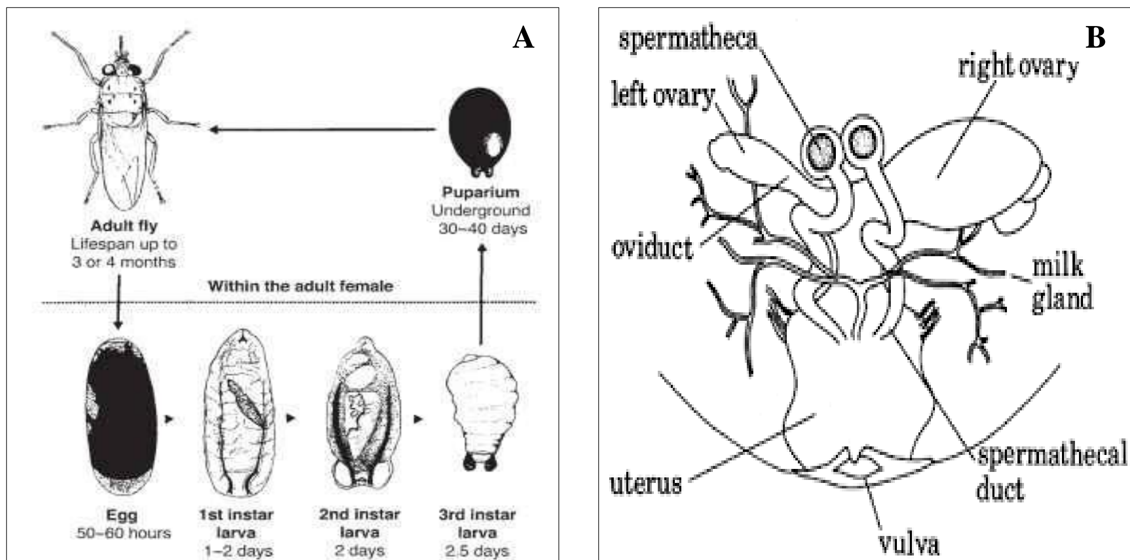

Figure 2. (A) Life cycle of the tsetse fly, (B) Viviparous reproduction cycle of tsetse flies. Female flies have ovaries connected to the uterus through oviducts. During the development of the uterus, the larva feeds on secretion from milk glands (www.fao.org).

After teneral young tsetse flies emerge from the puparia stage. The fly is considered "teneral" from emergence until its first blood meal. Due to a normal vertebrate blood diet, tsetse flies lack some necessary nutrients, and their development and maintenance depend on supplementation from associated endosymbionts [30]. These endosymbionts are required to complete construction of the tsetse reproductive system [31] and are vertically transmitted from mother to offspring [32].

1.3.3. The feeding process of tsetse flies

Both females and males feed on vertebrate blood, and feeding consists of three steps: host determination, landing, and feeding. Disruption of any of these steps could help to reduce trypanosome transmission and contributes to disease control. Completed feeding or success of feeding is defined when the abdomen of the flies become fully swollen with blood. Feeding achievement depends on the fat and haematin content of blood meals [33], ambient temperature, the presence of humans around the feeding areas [34] and other fly and host defenses [35].

Host determination is the first challenge and is achieved via a combination of odor and visual signals. Tsetse flies look for their host for roughly 25 min/day [36]. The method and length of seeking hosts vary depending on the geometry of habitat, species of the tsetse fly and sex [37]. Hosts can be identified by antennae at a distance of 60-120 m [38]. Three types of odor

attractants are important for tsetse flies [39] (i) odors are associated with animal breath (e.g. acetone [40], octanol, fluid mixtures in rumens [41] and carbon dioxide [42]; (ii) odor associated with urine (e.g. phenols) [43] and (iii) odor associated with skin secretions (e.g. sebum) [44]. Moreover, all these chemical compounds can be used to attract tsetse flies to traps [45, 46].

1.4. Biology of *Trypanosoma spp.*

1.4.1. Classification of trypanosomes

Trypanosomes are classified in the order *Kinetoplastida*, family Trypanosomatidae [18]. All Trypanosomes belong to the genus *Trypanosoma,* these parasites have been divided by the modes of transmission into two groups such as *Stercoraria* and *Salivaria* (**Table 1).** The first group **Stercoraria** causes American trypanosomosis in humans or "Chagas disease", and is transmitted from mammals, birds, reptiles, and amphibians to humans through the feces of the triatomine bug (*Rhodnius pallescens*) as the primary vector in South America [47]. The second group is Salivarian trypanosomes, which mainly infect vertebrates via the bite of insect vectors. The most important trypanosomes are *Trypanosoma congolense, T. vivax,* and *T. brucei,* each with a different lifecycle, infecting livestock and each contributing to AAT.

1.4.2. Lifecycle of African trypanosome

The lifecycle of African and American trypanosomes are entirely different. These *Trypanosoma* parasites start their lifecycle by first colonizing the midgut of tsetse host and then migrate to the salivary gland when they become ready to be transmitted to mammalian hosts by an infected tsetse bite (this stage is known as a metacyclic trypomastigote) (**Figure 3**)[48] (https://vimeo.com/200798320, courtesy of Jan Van Den Abbeele, ITM). When infected tsetse flies bite the host (human or animal), the metacyclic forms are injected into the body, and the parasite travels through the blood stream. Upon biting an infected person, the tsetse fly will also ingest trypomastigotes from the human/animal body, and the cycle can then perpetuate [49]. The lifecycle of each trypanosome is group-specific. Only six of *Glossina* species are a vector of trypanosomes. *Glossina. palpalis, G. tachinoides, G. fuscipes* and *G. m. morsitans* transmit *T. brucei gambiense,* the causative agent of Western African sleeping sickness. *G. swynertoni* and *G. pallidipes* transmit *T. brucei rhodesiense* which cause the Eastern African sleeping sickness, **[3]**.

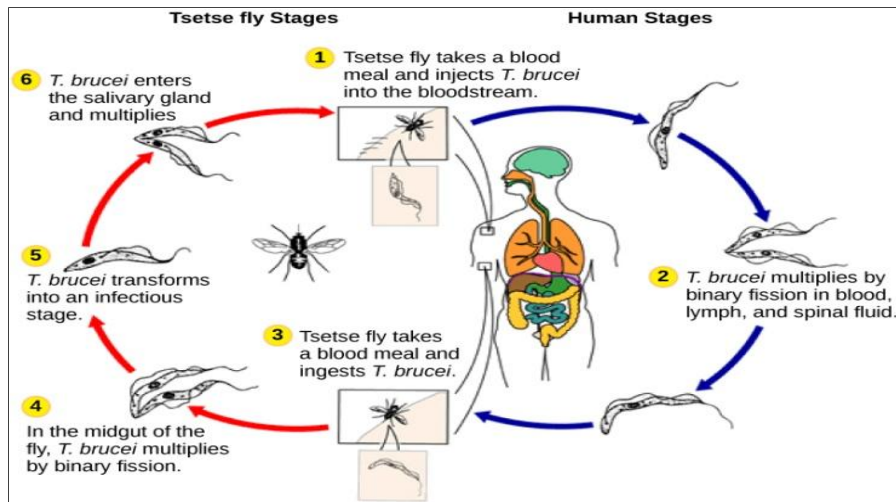

Figure 3. Life cycle of African trypanosomosis (*T. b. brucei*)
(https://www.cdc.gov/parasites/sleepingsickness/biology.html)

1.5. Current treatment strategies

African trypanosomosis has significant effects on the agricultural economies of sub-Saharan countries with overwhelming influence on nutrition and public health. African trypanosomoses are challenging to control, and so far only a few anti-trypanosome drugs are available for treatment. However, the drugs are costly [50], and the developments of new ones are exceptionally slow [51]. Currently, the available trypanocidal drugs are, pentamidine, suramin, melarsoprol, eflornithine, respectively [52, 53]. A primary challenge of these drugs is their toxicity triggers severe side effects and can cause death [50]. They can also enhance the drug resistance of the parasite [53]. Thus, less toxic, cheap and more effective drugs are needed. Control of disease vectors is, therefore, highly important and is probably the most sustainable control method.

1.6. Control of Tsetse Flies

Tsetse flies are suitable for eradication/and or suppression due to their low reproduction (*k* -strategists) and dissemination rates in comparison with other insects [24]. In addition, they have decreased genetic variability, and their habitat restrictions reduce their capacity to exploit and adapt themselves to different control methods [54]

Vector control is the most applicable and powerful approach to disease control [22, 24, 55]. Primary tsetse control methods based on the removal of vegetation or destroying of host game animals were very efficient. However, these control methods caused adverse and unacceptable

effects on the environment [56, 57]. Either from the ground or air, residual insecticides (e.g., DDT, dieldrin, endosulfan) sprayed to control tsetse flies caused several undesirable effects to fragile ecosystems and currently are not acceptable due to an increased awareness of health and environment concerns [58]. To reduce the adverse effects on the environment and nontarget organisms, several other control methods have been used to control tsetse population such as the sequential aerosol technique (SAT), traps and insecticide-impregnated targets [59], and live bait technologies [60]. Each of these methods has succeeded in decreasing the local tsetse populations [24], but each has their limitations such as (i) they do not protect cleared areas from the reinvasion by tsetse flies from neighboring territories [61], (ii) the methods depend on the availability of funds which means that methods are not sustainable [62]. Therefore, there is a need to explore other advanced techniques to control tsetse flies and manage the disease of trypanosomiasis like the Sterile Insect Technique (SIT).

1.6.1. Sterile Insect Technique

The Sterile Insect Technique (SIT) is a vector control method that has been successfully used against different species of tsetse flies.The technique is based on the production of large numbers of the sterile males, [63, 64], and the release of them to compete with wild males in mating with wild virgin females in order to suppress the targeted population [65, 66] (**Figure 4**). SIT is environmentally friendly and effective when it is applied in the framework of an area-wide integrated pest management control (AW-IPM) [65]. The continuous release of sterile males reduces the size of the target vector population and finally break it down [66].

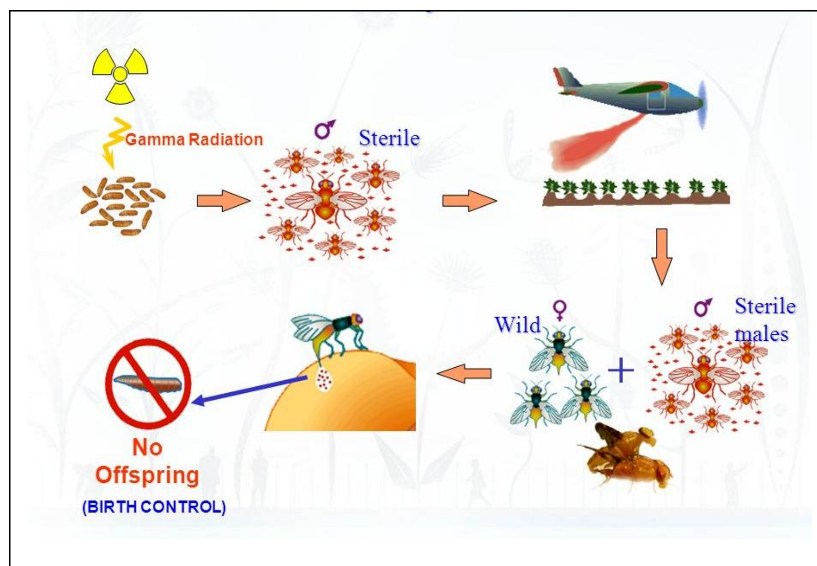

Figure 4. Sterile Insect Technique (https://www.slideshare.net/Shanura/nuclear-techniques-in-food-and-agriculture)

SIT has no significant antagonistic effect on non-target organisms and the environment, and it is species-specific [67]. Over time, taking into consideration the cost of negative side effect on the environment and human health, SIT appears more economic than the use of chemical insecticides. Insecticides are only efficient when the size of the target population is low, meaning that information about the biology and ecology of the target insect needs to be studied thoroughly [68]. The SIT method can be successfully used in combination with other control tactics such as parasitoids, predators, and pathogens to suppress targeted populations of economic interest [67].

The SIT was successfully implemented on the island of Unguja (Zanzibar) against *G. austeni*, and resulted in sustainable eradication. The island has been *G. austeni* free since 1997. The first full-scale SIT implementation was against *G. m. morsitans* species in Tanzania from 1977 to 1979 [69-71], *G. p. gambiensis* and *G. tachinoides* in the Sideradougou are in Burkina Faso and *G. p. palpalis* in the Lafia area of Nigeria [72, 73]. However, the programs in Burkina Faso and Nigeria were not implemented according to AW-IPM rules and tsetse cleared area was re-invaded by tsetse flies. Another SIT program in the frame of AW-IPM was started in 1997 to eradicate the *G. pallidipes* and *G. f. fuscipes* species in the Ethiopian Southern Rift Valley region [74].

In general, tsetse SIT campaigns face various obstacles including the mass production of sterile flies which could mainly be due to (i) the need for adaptations of the target insect species to mass rearing conditions [75], pathogenic infections that cause decreased colony productivity [76], (ii) compromised performance of the mass reared flies (e.g. feeding behavior, flight ability, survival, etc.) due to the mass rearing conditions (i.e. crowding, loss of important gut microbiota) and impact of sterilization, handling which affect the flies performance after release into the field [77], (iii) the high costs due to the low production rate and the hematophagous feeding behaviour and lack of efficient sex separation tools [78] and (iv) the ability of tsetse sterile males to transmit disease in the program area.

Quality of the released insects is one of the most important parameters of SIT as the sterile males must be as fast and competitive as their wild counterparts for mating [68]. Female tsetse flies use to maintain the tsetse colonies in mass rearing facilities, and males use for release during the SIT. Mass-rearing of tsetse colonies revealed that *G. pallidipes* colonies are susceptible to a virus infection that causes salivary gland hypertrophy syndrome (SGH) and cause a reduction in the fitness, fertility, and fecundity of the flies, leading to a decline of the

colonies within a few generations [79]. In this thesis, the virus impact was investigated on several tsetse species. Virus interaction with tsetse symbionts was also investigated.

Following the success of the SIT programme in Zanzibar, the African Union took the initiative of The Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) to eradicate tsetse and trypanosomiasis. The IAEA governor board also took the strategic resolution to support the PATTEC objectives. To date, SIT programs have been implemented in areas without human sleeping sickness. However, future projects could contain HAT endemic areas. In such projects, it would be unacceptable to release sterile males capable of transmitting the parasite to humans. Therefore, attempts to produce tsetse strains refractory to trypanosome infection to be used for SIT would be welcomed. To achieve this goal, a thorough understanding of the underlying mechanisms involved in vector competence is required.

1.7. Insect Symbiosis

Symbiosis (Greek *σύν* = together and *βίωσις* = living) is a well-known phenomenon in nature and extends over all domains of life. Symbiosis is '*the living together of unlike organisms*' according to the German microbiologist and mycologist Heinrich Anton de Bary (1879). Symbiosis is unique and has significant consequences in evolution and biodiversity. Insects have established both endo- and ectosymbiosis. Symbiotic microorganisms influence the different parts of their insect's physiology, including their development, nutrition, reproduction, speciation defense against natural enemies and host preferences [80].

Insect symbiotic associations can be divided into at least three categories and classified according to the type of interaction, *i.e.*, mutualism, commensalism, and parasitism [81]. Mutualism is both host and symbiont reciprocally provide an advantageous relationship to each other, while in the case of a commensal relationship, symbionts only use the host without any benefit or damage [81]. Finally, in parasitic relationships, the host is negatively affected by the parasite (parasitism), and the parasite may manipulate the reproductive properties of their hosts, inducing phenomena such as parthenogenesis, feminization, male killing and cytoplasmic incompatibility (CI) [82].

Symbiotic relationships comprise two forms; ectosymbiosis, where one organism lives externally on another one and endosymbiosis, is one organisms lives inside another one, i.e., mitochondria and chloroplasts have endosymbiotic relationships, even though both organelles

originated from bacterial endosymbionts [83]. Insect and bacterial endosymbionts live together, and this relationship can be obligate or facultative. In an obligate endosymbiotic relationship, the endosymbiont's presence is necessary for host survival. On the contrary, facultative endosymbionts are not essential for survival, although they might be still beneficial to the host [84].

1.7.1. Tsetse symbiosis

Tsetse flies are dependent on their microbial flora for providing nutrients that are not present in their restricted blood diet. They harbor three distinct, maternally-transmitted bacterial endosymbionts, *Wigglesworthia glossinidia* [85], *Sodalis glossinidius* and *Wolbachia pipienti*s [86] (**Figure 6**). It was shown that the symbiont association in tsetse also affects many aspects of host physiology [87] Recently, a fourth facultative endosymbiotic bacteria, *Spiroplasma, was* found in some tsetse species.

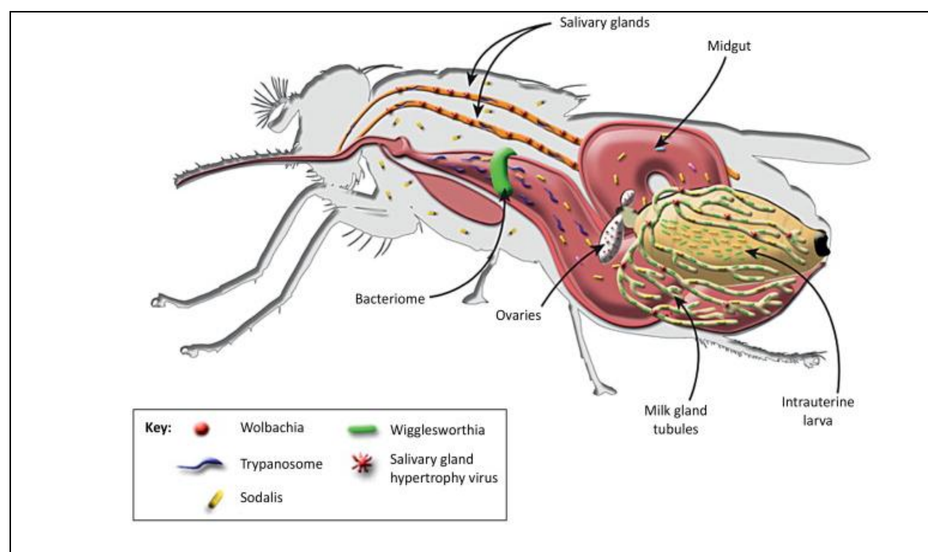

Figure 6. Tsetse microbiota and pathogens [88]

1.7.1.1. The primary symbiont *Wigglesworthia glossinidius*

Wigglesworthia (γ-proteobacteria) is an obligatory primary (P) endosymbiont in tsetse host. The association between *Wigglesworthia* and its host is ancient (50- 80 million years), It is vertically transmitted from one generation to the next [31, 89]. This symbiont is strictly resided in a specialized tsetse host organ called the bacteriome and is inherited from female to offspring through the milk gland during larval development [90, 91]. It has two key functional roles in *Glossina* species. Firstly, the proteobacteria provide all essential vitamins and supplementation of nutrients- lacking in the blood diet of tsetse flies [92]. Female fecundity

and larval development is sustained through certain metabolites found in *Wigglesworthia* [86]. Secondly, *Wigglesworthia* is necessary for the immune system to function correctly in adults during the development of immature progeny [93] and the flies are more susceptible to trypanosome infection [94].

1.7.1.2. The secondary symbiont *Sodalis glossinidius*

The commensal γ-proteobacterium *Sodalis glossinidius* is harbored in all tsetse flies in the insect colonies but is heterogeneous in natural populations [95]. It was first identified as a rickettsia-like organism (RLO) [96] and is a member of the *Enterobacteriaceae* family [31]. *Sodalis* is closely related to the weevil primary symbiont *Sitophilus oryzae* [97] and considered as secondary (S) symbiont of *Glossina spp.* This bacterium is polytrophic and is found intra- and extracellularly in the tsetse gut, hemolymph and salivary glands, milk glands and uterus. It is also transmitted into the tsetse's intrauterine larva in mother's milk secretions [90, 98]. Unlike *Wigglesworthia*, *Sodalis* from different tsetse species are closely related, thus indicating this bacterium's recent association with its tsetse host. *Sodalis* exhibits genotypic traits similar to those found in several free-living microbes and can be cultured outside of tsetse [90, 99, 100]. Furthermore, *Sodalis* co-habit the tsetse gut along with pathogenic trypanosomes and is amenable to genetic manipulation. So far, there is no clear information about the functional role of *Sodalis* for the tsetse host. However, it has been suggested that the susceptibility to trypanosomes may increase with the increasing density of *Sodalis* in the fly's gut [101-103]. Also, recent data suggest that the tsetse fly midgut's microbiota (*Sodalis* and *Wigglesworthia*) can also modulate trypanosome development and host longevity [104]. These characteristics make *Sodalis* an ideal candidate for use in tsetse paratransgenesis.

1.7.1.3. The third symbiont *Wolbachia*

The third symbiont of tsetse is *Wolbachia* which is an obligatory γ-proteobacterium that infect up to 40 % of arthropods [105-107] including insects, terrestrial crustaceans, spiders, scorpions and springtails, and filarial nematodes species [82]. *Wolbachia* prevalence in natural populations is variable, i.e., not all natural populations are infected with it [108]. *Wolbachia* is intracellular and mainly transmitted maternally to offspring through the egg cytoplasm [89]. *Wolbachia* establishes both somatic and gonadal infections and can manipulate many aspects of the biology, physiology, ecology, and evolution of their hosts [82, 109].

Wolbachia causes some reproductive phenotypes in many arthropods, such as cytoplasmic incompatibility (CI), male killing, feminization, and parthenogenesis [82, 109]. Furthermore, as part of their co-evolutionary physiological interrelations at the cellular and metabolic levels, it can also manipulate host fitness and fecundity, immunity and longevity, development and even sexual behavior. It has been a point of interest to survey the potential capacity of natural *Wolbachia* infections to functionally interfere with trypanosome [110]. Therefore, investigating the potential interaction between *Wolbachia* infections and trypanosomes is important. In addition, there is evidence of chromosomal symbiont-insertions in some tsetse species [111-114]. Furthermore, the presence of *Wolbachia* in some insect species may provide antiviral protection to infections with and transmission of certain pathogens such as *Chikungunya*, *Plosmodium*, *Dengue, malaria,* and *filariasis* [115, 116]. Therefore, *Wolbachia* attracts an applied-research interest as a novel biocontrol agent for arthropod pests and vectors such as mosquito-transmitted malaria or even tsetse fly-transmitted trypanosomosis.

1.7.1.4. The fourth tsetse symbiont *Spiroplasma*

Spiroplasma, which are recently identified symbionts, are wall-less, motile, helical, gram-positive bacteria that associate both endocellular and extracellularly with a variety of arthropods, (particularly insects) and are a fourth tsetse bacterial symbiont in *G. f. fuscipes* and *G. tachinoides* (palpalis sub-group) [117]. This bacterium has also been recently identified as a member of the bacteriome of *G. p. gambiensis* flies from sleeping sickness foci in Cameroon [118]. *Spiroplasma* bacteria cause female-biased sex ratios of their hosts including *Drosophila* flies, ladybird beetles, and butterflies and connected with reproductive changes such as male killing [119, 120].

1.8. Tsetse Pathogen-*Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV)

Salivary Gland Hypertrophy (SGH) was first observed in *G. pallidipes* in the 1930s [121]. SGH was associated with a virus-like particles detected in the cytoplasmic vacuoles of the salivary glands and midgut epithelial cells of *G. m. morsitans* and *G. f. fuscipes* species [122-126]. The clear association between the SGH and "virus-like particles" and the demonstration of the virus as the causative agent for SGH was reported by Jaenson [127].

Laboratory tsetse flies and a number of natural populations carry a nuclear shaped double-stranded DNA (dsDNA) virus, *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHVs) [128]. GpSGHV replicates in salivary glands cells. The viral infection in most cases are asymptomatic but in certain cases can be symptomatic with SGH and can transmit vertically from females to offspring either via milk glands or transovum [129, 130]. If the infection is symptomatic, swelling of the salivary glands (SGs) and SG hypertrophy (SGH) syndrome are observed. GpSGHV can also be transmitted horizontally during blood feeding on an *in vitro* membrane [131, 132]. The virus is associated with testicular degeneration and ovarian abnormalities [129, 130, 133] and influence survival, fertility and fecundity of naturally and experimentally infected flies [134]. GpSGHV induces various pathologies in different tsetse species (Abd-Alla et al., 2016) and was involved in *G. pallidipes* colony collapses in Seibersdorf Austria in 1978 and 2002 [76]. SIT requires the production of large tsetse colonies. Thus, understanding the virus impact on tsetse species and their fecundity is crucial. Although positive correlations between virus copy number and symptomatic infection have been found, other unknown parameters such as genetics, and interaction with tsetse microbiota cannot be excluded. Understanding GpSGHV interaction with tsetse microbiota is crucial not only for *G. pallidipes* but also on other tsetse species.

1.9. Tsetse paratransgenesis

Recently, possible management of the trypanosomosis disease by genetically altering the symbionts in tsetse and causing them to produce anti-trypanosomal factors which can affect the establishment of midgut trypanosomal infections or trypanosome differentiations was reported [135, 136] [93, 137]. Cultured midgut symbionts *Sodalis* of *G. m. morsitans* can be genetically transformed to express desirable genes [138]. After introducing the transformed *Sodalis* into tsetse, it is assumed that they can promote the natural spread of the engineered refractory fly phenotypes [106, 138].

1.10. Effects of radiation on symbiont and pathogens

Tsetse males are sterilized through the exposure to radiation doses and used for release in target areas of the SIT program. Radiation treatment affects the bacterial community associated with tsetse males which might has some side effects on male performance. Therefore, irradiation might also influence the tsetse fly's physiology and vectorial competence. Understanding the effect of radiation on tsetse and their symbionts might help us in designing methods that might increase the efficiency of SIT. Paratransgenesis is a

promising approach to produce tsetse males strain refractory to trypanosome infection through modifying one of the associated symbiont. Using tsetse males that are refractory to trypanosome infection in SIT would reduce or eliminate the risk associated with the release of males in a tsetse SIT program. To this end, the impact of radiation treatment on the modified symbiont needs to be studied.

Aim of the Thesis

The work presented in this PhD thesis was performed at the Insect Pest Control Laboratory at the Joint FAO/IAEA Divisions of Nuclear Techniques in Food and Agriculture in Seibersdorf (Vienna, Austria) under the supervision of Prof Dr. Adly M. M. Abdalla and Prof Dr. Robert L. Mach from Vienna University of Technology.

The aim of this work is (i) to develop new molecular identification tools to distinguish tsetse species, (ii) improve tsetse mass rearing through research on symbionts and pathogens and (iii) to investigate the impact of radiation on tsetse, tsetse symbionts, and males vectorial capacity.

The thesis is a cumulative work and consist of eight chapters. The introduction gives an outline of current status and description about tsetse, trypanosomiasis, tsetse symbionts and pathogens, gamma irradiation, mating behavior and vector control strategies (Chapter 1). The thesis is the combination of several research publication as a body of the work and presented in six chapters. The final part is the General Discussion and Conclusion (Chapter 8) which provides a synopsis on the extent to which different research objectives explained in this thesis were accomplished, highlights of the original studies and future hypothesis in an attempt to explain some of the important features of symbiont and pathogenic relations of tsetse flies.

The overall objective of this thesis was to enhance and improve the tsetse suppress/eradicate program to control tsetse and trypanosomes by a combined approach of paratransgenesis-SIT. The first aim was to accurately identify the target tsetse species for SIT using molecular tools instead of morphometric tools. This was conducted by evaluating different molecular tools that can be applied for species delimination of different *Glossina* species (**Publication #1-Chapter 2**). The second aim was an investigation of the prevalence and coinfection dynamics between *Wolbachia*, trypanosomes, and SGHV in four tsetse species (*Glossina palpalis gambiensis*, *G. tachinoides*, *G. morsitans submorsitans*, and *G. medicorum*) that were collected from 46 geographical locations in Burkina Faso, Mali, Ghana, Guinea, and Senegal (West Africa) between 2008 and 2015 (**Publication #2- Chapter 3**). The third aim was to asses the susceptibility of six Glossina species (*G. pallidipes, G.brevipalpis, G. m. morsitans, G. m. centralis, G. f. fuscipes* and *G. p. gambiensis*) to SGHV infections species (**Publication #3-Chapter 4**). The fourth aim was to evaluate in much more detail the impact of GpSGHV

on the performance and quality control parameters *G. f. fuscipes* (**Publication #4- Chapter 5**). The fifth aim was to investigate the feasibility of combining the paratransgenesis approach with SIT by analyzing the impact of ionizing radiation on the density of *Sodalis* and the vectorial capacity of sterilized tsetse males (**Publication # 5- Chapter 6**). The sixth aim was to investigate whether disruption of the microbiota through antibiotic or irradiation treatment affects cuticular hydrocarbon profiles, and possibly mate choice behavior in the tsetse fly, *Glossina morsitans morsitans* (**Publication# 6- Chapter 7**). All of the performed experiments intended to improve current mass production of sterile males, release and reduce the risk associated with the SIT program in sub-Saharan Africa.

Chapter 2

A nuclear, mitochondrial and *Wolbachia*-based approach for the rapid and accurate identification of tsetse species

A nuclear, mitochondrial and *Wolbachia*-based approach for the rapid and accurate identification of tsetse species

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Abstract

Tsetse flies (Diptera: Glossinidae) are solely responsible for the transmission of African trypanosomes, causative agents of sleeping sickness in humans and nagana in livestock. Due to the lack of efficient vaccines and the emergence of drug resistance, vector control approaches, such as the sterile insect technique (SIT) as a component of integrated pest management strategies, remain the most effective way for controlling the disease. SIT is a species-specific approach and therefore requires accurate species of natural populations of the target pest species. However, the presence of morphologically similar species (species complexes and sub-species) in tsetse flies challenges the development and successful implementation of SIT-based population control. In this study, we evaluated different molecular tools that can be applied for the delimitation of different *Glossina* species using tsetse samples derived from laboratory colonies, natural populations and museum specimens. The combined use of mitochondrial markers, nuclear markers (including internal transcribed spacer 1 (ITS1) and different microsatellites), and bacterial symbiotic markers (*Wolbachia* infection status), as well as relatively inexpensive techniques such as PCR, agarose gel electrophoresis, and, to some extent, sequencing, provided a rapid, cost effective, and accurate identification of several tsetse species. The effectiveness of SIT benefits from the fine resolution of species limits in nature. The present study supports the quick identification of large samples using simple and cost effective universalized protocols, which can be easily applied by countries/laboratories with limited resources and expertise.

Introduction

Tsetse flies are responsible for the cyclic transmission of trypanosomes, causative agents of sleeping sickness or human African trypanosomosis (HAT) in humans and nagana or African animal trypanosomosis (AAT) in livestock [1,2]. There are about 31 tsetse fly species and sub-species in *Glossina* genus (Diptera: Glossinidae), distributed in 37 sub-Saharan African countries. However, only 8-10 of these species are of economic importance [3].

Due to the lack of vaccines against trypanosomes and increasing resistance of the AAT parasites to available drugs [4,5], vector control remains the most effective way of managing African trypanosomosis [6]. Some of the vector control strategies that have been applied for the control of trypanosomosis include the use of sequential aerosol technique (SAT) [7], stationery attractive devices, live bait technique and sterile insect technique (SIT) [8–10]. The SIT involves production of the target insect species in large number in specialized rearing facilities followed by sterilization of the males by irradiation [11]. The sustained and systematic release of the sterile males over the target area in large numbers out-competes the wild male population for mating with wild females. Mating of mass -produced sterile males with wild females leads to infertile no offspring and subsequent decrease of the targeted population [12]. SIT is a species-specific and environmental friendly control method that has been successfully applied for the eradication of a population of *Glossina austeni* from Unguja Island in Zanzibar [13].

The correct species identification is of critical importance for successful SIT applications. Several methods have been applied to identify tsetse species, including morphological characters such as external genitalia of males, their habitat requirements and host preference [10]. Based on these characters, the *Glossina* species are divided into three distinct taxonomic groups: *morsitans*, *palpalis* and fusca [14]. However, delimitation of closely related species and/or subspecies remains challenging.

In addition to morphological taxonomic identification of *Glossina* species, molecular and genetic markers have also been used in the last decades. Nuclear markers, such as ITS1 and ITS2, were reported to distinguish some of the species based on the size and/or specificity of the amplicons, as revealed by both agarose gel electrophoresis and sequencing [15–18]. Microsatellite markers have also been developed for different *Glossina* species and have provided encouraging results regarding their potential use in phylogenetic analysis and species identification [19–23]. Mitochondrial markers, including cytochrome oxidase 1 (COI),

cytochrome oxidase 2 (COII), cytochrome b (CYTB), 16S rRNA, and NADH dehydrogenase 2 (ND2), have also been implemented for the phylogenetic analysis of *Glossina* species, based on DNA sequencing [15–17,24–27]. The availability of polytene chromosomes in *Glossina* and the development of polytene chromosome maps provide additional genetic tools that can shed light on specific chromosomal banding pattern changes and / or rearrangements that could provide diagnostic characters for species identification [28–31].

A previously neglected parameter regarding speciation is the development of intimate relationships of the tsetse fly with bacterial symbionts, such as *Wigglesworthia*, *Sodalis,* and *Wolbachia*, that may alter the host's behavior [32–35]. *Wolbachia* is obligatory intracellular and maternally transmitted and is known to cause reproductive alterations and cytoplasmic incompatibility (CI) [36]. CI is mainly expressed as embryonic mortality when an infected male mates with an uninfected female (unidirectional CI) [37] or when the male and female crossed harbor different and mutually incompatible *Wolbachia* strains (bidirectional CI) [38]. Such incompatibilities lead to restriction of gene flow among natural populations and can be both 'accelerators' and diagnostic markers of speciation [39]. Another aspect of symbiosis that could be exploited is the presence of ancient, species-specific, horizontal gene transfer events in the host's chromosomal DNA. Such events have been demonstrated in *Glossina*, through the presence of fixed chromosomal introgressions of *Wolbachia* only in *Glossina morsitans morsitans* up to now and can provide additional diagnostic markers [40,41].

Regarding the delimitation of closely related species and given that speciation can be driven through different or combined forces, integrative taxonomy suggests the utilization of multidisciplinary approaches for the inference of robust conclusions regarding species limits and phylogenetic relationships [42–46]. The utilization of a single marker, or a single class of tightly linked markers (such as mitochondrial genes), although easy to universally apply, is not expected to provide beyond doubt species identification [47,48]. The fact that the phylogenetic signal of mitochondrial markers can be masked or altered by the presence of reproductive symbionts, such as *Wolbachia* (through, for example, mitochondrial sweeps) and the limitation that mitochondrial markers are unable to identify hybrids among closely related species (important in hybridizing zones of closely related species) also points to the need for 'the more, the better' approaches in species delimitation [49]. Previous studies also in tsetse flies have documented that different classes of markers may provide either a differential depth of analysis or even contradicting results [15,17,50].

Besides robustness, it is critical to develop diagnostic tools that can be applied quickly, easily, massively and cost effectively. This can be done by integrating different classes of markers and by utilizing different resolution techniques, such as gel electrophoresis and sequencing. Such integrated approaches allow the screening of many samples and many individuals per sample with reduced cost in a relatively short time and without the need of highly specialized equipment/skills.

Here we report the evaluation of different classes of molecular markers (nuclear ITS1, nuclear microsatellites, mitochondrial genes, and the *Wolbachia* infection status) for the identification of tsetse taxa. We evaluated these tools against tsetse laboratory colonies that were used as reference material. At the same time, we tried to correlate our data with previously published sequences and data from tsetse museum specimens and, finally, we evaluated the discriminative power of ITS 1 amplicon electrophoresis through the genotyping of an extended collection of samples derived from nature. Based on our findings, we recommend a set of markers and analytical approaches that can quickly and cost effectively support the morphometric taxonomy or even stand alone to identify *Glossina* species.

Materials and Methods

Laboratory colonies

Glossina species maintained at the Insect Pest control Laboratory (IPCL) of the Joint FAO/IAEA Programme of Nuclear Applications in Food and Agriculture (NAFA) were used in this analysis. The species were *G. pallidipes, G. morsitans morsitans, G. morsitans centralis, G. palpalis gambiensis, G. fuscipes fuscipes,* and *G. brevipalpis*. Identification of the samples to species was based on standard morphological characters [14]. As morphological characters are not reliable for subspecific identification the subspecific laboratory colonies were assigned based on the conventional designation for the place of origin. Details of the *Glossina* species and colonies used in this study are provided in **Table 1.** All the tsetse colonies are fed on heated, defribrinated bovine blood for 10-15 min, three days per week using an *in vitro* membrane feeding technique [51].

Museum specimens

Glossina specimens were obtained from Mr Nigel P. Wyatt, Department of Entomology, Natural History Museum, London, UK (loan no. 2011-159) and comprised of representatives of the following *Glossina* taxa: *G. morsitans morsitans, G. morsitans centralis,* and *G. palpalis gambiensis*. These specimens were collected between 1915 and 1952 and were assigned to the respective taxa based on morphological characters (**Table 1**).

Natural populations

A total of 2634 individual tsetse flies, representing 30 taxon/geographical locations combinations from five countries in West Africa (Burkina Faso, Ghana Guinea, Mali, and Senegal), were included in this analysis. These samples were collected during different periods between 1994 and 2014 (**Table 1**) and were used as a ‘blind test’ to verify their species status using the ITS1 PCR amplicons, plus the *Wolbachia* infection, where necessary/applicable.

Table 1: *Glossina* samples used in this study

***Glossina* species**	**Origin**	**Original collection date**	**Details**	**Type[1]**	**No**
G. pallidipes	Uganda (Tororo)	1975	1978 IPCL (from Institute of Experimental Entomology, Amsterdam, The Netherlands)	L	8
	Ethiopia (Arba Minch)	1997-2001	2005 IPCL (Arba Minch colony)	L	8
G. m. morsitans	Zimbabwe	1968	1972 IPCL (from Bristol laboratory colony)	L	8
G. m. centralis	Tanzania	N/A	1999 IPCL	L	8
G. p. gambiensis	Burkina Faso	1972	2005 IPCL (from CIRDES laboratory colony)	L	8
	Senegal (Pout)	2009	2009 IPCL	L	8
G. f. fuscipes	Central Africa Republic	1986	2009 IPCL	L	8
G. brevipalpis	Kenya (Shimba hills)	1987	2002 IPCL	L	8
G. tachinoides	Burkina Faso	N/A	CIRDES	L	12
G. m. submorsitans	Burkina Faso	N/A	CIRDES	L	12
Total					**88**
G. m. morsitans	Tanganyika Terr (Morogoro, Uluguru)	1915	Dr. A. G. Wilkins	M	1
	Tanganyika (Korogwed Handeni)	1952	16-IX-52 Brit. Mus. 1959-638 Dr. E. Burtt	M	1
	Tanganyika Terr: (Morogoro, Uluguru)	1921	Dr. A.G. Wilkins Pres. by Imp. Bur. Ent. Brit. Mus. 1921-152.	M	2
G. m. centralis	Tanganyika Terr.	1923	Brit. Mus. 1923-269	M	1
	Sedamara (Mbulu)	1950	26.9.50 London School of Hygiene & Tropical Medicine coll. BMNH	M	1
G. p. gambiensis	Sierra Leone (Scarcies, Kambia)	1946	Nash & Walton, 26/1/46	M	1
Total					**7**
G. pallidipes	Ethiopia (Arba Minch)	2014		F	30
	Uganda (Lukoma – Bavuma)	2013		F	27

Glossina species	Origin	Original collection date	Details	Type[1]	No
	Kenya (BioRI-KALRO)	2008		F	3
	Zambia (Mfuwe)	2007		F	3
	Zimbabwe (Ruckomechi)	2006		F	3
	Zimbabwe (Makuti)	2006		F	1
	Tanzania (Tanga)	2005		F	2
G. m. morsitans	Zambia (Mfuwe)	2007		F	1
	Zimbabwe (Ruckomechi)	2006		F	1
	Zimbabwe (Makuti)	2006		F	1
	Tanzania (Usinge)	2013		F	9
	Kenya (BioRI-KALRO)	2008		F	1
G. m. centralis	Angola (Guissakina)	2013		F	25
	Tanzania (Ugalla)	2013		F	60
*G. m. submorsitans**	Burkina Faso (Comoe)	2009		F	277
*G. p. gambiensis**	Senegal (Sebikotane)	2009		F	3
	Senegal (Sebikotane)	2013		F	9
	Senegal (Kayar)	2010		F	3
	Senegal (Kayar)	2013		F	17
	Senegal (Niokolo-Koba)	2012		F	3
	Senegal (Niokolo-Koba)	2013		F	30
	Senegal (Pout)	2009		F	11
	Senegal (Pout)	2013		F	30
	Burkina Faso (Comoe)	2008		F	1152
	Mali	2010		F	8
	Guinea	2010		F	1
G. f. quanzensis	Angola (Guissakina)	2013		F	3
	Uganda	2013		F	52
G. brevipalpis	Mozambique (Maputo GR)	2013		F	6
*G. swynnertoni**	Tanzania (Ikorongo GR)	2015		F	24

Glossina species	Origin	Original collection date	Details	Type[1]	No
G. medicorum	Burkina Faso (Comoe)	2009		F	86
*G. tachinoides**	Burkina Faso (Comoe)	2009		F	792
	Ghana	2009		F	7
G. austeni	Mozambique (Maputo G)	2013		F	7
	Tanzania (Jozani)	1994		F	1
	Zanzibar (Unguja island)	1995		F	5
	South Africa (Zululand)			F	1
Total					**2695**

[1]Type: L = Laboratory colony; M = Museum specimen; F = Field collection

N: Number of individuals tested

N/A = not available; CIRDES = Centre International de Recherche-Développement sur l'Elevage en zone Subhumide, Bobo Dioulasso, Burkina Faso; IPCL = Insect Pest Control Laboratory

*these collections included false assigned individuals (see also **Table 4**)

DNA extraction, PCR, and sequencing

Flies derived from laboratory colonies and natural populations

DNA from teneral adult flies of each laboratory colony was isolated using the Qiagen DNeasy kit (Qiagen, Valencia, CA), following the manufacturer's instructions. DNA samples were stored at 4 ^{o}C until their use and at -20 ^{o}C for long term. Samples collected from the field were sorted by species, labelled, kept in 95% ethanol (or propylene-1,2-diol), and shipped to the IPCL for downstream analysis. DNA extraction was performed as described for the laboratory colonies. For all PCR amplifications, 1.1X pre-aliquoted PCR master mix was used (ABgene, UK). In 22.5 µl of the mix, 1.5 µl of DNA template and 1µl of forward and reverse primer were added (10µM each). Nuclear (ITS1 and microsatellite), mitochondrial (*COI*, 16S *rRNA,* and 12S *rRNA*), and symbiotic markers (*Wolbachia* 16S *rRNA* gene) that were used in the present study are shown in **Table 2**. PCR conditions to amplify COI, 16S rRNA and ITS1 genes were as described previously [16]. Primers 12SCFR and 12SCRR were used to amplify a 377 bp fragment of the *12S rRNA* mitochondrial gene, as described in previous publications [52]. PCR conditions to detect the presence of cytoplasmic or nuclear *Wolbachia* 16S rRNA were as described previously using the *Wolbachia* specific primers wspecF and wspecR [52]. PCR conditions for the different sets of microsatellite markers were as described in the respective publications [16,19,21,22,53,54]. PCR products were analysed on 1.5% agarose gels by electrophoresis and visualized using ethidium bromide. Amplicons of the mitochondrial genes were purified using QIAquick PCR kit (Qiagen Valencia, CA) and sequenced by MWG (MWG-Biotech AG, Germany). Forward and reverse sequences with good quality read were assembled and aligned using SeqMan Pro software (Lasergene 7.0, Dnastar Inc). The consensus sequences for each gene were aligned and trimmed using the ClustalW algorithm in MEGA version 6.0.

Table 2: A list of the molecular markers and primers used in this study

Molecular marker	Marker		Primer name	Primer sequence 5'-3'	Reference	Method of analysis
Nuclear markers	ITS1		*Glossina*ITS1_for	GTGATCCACCGCTTAGAGTGA	(Dyer *et al.*, 2008)	Gel electrophoresis
			*Glossina*ITS1_rev	GCAAAAGTTGACCGAACTTGA		
	Microsatellite markers	A10	A10 F	GCAACGCCAAGTGAAATAAAG		
			A10 R	TACTGGGCTCGCGTACATAAT		
		Gmm14	Gmm14 F	CACACCCTGGATTACAAA	(Baker & Krafsur, 2001)	
			Gmm14 R	TGAAATGCAACCCTTCTT		
Mitochondrial markers	COI		COI	TTGATTTTTTGGTCATCCAGAAGT	(Simon *et al.*, 1994)	Sequencing
			CULR	TGAAGCTTAAATTCATTGCACTAATC		
	16S rRNA		NI-J-12585	GGTCCCTTACGAATTTGAATATATCCT		
			LR-N-12866	ACATGATCTGAGTTCAAACCGG		
	12S rRNA		12SCFR	GAGAGTGACGGGCGATATGT	(Doudoumis *et al.*, 2012)	
			12SCRR	AAACCAGGATTAGATACCCTATTAT		
Symbiotic markers	Wolbachia	16S rRNA	WspecF	YATACCTATTCGAAGGGATAG		Gel electrophoresis
			WspecR	AGCTTCGAGTGAAACCAATTC		

Museum specimens

Before DNA extraction, *Glossina* specimens were surface-sterilized by immersing in 80% ethanol and then rinsed with sterile PBS twice. DNA was extracted using Nucleospin Tissue Kit (Macheray-Nagel) following the manufacturer's instructions. DNA integrity was assayed by amplifying part of the mitochondrial 12S *rRNA* gene as described above. DNA samples were stored at 4 ^{o}C until their use and at -20 ^{o}C for long term PCR amplifications were performed in reactions containing 10 ng DNA, 10 pmol of each primer, 0.5 units KAPA Taq (KAPA Biosystems), 1x KAPA buffer A (KAPA Biosystems), 0.25 mM deoxynucleotide triphosphate mixture (dNTPs) and water to a final volume of 20 µl. Amplification was performed in a PTC-200 Thermal Cycler (MJ Research), using the following cycling conditions: 95°C for 5 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 54 °C, 1 min at 72 °C and a final extension of 10 min at 72 °C. PCR reactions were electrophoresed on a 1.5% agarose gel. Negative samples were reamplified by PCR using 2 µl of the first PCR reaction as template and the same set of primers and conditions for 35 cycles. Positive samples of the first or the second PCR reaction were further analyzed by double stranded sequencing with both forward and reverse primers. A dye terminator-labelled cycle sequencing reaction was conducted with the BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems). Reaction products were analyzed using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Gene sequences generated in this study were assembled and manually edited with SeqManII by DNAStar (Lasergene). For each sample, a majority-rule consensus sequence was created.

Phylogenetic analysis

Phylogenetic analysis was performed using MEGA 6.0 software [55], using Maximum-Likelihood (ML) based on the General Time Reversible model with gamma distributed rates with 1000 bootstrap replications. *Musca domestica* sequences, which are closely related to *Glossina* genus, were used as outgroup for each of the analysed genes (gi|514058521, COI; AY573084.1, 12S rRNA).

Results

Evaluation of the discriminating power of different molecular tools

For the initial evaluation of the available molecular tools, ten laboratory colonies were used and eight to twelve individuals were genotyped per colony (**Table 1**).

Mitochondrial markers: COI **and 16S** *rRNA*

Sequence datasets generated for each of the mitochondrial genes (616 bp for *COI* and 207 bp 16S *rRNA*) were aligned for all ten *Glossina* laboratory colonies. The phylogenetic reconstruction for each of the mitochondrial markers clearly clustered the three taxonomic groups of *Glossina* (*palpalis, morsitans* and *fusca* groups). *COI* was more informative than 16s *rRNA* and was selected as a representative gene of the mitochondrial DNA (**Figure 1**). However, clustering in sub species and closely related species level was not always accurate, as in the case of *G. m. morsitans* and *G. m. centralis*. Within some taxa, distinct haplotypes were observed using either the *COI* gene (**Figure 1**) or the 16S *rRNA* gene (data not shown). For instance, *G. m. centralis, G. pallidipes* from Ethiopia, *G. f. fuscipes,* and *G. p. gambiensis* from Senegal were found to have three haplotypes each (H1, H2, H3) for the *COI* dataset.

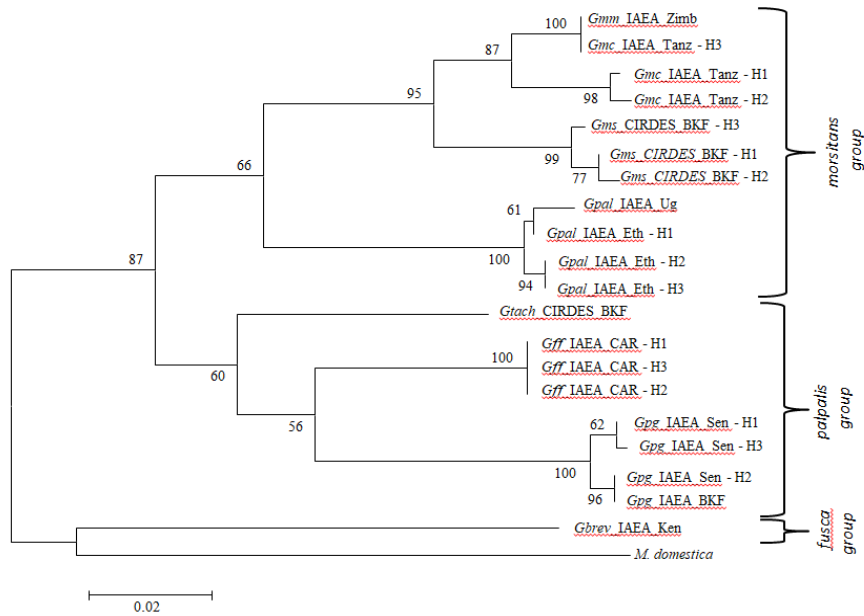

Figure 1 Molecular Phylogenetic analysis of laboratory populations by Maximum Likelihood method, using a COI gene fragment. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-2065.3726) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 600 positions in the final dataset. *Musca domestica* was used as outgroup. The numbers at each node represent bootstrap proportions based on 1000 replications. All abbreviations used in the Figures are shown in **Table S5**.

Nuclear markers: ITS1 and microsatellite markers

Variation in the length of the ITS1 amplicon was observed across the different *Glossina* laboratory coloniess, consistent with the species identification (**Table 3**, **Figure 2**). Based on size and/or number of amplicons, as revealed by agarose gel electrophoresis, most of the taxa were successfully separated. Among eight screened taxa, only *G. m. centralis/G. m. submorsitans* and *G. m. morsitans/G. brevipalpis* could not be separated from each other. However, sequencing analysis showed that there was a three bp difference between the amplicons of *G. brevipalpis* (778 bp) and *G. m. morsitans* (775 bp). This difference can be used to differentiate among them, using an appropriate fragment analysis approach. To further evaluate the discriminative power of ITS1, field collection representing *G. swynnertoni* (Tanzania) was added in this analysis. This sample shared the ITS1 pattern of the *G. m. morsitans/G. brevipalpis* group (~775 bp) (**Figure 2**).

Table 3: Analysis of ITS1 sequence length, microsatellite markers and *Wolbachia* status in *Glossina* laboratory populations

Glossina species	Country of origin (Location)	No	ITS1 expected size	Wolbachia		Microsatellites		Correctly identified samples
				cytoplasmic	chromosomal	A10	Gmm14	
G. pallidipes	IPCL Uganda	8	920	0.0 % (0/8)	0.0 % (0/8)	-	+	8/8
	IPCL Ethiopia	8		12.5 % (1/8)	0.0 % (0/8)	-	+	8/8
G. m. morsitans	IPCL	8	775	75 % (6/8)	100 % (8/8)	-	+	8/8
G. m. centralis	IPCL	8	~800 + ~150	100 % (8/8)	0.0 % (0/8)	-	+	8/8
G. m. submorsitans	CIRDES	12	~800 + ~150	0.0 % (0/12)	0.0 % (0/8)	-	+	12/12
G. p. gambiensis	IPCL POUT	8	543	0.0 % (0/8)	0.0 % (0/8)	+	+	8/8
	IPCL CIRDES	8		0.0 % (0/8)	0.0 % (0/8)	+	+	8/8
G. f. fuscipes	IPCL Central Africa Republic	8	618	12.5 % (1/8)	0.0 % (0/8)	Partial	+	8/8
G. brevipalpis	IPCL	8	778	75 % (6/8)	0.0 % (0/8)	-	-	8/8
G. tachinoides	CIRDES	12	597	0.0 % (0/12)	0.0 % (0/8)	-	+	12/12

-: no amplicon detected

+: the expected amplicon was detected in all individuals screened

Partial: the expected amplicon was detected, but not in all individuals screened

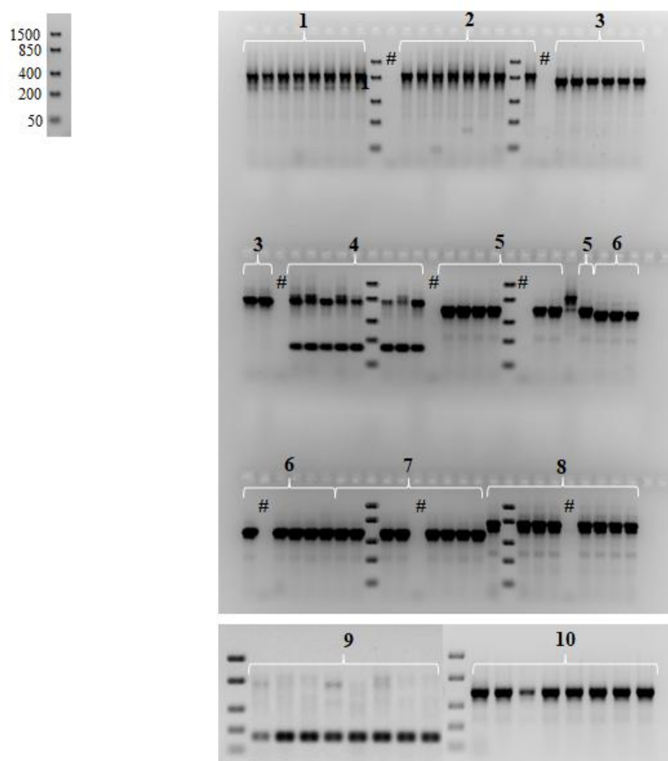

Figure 2 Agarose gel electrophoresis (2.5% agarose) showing the different band sizes of ITS1 gene amplicons for the different tsetse laboratory populations. Eight to twelve flies per laboratory population were analyzed. All abbreviations used in the Figures are shown in **Table S5**. The DNA ladder used to determine the size of the analyzed PCR products is also shown. #: Negative control during DNA extraction; -: negative PCR control; +: positive PCR control (G. pallidipes DNA). Numbers indicate the respective colonies: 1: Gpal_IAEA_Ug; 2: Gpal_IAEA_Eth; 3: Gmm_IAEA_Zimb; 4: Gmc_IAEA_Tanz; 5: Gff_IAEA_CAR; 6: Gpg_IAEA_BKF; 7: Gpg_IAEA_Sen; 8: Gbrev_IAEA_Ken; 9: Gms_CIRDES_BKF; 10: Gtach_CIRDES_BKF.

A set of 36 previously published microsatellite markers was tested against 1-3 individuals of the ten laboratory populations (**Table S1**). The analysis was carried out only with agarose gel electrophoresis and showed that there are microsatellite markers producing species-specific amplicons in the expected size range. As an example, microsatellite marker A10, which had been designed for *G. f. fuscipes* and was reported to be specific for *G. p. gambiensis,* produced the expected amplicon in all *G. p. gambiensis* individuals plus some of the *G. f. fuscipes* samples but gave no amplicons in all other taxa (**Figure 3A**). Also, microsatellite marker Gmm14 amplified in all taxa analyzed except *G. brevipalpis* (**Figure 3B**).

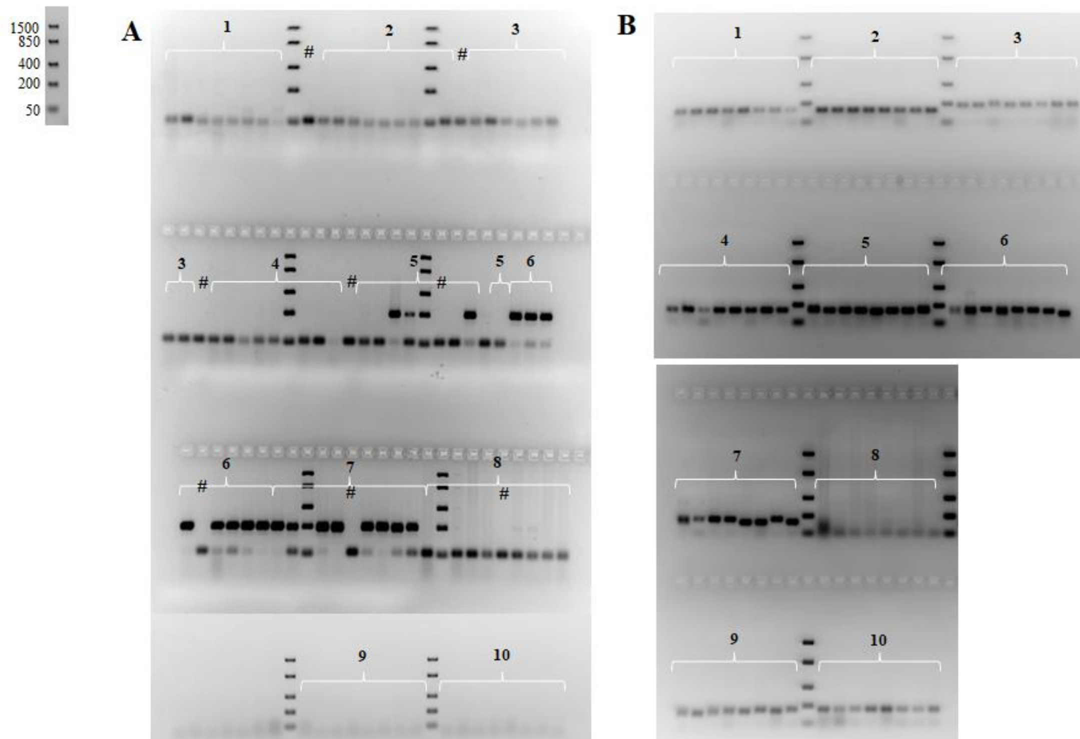

Figure 3 Agarose gel electrophoresis (2% agarose) presenting the PCR amplifications of microsatellite markers A10 (A) and Gmm14 (B) for the different laboratory populations. Eight to twelve flies per laboratory population were analyzed. All abbreviations used in the Figures are shown in **Table S5**. The DNA ladder used to determine the size of the analyzed PCR products is also shown. #: Negative control during DNA extraction; -: negative PCR control; +: positive PCR control (*G. pallidipes* DNA). Numbers indicate the respective colonies: 1: *Gpal*_IAEA_Ug; 2: *Gpal*_IAEA_Eth; 3: *Gmm*_IAEA_Zimb; 4: *Gmc*_IAEA_Tanz; 5: *Gff*_IAEA_CAR; 6: *Gpg*_IAEA_BKF; 7: *Gpg*_IAEA_Sen; 8: *Gbrev*_IAEA_Ken; 9: *Gms*_CIRDES_BKF; 10: *Gtach*_CIRDES_BKF.

Table 3: Analysis of ITS1 sequence length, microsatellite markers and *Wolbachia* status in *Glossina* laboratory populations

Glossina species	Country of origin (Location)	No	ITS1 expected size	Wolbachia		Microsatellites		Correctly identified samples
				cytoplasmic	chromosomal	A10	Gmm14	
G. pallidipes	IPCL Uganda	8	920	0.0 % (0/8)	0.0 % (0/8)	-	+	8/8
	IPCL Ethiopia	8		12.5 % (1/8)	0.0 % (0/8)	-	+	8/8
G. m. morsitans	IPCL	8	775	75 % (6/8)	100 % (8/8)	-	+	8/8
G. m. centralis	IPCL	8	~800 + ~150	100 % (8/8)	0.0 % (0/8)	-	+	8/8
G. m. submorsitans	CIRDES	12	~800 + ~150	0.0 % (0/12)	0.0 % (0/8)	-	+	12/12
G. p. gambiensis	IPCL POUT	8	543	0.0 % (0/8)	0.0 % (0/8)	+	+	8/8
	IPCL CIRDES	8		0.0 % (0/8)	0.0 % (0/8)	+	+	8/8
G. f. fuscipes	IPCL Central Africa Republic	8	618	12.5 % (1/8)	0.0 % (0/8)	Partial	+	8/8
G. brevipalpis	IPCL	8	778	75 % (6/8)	0.0 % (0/8)	-	-	8/8
G. tachinoides	CIRDES	12	597	0.0 % (0/12)	0.0 % (0/8)	-	+	12/12

-: no amplicon detected
+: the expected amplicon was detected in all individuals screened
Partial: the expected amplicon was detected, but not in all individuals screened

Wolbachia 16S rRNA

The presence of *Wolbachia* was analyzed with a *Wolbachia* specific 16S *rRNA* based PCR. The prevalence of *Wolbachia* infections differed significantly between the different laboratory colonies. A fixed cytoplasmic *Wolbachia* infection (with strong PCR amplicons) was detected only in *G. m. centralis.* High infection prevalence (with strong PCR amplicons) was observed in *G. brevipalpis* and *G. m. morsitans*. Sporadic infections (with weak PCR amplicons) were observed in *G. pallidipes* and *G. f. fuscipes.* However, *G. m. morsitans* presented the fixed chromosomal insertion (296 bp amplicon) previously reported [52] that was present in none of the other laboratory colonies. The remaining taxa/colony (*G. m. sub-morsitans, G. p. gambiensis,* and *G. tachinoides*) did not give any amplicon indicative of either active cytoplasmic infection or chromosomal insertion of *Wolbachia* (**Table 3**, **Figure 4**).

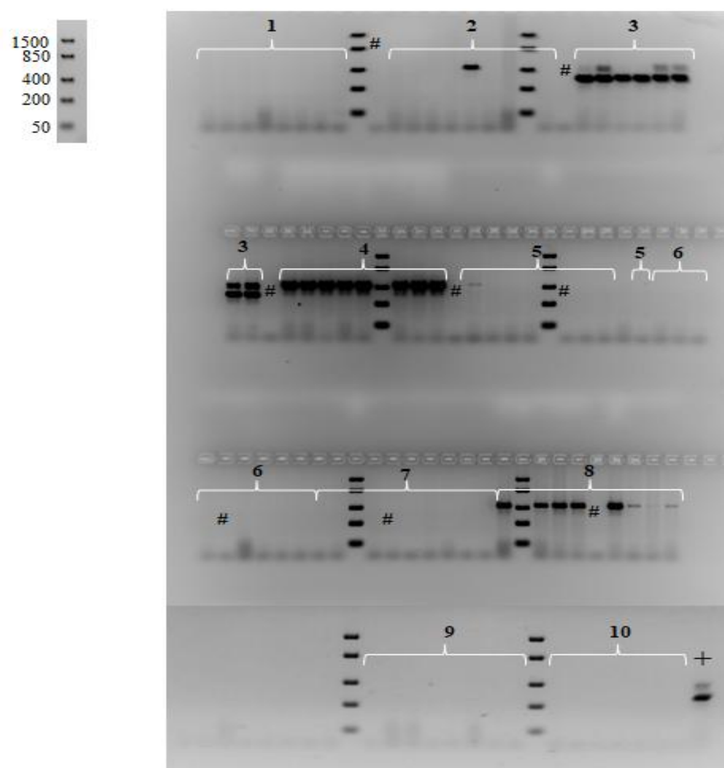

Figure 4 Agarose gel electrophoresis (2% agarose) showing the *Wolbachia* amplicons for the different laboratory populations. The presence of the 438 bp amplicon is indicative of an active (cytoplasmic) *Wolbachia* infection, while the 296 bp amplicon is indicative of the presence of the partial sequence of the *Wolbachia* 16S *rRNA* gene that is integrated into the tsetse genome. Eight to twelve flies per laboratory population were analyzed. All abbreviations used in the Figures are shown in **Table S5**. The DNA ladder used to determine the size of the analyzed PCR products is also shown. #: Negative control during DNA extraction; -: negative PCR control; +: positive PCR control (*G. pallidipes* DNA). Numbers indicate the respective colonies: 1: *Gpal*_IAEA_Ug; 2: *Gpal*_IAEA_Eth; 3: *Gmm*_IAEA_Zimb; 4: *Gmc*_IAEA_Tanz; 5: *Gff*_IAEA_CAR; 6: *Gpg*_IAEA_BKF; 7: *Gpg*_IAEA_Sen; 8: *Gbrev*_IAEA_Ken; 9: *Gms*_CIRDES_BKF; 10: *Gtach*_CIRDES_BKF

Correlation with museum specimens

Due to low DNA quality, only few amplicons were obtained from museum specimens and only for the 12S *rRNA* gene. Therefore, representative samples from all laboratory coloni were also sequenced for the 12S *rRNA* gene. Despite the limited resolution provided, the laboratory colonies correlated well with the museum specimens (**Figure 5**).

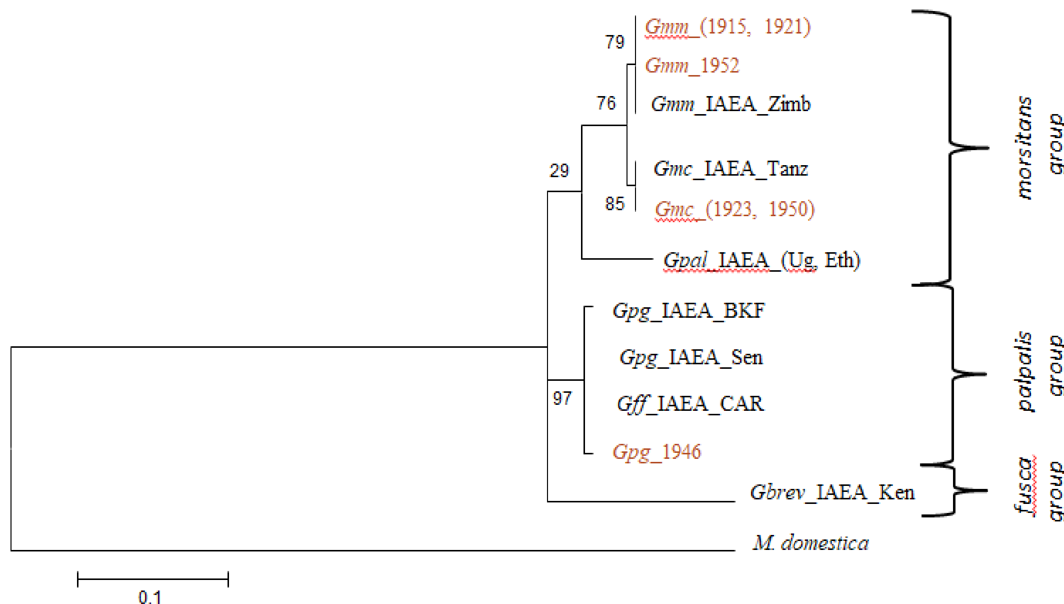

Figure 5 Molecular Phylogenetic analysis of laboratory populations and museum specimens by Maximum Likelihood analyses, using the 12S *rRNA* gene sequence.The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-629.9965) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 180 positions in the final dataset. The numbers at each node represent bootstrap proportions based on 1000 replications. Laboratory populations are in black and Museum specimens are in brown. *Musca domestica* was used as outgroup. All abbreviations used in the Figures are shown in **Table S5**.

Evaluation of COI as a 'stand-alone' marker for species identification

COI gene sequence was used to a) correlate our reference laboratory colonies with published sequences of different taxa and b) identify selected samples from the field that were available in IPCL DNA base. In general, laboratory colonies were well correlated both to previously published sequences (**Figure 6A**) and samples field collections available in our DNA base

(**Figure 6B**). On the other hand, *COI* cannot clearly resolve closely related species (subspecies or complex species), as was the case of the *G. morsitans* subspecies and *G. f. quanzensis* from Angola (which is more closely related to the *G. p. gambiensis* samples, rather than the rest of the *G. fuscipes* samples (**Figure 6B**).

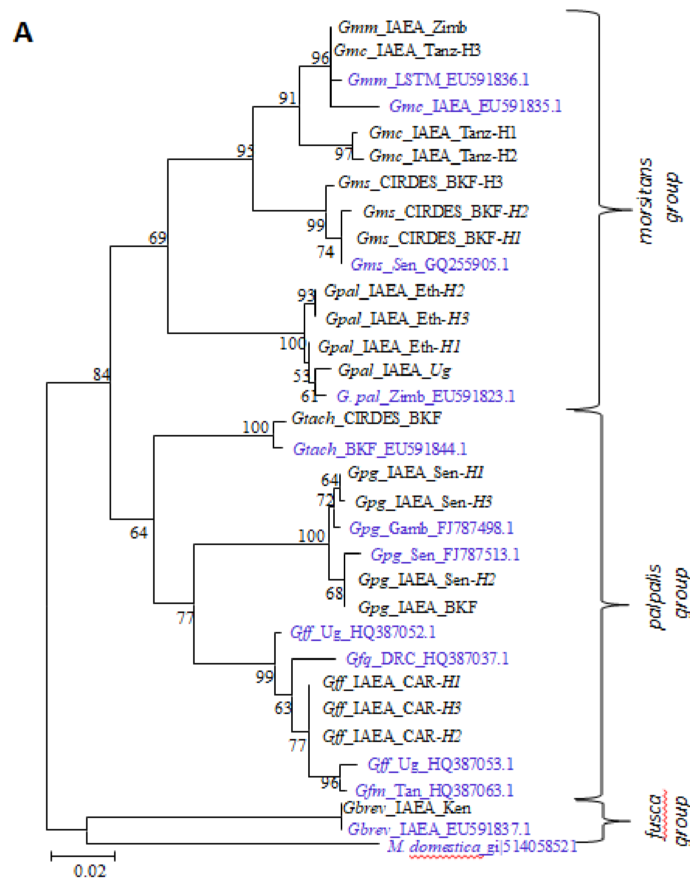

Figure 6 A Molecular Phylogenetic analysis of laboratory populations, published sequences, and selected samples from collections deriving from wild by Maximum Likelihood method -Using a *COI* gene fragment. A: The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-2609.6833) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 33 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 600 positions in the final dataset. Samples derived from laboratory populations of the present study are in black and different tsetse sequences available in the NCBI database are in blue. *Musca domestica* was used as outgroup. All abbreviations used in the Figures are shown in **Table S5.**

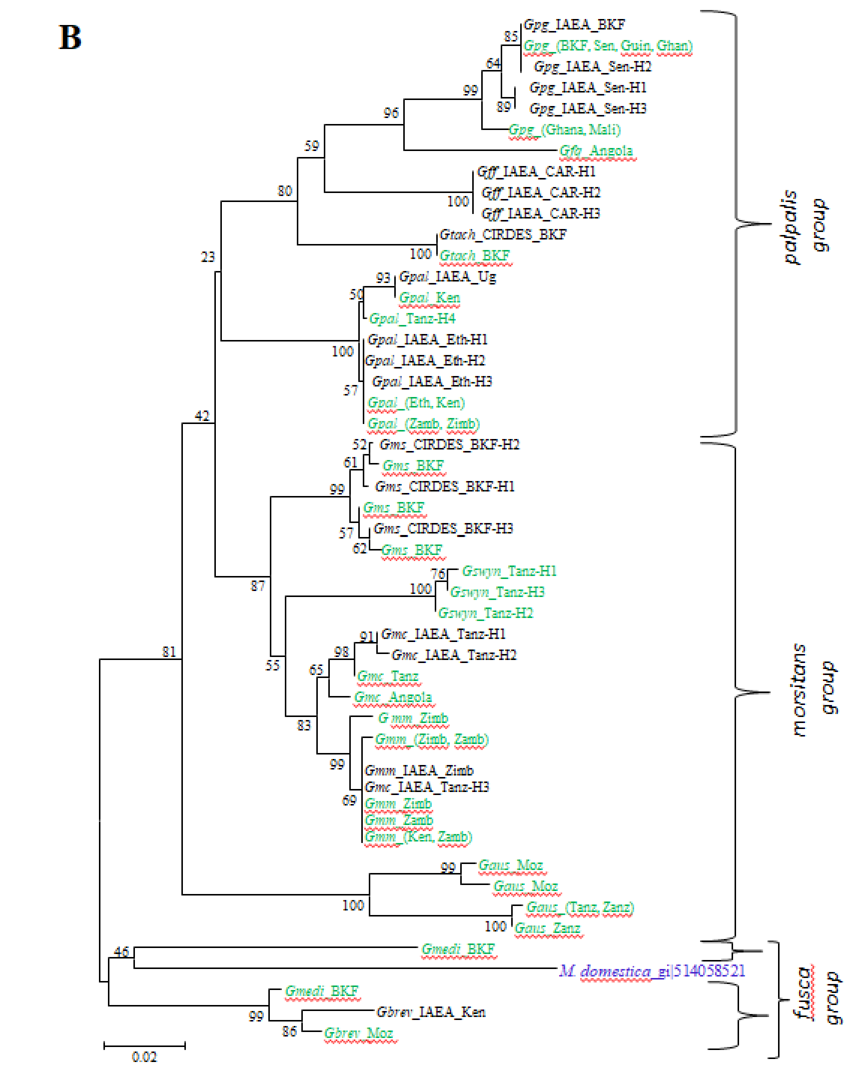

Figure 6 Molecular Phylogenetic analysis of laboratory populations, published sequences, and selected samples from collections deriving from wild (B), by Maximum Likelihood method - Using a *COI* gene fragment B: The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-2044.8169) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 49 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 362 positions in the final dataset. *Musca domestica* was used as outgroup. All abbreviations used in the Figures are shown in **Table S5**. Samples derived from laboratory populations of the present study are in black and samples collected from the field are in green.

Development of a multi-marker species identification approach

Based on the initial data derived from the laboratory colonies, we focused on the discriminative power of the combined use of ITS1, microsatellite markers Gmm14/A10, and

the *Wolbachia* status (both cytoplasmic and chromosomal), utilizing only agarose gel electrophoresis. Previous findings as well as the findings of this study, suggested that the length of the ITS1 amplicon should be sufficient to identify most of the taxa analyzed, except two cases: the *G. m. centralis/G. m. submorsitans* group and the *G. m. morsitans/ G. brevipalpis* (**Figure 2**). To differentiate *G. m. centralis* from *G. m. submorsitans*, we used the *Wolbachia* infection status (cytoplasmic) (**Figure 4**). To differentiate *G. m. morsitans* from *G. brevipalpis*, we used the *G. m. morsitans* – specific chromosomal introgression of the *Wolbachia* 16S *rRNA* gene (**Figure 4**). These results are summarized in **Table 3** and the approach used to differentiate among the available taxa is summarized in **Figure 7**. Following this approach, without using any morphological data, all ten laboratory colonies (representing 8 taxa) were accurately resolved.

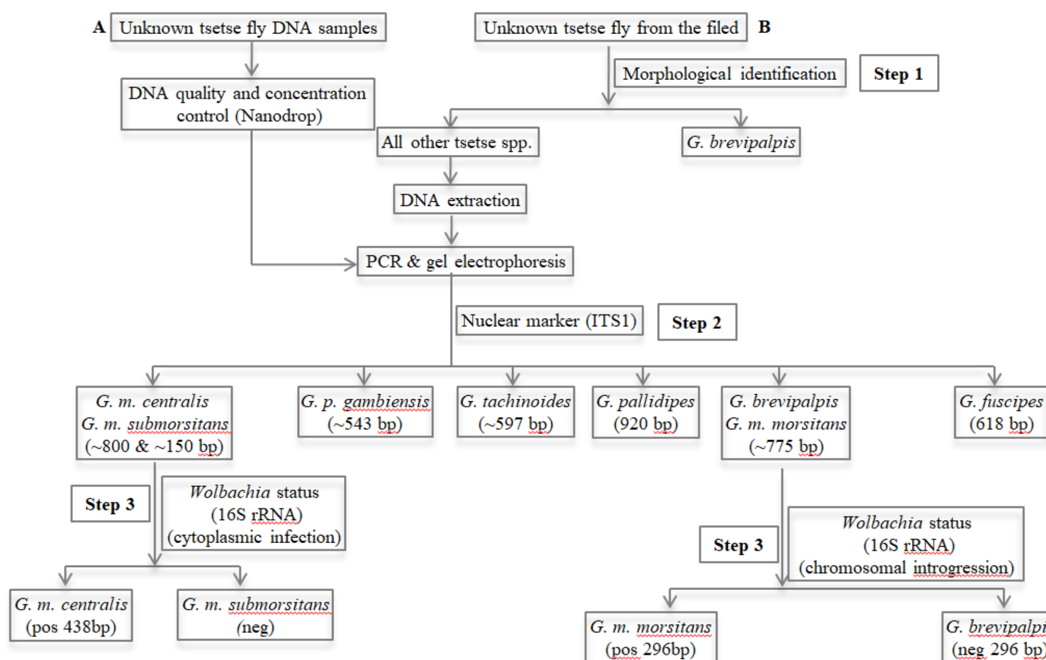

Figure 7 A multi-marker based approach to distinguish tsetse species, based on agarose gel electrophoresis. This approach relies on the amplicons (size and number) of ITS1 and the presence/absence of the *Wolbachia* specific *16S rRNA* amplicons (both cytoplasmic and chromosomal).

The 'blind test' using ITS1, selected microsatellite markers, and Wolbachia

To further test the resolution power of this combined approach, a 'blind test' of randomly selected DNAs available at the DNA base of the IPCL was performed. The first step was the application of the ITS1 assay. A total of 2695 individuals were genotyped and 2662 (98.78 %)

were assigned to the expected taxon (**Table 4**), based on the information available upon collection. For 33 individuals, there was a discrepancy between data obtained upon collection and ITS1 profile. More specifically, for 0.57 % of the *G. p. gambiensis* samples (7 out of 1267), 7.94 % of the *G. m. submorsitans* samples (22 out of 277), 0.13 % of the *G. tachinoides* samples (1 out of 799), and 12.5 % of the *G. swynnertoni* samples (3 out of the 24), data from collection sites were not in agreement with the molecular identification (**Table 4**). These samples were revisited and the *Wolbachia* infection status, the amplicon profile of microsatellite markers A10 and Gmm14, and the sequencing data of *COI* gene were also used. The combined use of the four classes of markers, along with data of the geographical distribution of *Glossina* species verified the taxon of these samples, showing that they were cases of either misidentification in the field or subsequent mislabeling (**Table 4**). Therefore, all samples were correctly identified with the combined use of these markers. In this analysis, four field collected samples representing four additional taxa were included (*G. austeni, G. f. quanzensis, G. medicorum,* and *G. swynnertoni*). For these taxa, there were no laboratory colonies available to use as reference. The estimated size of ITS1 amplicons were in accordance with that expected from previous studies. The pattern of ITS1 is sufficient to differentiate both *G. austeni* (amplicon of 633 bp) from all other taxa of this study, although this amplicon size is very similar to the *G. fuscipes* amplicon size (633 bp). *G. f. quanzensis* could not be differentiated from *G. f. fuscipes*, based on the single agarose gel electrophoresis of the ITS1 amplicon. *G. medicorum* gave two amplicons, with the one having a size between 600 and 700 bp, and the other being close to the one expected from previous studies (~880 bp). However, in our samples, the amplicon of lower molecular weight (600 -700 bp) was more robust and consistent than the expected one. *G. swynertoni* provided a unique combined profile: (a) the COI sequencing data place these samples close to *G. m. centralis* and *G. m morsitans* (**Figure 6B**), (b) the ITS1 profile (amplicon size) is similar or identical to *G. m. morsitans* and *G. brevipalpis* and (c) the *Wolbachia* infection status (complete absence of both cytoplasmic and chromosomal amplicons). Due to the lack of reference laboratory colonies, the *G. swynertoni* samples were not included in the approach described in **Figure 7**.

Table 4. Validation of Tsetse species from field collected samples using Glossina ITS1

Tsetse field collected species	Expected band size	Tested flies	Correctly identified flies		Misidentified flies		Band size of the misidentified samples	Corrected identification
			N	%	N	%		
G. pallidipes	920	69	69	100	0	0	-	-
G. m. morsitans	775	13	13	100	0	0	-	-
G. m. centralis	800 + 150	85	85	100	0	0	-	-
G. p. gambiensis	543	1267	1260	99.44	7	0.56	800 + 150	*G. m. submorsitans*[1]
G. f. fuscipes	~618	52	52	100	0	0	-	-
G. f. quanzensis	~618	3	3	100	0	0	-	-
G. m. submorsitans	800 + 150	277	255	92.06	22	7.94	597	*G. tachinoides*[2]
G. brevipalpis	775	6	6	100	0	0	-	-
G. tachinoides	597	799	798	99.87	1	0.13	800 + 150	*G. m. submorsitans*[3]
G. austeni	~700	14	14	100	0	0	-	-
G. medicorum	~850+~650	86	86	100	0	0	-	-
G. swynnertoni	~775 bp[5]	24	21	87.5	3	12.5	920	*G. pallidipes*[4]
total		2,695	2,662	98.78	33	1.22		

In grey scale: field collections lacking reference laboratory populations.
In bold: field collections where discrepancies between data deriving from collection sites and molecular identification was observed.
[1]Based on the ITS1 profile, non-amplification of microsatellite A10, complete absence of the cytoplasmic infection of *Wolbachia,* and the geographical distribution of tsetse species, these 7 samples were identified as *G. m. submorsitans*.
[2]Based on the ITS1 profile, amplification pattern of both A10 and Gmm14 microsatellite markers, absence of cytoplasmic and chromosomal *Wolbachia*, and the geographic distribution of tsetse species, these individuals were identified as *G. tachinoides.*

[3]Based on the ITS1 profile, amplification of both A10 and Gmm14 microsatellite, absence of cytoplasmic and chromosomal *Wolbachia*, and the geographic distribution of tsetse species, these individuals were identified as *G. m. submorsitans.*
[4]Based on the ITS1 profile, COI profile, amplification pattern of both A10 and Gmm14 microsatellite markers, absence of cytoplasmic and chromosomal *Wolbachia*, and the geographic distribution of tsetse species, these individuals were identified as *G. pallidipes.*
[5]For *G. swynnertoni*, there was no ITS1 amplicon expected from previous studies. The one generated in the present study is stated as 'expected'.

Of special interest is the combined use of ITS1 and *Wolbachia* to differentiate among the subspecies of *G. morsitans.* As described, *G. m. morsitans* has a distinct ITS1 profile and the presence of the chromosomal introgression of *Wolbachia. G. m. centralis* and *G. m submorsitans*, which share the same characteristic ITS1 pattern can be differentiated by the presence of an active *Wolbachia* infection. To support this, 85 field collected individuals belonging to *G. m. centralis* (Angola and Tanzania), that had the same ITS1 profile, were also 100 % infected with *Wolbachia* (**Table 5**). Regarding *Wolbachia* status of the other field collected samples, *G. austeni* was 100 % infected, *G. brevipalpis* did not show a fixed infection pattern (in a small sample size though with strong PCR amplicons in some of the individuals), and three other taxa also presented non-fixed infection patterns and with weak PCR amplicons (*G. f. fuscipes, G. f. quanzensis,* and *G. p. gambiensis*). *G. pallidipes* did not show any evidence of *Wolbachia* infection (**Table 5**)

Table 5 *Wolbachia* status of selected *Glossina* field collections

Field collected tsetse species	Wolbachia status				
	Cytoplasmic			Chromosomal	
	N	%	Estimation	N	%
G. pallidipes	0/57	0	no PCR amplicon,no infection	0/57	0
G. m. centralis	85/85	100	strong PCR amplicons, fixed infection	0/85	0
G. p. gambiensis	15/78	19.2	weak PCR amplicons, sporadic	0/78	0
G. f. fuscipes	2/52	3.8	weak PCR amplicons, sporadic	0/52	0
G. f. quanzensis	1/3	33.3	weak PCR amplicons, sporadic	0/3	0
G. brevipalpis	3/6	50	strong PCR amplicons, not fixed infection	0/6	0
G. austeni	7/7	100	strong PCR amplicons, fixed infection	0/7	0

Discussion

The present study clearly suggests that the combined use of ITS1, selected microsatellite markers, and *Wolbachia* status (cytoplasmic infection and chromosomal introgression) provides a reliable and cost-effective approach that can be applied for the identification of many *Glossina* taxa, without need of sequencing.

Sequencing of some of the mitochondrial genes supports the phylogeny of three *Glossina* groups. Different haplotypes within some species were revealed for the COI gene sequence. Although the sequencing of the mitochondrial markers showed differences among the *Glossina* species and even within populations of different geographical areas, these sequences alone could not distinguish among some taxa. For instance, the *G. m. centralis H3* COI and 16S *rRNA* gene sequences were similar to the *G. m. morsitans* sequences. Additionally, mitochondrial markers can be considered as 'compromised' in cases of closely related species. In such cases, mitochondrial haplotypes may have a completely different phylogenetic history than nuclear DNA. Moreover, the distinct patterns of *Wolbachia* infections in the different *Glossina* taxa make the use of mitochondrial markers even more questionable. For these reasons, sequencing of mitochondrial markers was not included as a tool in the approach followed in the present study.

The ITS1 sequence length variation proved quite a reliable marker in species level. The ITS1 amplicons generated from this study are in accordance with previously published ITS1 sequenced species [15–17] (**Table S2**). Some ITS1 amplicons, representing sequence variants from different taxa (from reference laboratory colonies only), were sequenced to confirm the actual amplicon size (data not shown). In all cases, sequences matched the published ITS1 sequences [16].

The main objective of this study was to develop and evaluate a convenient and cost-effective approach to identify *Glossina* species at the molecular level (i.e. PCR and gel electrophoresis). Taking together results from laboratory and field samples, the ITS1 amplicon produced eight size variants that could easily be recognized in 2.5 % agarose gel electrophoresis. These profiles successfully identified five species (*G. pallidipes, G. p. gambiensis, G. tachinoides, G. austeni,* and *G. medicorum*). The three remaining ITS1 profiles clustered seven taxa in three different groups. The *G. m. morsitans / G. swynnertoni / G. brevipalpis* group, the *G. m. centralis / G. m. submorsitans group,* and the *G. f. fuscipes / G. f. quanzensis* group. To provide further analysis, several microsatellite markers were screened to identify some taxon-specific markers that could be used as diagnostic markers among specific taxa and we coupled this with 'symbiotic markers' that is the *Wolbachia* status. Cross-species amplification of microsatellite markers is an indication of the phylogenetic relation among different taxa and more closely related taxa are expected to share a higher number of cross amplified markers and this also can be regarded as an indicator of their genetic proximity. This property has been already exploited in *Glossina* species to avoid

the *de novo* development of markers (**Table S3**). As reported also by [16], microsatellite A10 can be used to distinguish *G. p. gambiensis* from *G. tachinoides* which showed similar (but not identical) ITS1 length. Moreover, microsatellite Gmm14 can successfully differentiate *G. brevipalpis* from all other taxa in this study, which was crucial since it shared an identical (or similar) ITS 1 profile with *G. m. morsitans* and *G. swynnertoni*. The two remaining 'black boxes' are the *G. m. morsitans / G. swynnertoni* and the *G. m. centralis / G. m. submorsitans* groups. However, based on our (and previous) data, they can be separated based on the *Wolbachia* profile. *G. m. morsitans* is up to now the only taxon that has a *Wolbachia* chromosomal insertion that gives a characteristic 16S *rRNA* amplicon of 296 bp and *G. swynnertoni* samples tested did not produce this amplicon. Regarding the last group, *G. m. centralis* has a fixed *Wolbachia* infection (cytoplasmic), while *G. m. submorsitans* seems to lack *Wolbachia.* Regarding the *G. fuscipes* subspecies, we did not have well characterized material besides *G. f. fuscipes.* Few field collected individuals were available for *G. f. quanzensis* that shared the same ITS1 profile with *G. f. fuscipes.* Dyer and her colleagues have developed ITS1 diagnostic primer pairs and diagnostic assays that can differentiate among the three subspecies of *G. fuscipes (fuscipes, quanzensis,* and *martinii*) [17]. Since we did not have reference laboratory material for the two of the three subspecies, we could not investigate the identification of these taxa further.

Among the ten laboratory colonies screened here, only *G. m. centralis* harbored a fixed *Wolbachia* infection and only *G. m. morsitans* showed a fixed chromosomal insertion. All other laboratory colonies were shown to be either *Wolbachia*-free (*G. pallidipes, G. p. gambiensis, G. m. submorsitans,* and *G. tachinoides*) or had varying levels of *Wolbachia* infection (*G. m. morsitans*, *G. f. fuscipes,* and *G. brevipalpis*). These data are in agreement with previous studies about the *Wolbachia* infection status of laboratory colonies and natural populations of *Glossina* species [32,41,52,56,57]. The presence of *Wolbachia* in some of the *G. pallidipes* flies from Ethiopia and its absence from all Uganda *G. pallidipes* flies suggests that geographical origin of a species might impact the *Wolbachia* infection status of the species. The presence or absence of *Wolbachia* infection in the same species from different geographical areas has been previously reported [32,41,52,56]; however, many of these cases are both low prevalence and low titer infections (**Table S4**). The biological, ecological and evolutionary significance of such infections remains to be resolved.

The horizontal gene transfer of *Wolbachia* was found fixed in *G. m. morsitans* laboratory colony, using the 16S *rRNA* gene-based PCR assay, in agreement with already published

results [40,41]. None of the other laboratory colonies and field collections of any other taxon showed evidence of the specific chromosomal insertion. We did not have material to expand our sampling of *G. m. morsitans* but all the material belonging to *G. m. centralis* and *G. m. submorsitans*, both laboratory and field collected, were negative.

Concluding Remarks

The integration of nuclear and symbiotic markers in this study could clearly discriminate among some different economically important *Glossina* taxa. The correct identification at least at the species level is critical for the application of SIT and requires large numbers of individuals, especially in cases of morphologically indistinguishable subspecies, complexes of species and sympatric species. We avoided using sequencing and/or specialized PCR assays (diagnostic primer pairs) to keep the identification test easy to apply, easy to analyze and cost effective. Although there are now modern tools available that can support molecular taxonomy (genome wide sequencing for example), they cannot yet be used cost effectively on numerous individuals. Therefore, our approach can be considered as adequate to support species identification, especially in African countries where quick decision making and planning may be needed, depending on the data derived from trap collections.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

AAA, AMMA and KB conceived and designed the experiments. AAA, IM, GDU, AS and GOSG performed the experiments. AAA, IM, GT, AP, AMMA, and KB interpreted the experiments. AAA, IM, and GDU drafted the manuscript. AAA, GT, MV, AP, AMMA, and KB have critically revised the manuscript. All authors have approved the version to be published and agreed to be accountable for all aspects of this work.

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Supplementary Material

Supplementary Table S1: The set of microsatellites markers tested for the identification of *Glossina* species. These markers were evaluated against different laboratory populations, considering the amplification of the expected PCR product

Microsatellite marker	**Species**							
	G. pallidipes	*G. m. morsitans*	*G. m. centralis*	*G. m. submorsitans*	*G. tachnoides*	*G. brevipalpis*	*G. f. fuscipes*	*G. p. gambiensis*
GffA3				**X**		**X**	**X**	**X**
GffA9					**X**		**X**	**X**
GffB101							**X**	**X**
GffA10 (or ‘A10’)		**X**				**X**		**X**
69.22Gpg		**X**	**X**			**X**	**X**	**X**
GffB8							**X**	**X**
GffA19a		**X**	**X**	**X**	**X**	**X**	**X**	**X**
GffA23b								
GpB6b	**X**							
GffA6		**X**	**X**	**X**	**X**	**X**	**X**	**X**
Gpc107	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
55.3Gpg					**X**	**X**	**X**	**X**
19.62Gpg					**X**	**X**	**X**	**X**
Gmm8	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
Gmm14	**X**	**X**	**X**	**X**	**X**		**X**	**X**
Gmm15		**X**	**X**	**X**				
Gmm22	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
Gmm5	**X**	**X**	**X**	**X**				
GpB115	**X**	**X**	**X**	**X**				
GpB20b	**X**	**X**	**X**	**X**		**X**	**X**	**X**
GpC5b	**X**	**X**	**X**					

Microsatellite marker	**Species**							
	G. pallidipes	*G. m. morsitans*	*G. m. centralis*	*G. m. submorsitans*	*G. tachnoides*	*G. brevipalpis*	*G. f. fuscipes*	*G. p. gambiensis*
Gmm9B		**X**						
GmsCAG6	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
GmcCA16c	**X**	**X**	**X**	**X**		**X**	**X**	**X**
GmsCAG2	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
GmsCAG29B	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
GpCAG133	**X**	**X**	**X**	**X**			**X**	**X**
Gff112							**X**	
Gpc101		**X**	**X**	**X**	**X**	**X**	**X**	**X**
GpD18b		**X**	**X**	**X**				
GpC10b	**X**	**X**	**X**	**X**	**X**		**X**	**X**
GpC26b	**X**	**X**	**X**	**X**				
Gmm127	**X**	**X**	**X**	**X**				
GffC107	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
GffD6	**X**	**X**	**X**	**X**	**X**		**X**	**X**
GffD109	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**

X: presence of the expected amplicon. In grey: markers selected for downstream genotyping purposes. One to three individuals per colony were used for the initial evaluation

Supplementary Table S2: ITS1 size variants as published in previous studies

Taxon	ITS1 size variant												Reference
	Original primer pairs							Diagnostic PCR assays with modified primers					
	880	778	919	633	597	618	543	~240	~240+ ~330	234+ 239	234+ 417	339	
G. medicorum	+												Dyer et al 2008
G. brevipalpis		+											
G. pallidipes			+										
G. austeni				+									
G. tachinoides					+								
G. f. quanzensis						+							
G. f. fuscipes						+							
G. p. gambiensis							+						
G. p. palpalis						+							
G. p. palpalis								+	+				Dyer et al 2009
G. f. quanzensis										+			Dyer et al 2011
G. f. martinii											+		
G. f. fuscipes												+	

Supplementary Table S3: Microsatellite markers' cross species amplification in different Glossina taxa as referred in previous publications.

SSR	Taxon														Reference
	pp	ff	tach	ms	mm	palli	swy	aus	brev	ongip	fuscipl	longi	mc	pg	
55.3	171-175	181-185	-	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	Solano et al. 1997
19.62	170-174	174-182	-	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	
69.22	194-200	192-192	-	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Gmm8	+	+	+	nt	125-131	+	+	+	-	-	-	nt	nt	nt	Baker and Krasfur 2001
Gmm14	**-**	**-**	**-**	**nt**	**153-211**	+	**-**	**-**	**-**	**-**	**-**	**nt**	**nt**	**nt**	
Gmm15	-	-	-	nt	185-195	+	-	-	-	-	-	nt	nt	nt	
Gmm22	-	-	-	nt	133-145	+	-	-	-	-	-	nt	nt	nt	
Gmm5B	-	-	-	nt	155-175	-	-	-	-	-	-	nt	nt	nt	
Gmm9B	-	-	-	nt	140-180	-	-	-	-	-	-	nt	nt	nt	
GmsCAG16	-	-	-	nt	120-140	-	-	-	-	-	-	nt	nt	nt	
GmsCA16C	+	+	+	nt	200-210	+	+	+	+	+	+	nt	nt	nt	
GmsCAG2	+	+	-	nt	130-145	+	+	+	-	-	-	nt	nt	nt	
GmsCAG17B	+	+	+	nt	+	+	+	+	-	-	-	nt	nt	nt	
GmsCAG29B	-	-	-	nt	175-190	+	-	-	-	-	-	nt	nt	nt	
GpCAG133	+	+	+	nt	185-205	+	+	+	-	-	-	nt	nt	nt	
Gmm127	+	+	+	nt	295-301	+	+	-	-	-	-	nt	nt	nt	
Pgp1	124	+	+	nt	nt	nt	nt	-	nt	nt	nt	nt	nt	nt	Luna et al 2001
Pgp8	192	+	-	nt	nt	nt	nt	+	nt	nt	nt	nt	nt	nt	
Pgp11	178	+	-	nt	nt	nt	nt	+	nt	nt	nt	nt	nt	nt	
Pgp13	201	+	+	nt	nt	nt	nt	-	nt	nt	nt	nt	nt	nt	
Pgp17	191	+	+	nt	nt	nt	nt	-	nt	nt	nt	nt	nt	nt	
Pgp20	194	+	+	nt	nt	nt	nt	-	nt	nt	nt	nt	nt	nt	
Pgp22	279	+	+	nt	nt	nt	nt	-	nt	nt	nt	nt	nt	nt	
Pgp24	215	+	+	nt	nt	nt	nt	+	nt	nt	nt	nt	nt	nt	
Pgp28	103	+	+	nt	nt	nt	nt	+	nt	nt	nt	nt	nt	nt	
Pgp29	237	+	+	nt	nt	nt	nt	+	nt	nt	nt	nt	nt	nt	
Pgp33	208	+	+	nt	nt	nt	nt	+	nt	nt	nt	nt	nt	nt	
Pgp34	364	+	+	nt	nt	nt	nt	-	nt	nt	nt	nt	nt	nt	
Pgp35	202	+	+	nt	nt	nt	nt	+	nt	nt	nt	nt	nt	nt	
Pgp38	225	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Pgp37	217	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
GpA19a	nt	+	nt	+	-	142-189	+	+	+	nt	nt	+	+	nt	Ouma et al 2003
GpA23b	nt	+	nt	+	+	172-215	+	+	+	nt	nt	+	+	nt	
GpB6b	nt	+	nt	+	-	187-224	+	+	+	nt	nt	+	+	nt	

SSR	Taxon														Reference
	pp	ff	tach	ms	mm	palli	swy	aus	brev	ongip	fuscipl	longi	mc	pg	
GpB20b	nt	+	nt	+	+	139-200	+	+	+	nt	nt	+	+	nt	
GpC5b	nt	+	nt	+	+	187-239	+	+	+	nt	nt	+	+	nt	
GpC10b	nt	+	nt	+	+	283-314	+	+	+	nt	nt	+	+	nt	
GpC26b	nt	+	nt	+	+	168-201	+	+	+	nt	nt	+	+	nt	
GpD18b	nt	+	nt	+	-	220-229	+	+	+	nt	nt	+	+	nt	
GpB115	nt	-	nt	+	+	133-177	+	+	-	nt	nt	+	+	_	
GpC101	nt	+	nt	+	+	186-230	+	+	+	nt	nt	+	+	+	Ouma et al 2006
GpC107	nt	+	nt	+	+	202-217	+	+	+	nt	nt	+	+	+	
A10	**-**	**nt**	**nt**	**nt**	**nt**	**nt**	**nt**	**nt**	**nt**	**nt**	**nt**	**nt**	**nt**	+	Dyer et al 2008
Gff_B8	nt	183-217	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Gff_C107	nt	189-245	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Gff_D6	nt	259-279	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Gff_D109	nt	153-177	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Gff_A3	nt	227-258	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Gff_A6	nt	257-267	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	Brown et al 2008
Gff_A9	nt	170-174	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Gff_A112	nt	121-133	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Gff_B101	nt	268-308	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	
Gff_A10	nt	184-213	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	

Allele size or allele range is only given for the taxon where SSRs were originally developed.
+: presence of amplicon
-: absence of amplicon
nt: not tested
in bold: the two microsatellite markers selected to be included in the genotyping approach presented in this study

Supplementary Table S4: *Wolbachia* status in different Glossina taxa as referred in previous publications.

Taxon	*Wolbachia*		Reference
	Cytoplasmic	Chromosomal	
G. m. morsitans	Low to fixed	Fixed	Doudoumis et al 2012
G. pallidipes	low	Absent	
G. austeni	Medium to fixed	Absent	
G. p. palpalis	Absent	Absent	
G. p. gambiensis	Absent to low	Absent	
G. brevipalpis	Low to medium	Absent	
G. f. fuscipes	Absent	Absent	
G. m. centralis	Fixed (small sample)	Absent	
G. f. fuscipes	Low to medium	not tested	Alam et al 2012

Supplementary Table S5: List of abbreviations for figures

Glossina taxa	**Abbreviation**	**Country of origin**	**Abbreviation**
Glossina austeni	Gaus	Angola	Ang
Glossina brevipalpis	Gbrev	Burkina Faso	BKF
Glossina fuscipes fuscipes	Gff	Central Africa Republic	CAR
Glossina fuscipes quanzensis	Gfq	Ethiopia	Eth
Glossina medicorum	Gmedi	Ghana	Ghan
Glossina morsitans morsitans	Gmm	Guinea	Guin
Glossina morsitans centralis	Gmc	Kenya	Ken
Glossina morsitans submorsitans	Gms	Mozambique	Moz
Glossina pallidipes	Gpal	Senegal	Sen
Glossina palpalis gambiensis	Gpg	Tanzania	Tanz
Glossina tachinoides	Gtach	Zambia	Zamb
Glossina swynnertoni	Gswyn	Zanzibar	Zanz
		Zimbabwe	Zimb

Chapter 3

Prevalence of trypanosomes, salivary gland hypertrophy virus and *Wolbachia* in wild populations of tsetse flies from West Africa

Prevalence of trypanosomes, salivary gland hypertrophy virus and *Wolbachia* in wild populations of tsetse flies from West Africa

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Abstract

Tsetse flies are vectors of African trypanosomes, protozoan parasites that cause sleeping sickness or human African trypanosomosis in humans and nagana or African animal trypanosomosis) in livestock. In addition to trypanosomes, four symbiotic bacteria *Wigglesworthia glossinidia*, *Sodalis glossinidius, Wolbachia, Spiroplasma* and one parasitic microbe, the salivary gland hypertrophy virus (SGHV), have been reported in different tsetse species. We evaluated the prevalence and coinfection dynamics between *Wolbachia*, trypanosomes, and SGHV in four tsetse species (*Glossina palpalis gambiensis*, *G. tachinoides, G. morsitans submorsitans*, and *G. medicorum*) that were collected between 2008 and 2015 from 46 geographical locations in West Africa, i.e. Burkina Faso, Mali, Ghana, Guinea, and Senegal. The results indicate an overall low prevalence of SGHV and *Wolbachia* and a high prevalence of trypanosomes in the sampled wild tsetse populations. The prevalence of all three infections varied among tsetse species and sample location. The highest trypanosome prevalence was found in *Glossina tachinoides* (61.1%) in Ghana and in *Glossina palpalis gambiensis* (43.7%) in Senegal. The trypanosome prevalence in the four species in Burkina Faso was lower, i.e. 39.6% in *Glossina medicorum,* 18.08%; in *Glossina morsitans submorsitans,* 16.8%; in *Glossina tachinoides* and 10.5% in *Glossina palpalis gambiensis*. For The trypanosome prevalence in *Glossina palpalis gambiensis* was lowest in Mali (6.9%) and Guinea (2.2%). The prevalence of SGHV and *Wolbachia* was very low irrespective of location or tsetse species with an average of 1.7% for SGHV and 1.0% for *Wolbachia*. In some cases, mixed infections with different trypanosome species were detected. The highest prevalence of coinfection was *Trypanosoma vivax* and other Trypanosoma species (9.5%) followed by coinfection of *T. congolense* with other trypanosomes (7.5%). The prevalence of coinfection of *T. vivax* and *T. congolense* was (1.0%) and no mixed infection of trypanosomes, SGHV and *Wolbachia* was detected. The results indicate a high rate of trypanosome infection in tsetse wild populations in West African countries but lower infection rate of both *Wolbachia* and SGHV. Double or triple mixed trypanosome infections were found. In addition, mixed trypanosome and SGHV infection exist however no mixed infections of trypanosome and/or SGHV with *Wolbachia* were found.

Background

Tsetse flies (*Glossina* sp.) are obligate blood feeding insects that transmit protozoan parasites (*Trypanosoma* spp.), the etiological agents of African trypanosomosis that cause sleeping sickness or human African trypanosomosis, (HAT) and nagana or African animal trypanosomosis, (AAT) in livestock [1, 2]. Both diseases cause many direct and indirect losses, which represent a major obstacle for sustainable development in endemic countries [3].

Trypanosomosis is enzootic in an area covering ca. 10 million km^2 in sub Saharan Africa and is transmitted by different species of tsetse flies that vary in their vectorial capacity for the different *Trypanosoma* species. In West Africa, HAT is caused by *Trypanosoma brucei gambiense*, that accounts for over 90 percent of the globally reported HAT cases [4] and is mainly transmitted by tsetse flies from the Palpalis group (*Glossina tachinoides, G. palpalis gambiensis* and *G. p. palpalis*) [5]. The AAT causative agents (*Trypanosoma vivax, T. congolense, T. brucei brucei* and *T. evansi)* are transmitted by a broader range of tsetse fly species which include, in addition to the above mentioned Palpalis group, also flies from the Morsitans group (*G. morsitans submorsitans* and *G. longipalpis*) [6, 7]. There are 11 different pathogenic trypanosomes that can be characterized by molecular methods using specific or common primers [6-8].

Due to the lack of effective vaccines and inexpensive drugs for HAT and the development of resistance of the AAT parasites against available trypanocidal drugs [9], vector control remains the most efficient strategy for the sustainable management of these diseases [10]. The sterile insect technique (SIT) is one control tactic that may be used as part of an area-wide integrated pest management (AW-IPM) program against tsetse fly populations [11, 12].

The SIT was successfully used as part of an AW-IPM strategy to sustainably eradicate a population of *G. austeni* from the Island of Unguja, Zanzibar in the 1990's [13] and allowed the eradication of tsetse flies from the agro-pastoral land in Sidéradougou, Burkina Faso and in Jos, Nigeria [14, 15]. The latter two programmes were however not sustainable, as they were not implemented following AW-IPM principles, and hence suffered from re-invasion of wild flies from neighbouring areas.

The integration of the SIT in AW-IPM strategies to manage populations of tsetse flies requires the production of large numbers of high quality sterile males that are released in the

target area to compete with wild males for matings with wild females of the targeted species. The mass production of the required males will depend on the successful establishment and maintenance of a large, healthy colony of the targeted species in large production facilities. In some tsetse species such as *Glossina pallidipes*, colonies that are infected with a hytrosavirus, the salivary gland hypertrophy virus (SGHV), suffer from low male and female fertility which makes the maintenance of these colonies very difficult or even impossible [16-18]. This obviously hampers the implementation of AW-IPM programmes that have an SIT component. Tsetse colonies of species that are susceptible to the negative effects of the SGHV require the implementation of some measurements to manage the virus infection to enable colony maintenance and growth [19, 20].

The successful establishment of a large colony of G. pallidipes will not only depend on the virus infection but can also be affected by the tsetse associated symbiotic bacteria. Tsetse flies harbour four main symbiotic bacteria: (i) *Wigglesworthia glossinidia*, an obligate symbiotic bacterium that is present in all tsetse species. Its removal from a tsetse fly using antibiotic supplements in the tsetse's diet results in the loss of fertility [21-23], (ii) The commensal *Sodalis glossinidius*, present in all individuals of laboratory-maintained tsetse lines but not abundant in natural populations. It has been detected in the haemolymph, salivary glands and milk gland of the tsetse fly but also in the midgut where it lives in close proximity with trypanosomes [24-26], (iii) *Wolbachia*, which is an obligate intracellular and maternally transmitted alphaproteobacterium that infects many arthropod and filarial nematode species [27, 28]. *Wolbachia* is responsible for the induction of a number of reproductive alterations and cytoplasmic incompatibility (CI) [27, 28]. *Wolbachia* infections occur in some tsetse fly species, both in the laboratory and in nature. Available data indicate that *Wolbachia* infections were heterogeneous in the field, ranging from 0 to 100% in natural populations of *G. austeni* and *G. brevipalpis* and from 9.5 to 100% in natural populations of *G. m. morsitans* [29]. It has been reported that the presence of *Wolbachia* is associated with reduced prevalence of infections with pathogenic viruses and *Plasmodium* [30-40]. Therefore, the presence of *Wolbachia* in tsetse species might also reduce trypanosome and SGHV infections and transmission, and (iv) *Spiroplasma* that was recently detected in *G. fuscipes* and *G. tachnoides* but its impact on tsetse fly performance remains unclear [41].

In support of the potential development of sustainable AW-IPM strategies that might include an SIT component against tsetse species in West Africa, we assessed the prevalence of

trypanosomes, SGHV and *Wolbachia* in a large number of wild specimens from five countries as well as the potential interactions among these three microbes.

Methods

Sampling tsetse

Adult tsetse flies of *G. palpalis gambiensis, G. tachinoides, G. morsitans submorsitans,* and *G. medicorum* were collected between 2008 and 2015 in 46 geographical locations from five countries in West Africa (Burkina Faso, Guinea, Ghana, Mali, Senegal) **(Table 1 and Table 2)**. The flies were collected using the biconical Challier-Laveissière trap [42] and the monoconical Vavoua trap [43, 44] set as previously described [45]. On average, 20 traps were deployed per location to collect a minimum of 10 adult flies per location that were sorted by species and sex [46] **(Figure 1)**. Collected flies were preserved in 95% ethanol, labeled and shipped to the FAO/IAEA Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria where they were stored at -20°C until further use. Species status was confirmed using molecular identification tools including internal transcribed spacers (ITS), mitochondrial DNA cytochrome oxidase subunit 1 and microsatellites (Augustinos 2018 this special issue).

Table 1: List of collections of tsetse adults that were analyzed to established the prevalence of Trypanosome, *Wolbachia* and Salivary gland hypertrophy virus (SGHV) in wild tsetse population in West African countries

Country	No. of locations	No. of collected flies	Collection year
Burkina Faso	10	2062	2008, 2010, 2013, 2015
Mali	10	364	2008, 2010, 2011, 20012, 2013
Senegal	7	128	2008
Ghana	11	234	2008
Guinea	8	314	2008, 2009
Total	**46**	**3102**	

Table 2. Geographic coordinates of tsetse collected samples.

Glossina species	Country (area)	Longitude	Latitude
G. tachinoides	Burkina Faso (Folonzo)	-4.60801757	9.92967851
	Burkina Faso (Sissili)	-2.098178	11.09447
	Burkina Faso (Comoe)	-4.58976269	9.89106718
	Burkina Faso (Arly)	-1.289104	11.612917
	Ghana (Bougouhiba)	-0.719172226	10.23885694
	Ghana (Walewale)	-0.79846	10.351613
	Ghana (Mortani)	-0.714119074	10.23479058
	Ghana (Fumbissi)	-1.386834989	10.47282856
	Ghana (Sissili Bridge)	-1.319208122	10.33035865
	Ghana (Grogro)	-1.883133222	10.08224767
	Ghana(Kumpole)	-1.270183374	10.25432141
	Ghana (Nabogo)	-0.979001606	9.692628234
	Ghana (Psikpé)	-1.081506423	10.44471897
G. palpalis gambiensis	Burkina Faso (Kénédougou)	-4.80305222	10.98166737
	Burkina Faso (Moussodougou)	-4.95	10.833333
	Burkina Faso (Folonzo)	-4.60801757	9.92967851
	Burkina Faso (Comoé)	-4.58976269	9.89106718
	Burkina Faso (Kartasso)	-5.253033	11.141786
	Burkina Faso (Bama)	-4.4	12.033333
	Sénégal (Tambacounda)	-13.667222	13.7768889
	Sénégal (Fleuve Gambi)	-13.23552282	13.02433926
	Sénégal (Mako)	-13.27338336	12.85430818
	Sénégal (Niokolo)	-13.16964933	13.06555831
	Sénégal (Fleuève Gambi)	-12.35811122	12.84670702
	Sénégal (Diaguiri)	-12.09137828	12.62932251
	Sénégal (Moussalla)	-17.37981432	12.9297035
	Mali (Baoule)	-8.62	12.88
	Mali (Banko)	-6.516667	12.1
	Mali (Siby)	-8.32664	12.377685
	Mali (Système Sénégal)	-11.103663	13.416551
	Mali (Système Niger)	-4.201945	14.466284
	Mali (Bani)	-4,202017	14,466353
	Mali (Bougouni)	-7.483333	11.416667
	Mali (Sikasso)	-5.666667	11.316667
	Mali (Kita)	-9.484723	13.04114
	Mali (Baguineda)	-7.776667	12.615278
	Guinea (Kangoliya)	-13.65584	9.96084
	Guinea (Dekonkore)	-10.016667	9.85
	Guinea (Bafing)	-7.524724	8.325205
	Guinea (Lemonako)	-11.566667	11.733333
	Guinea (Kerfala)	-9.461194	11.343966
	Guinea (Mimi)	-9.053083	10.400434
G. morsitans submorsitans	Burkina Faso (Folonzo)	-4.60801757	9.92967851
	Burkina Faso (Sissili)	-2.098178	11.09447
	Burkina Faso (Comoe)	-4.58976269	9.89106718
G. medicorum	Burkina Faso (Comoe)	-4.58976269	9.89106718
	Burkina Faso (Folonzo)	-4.60801757	9.92967851

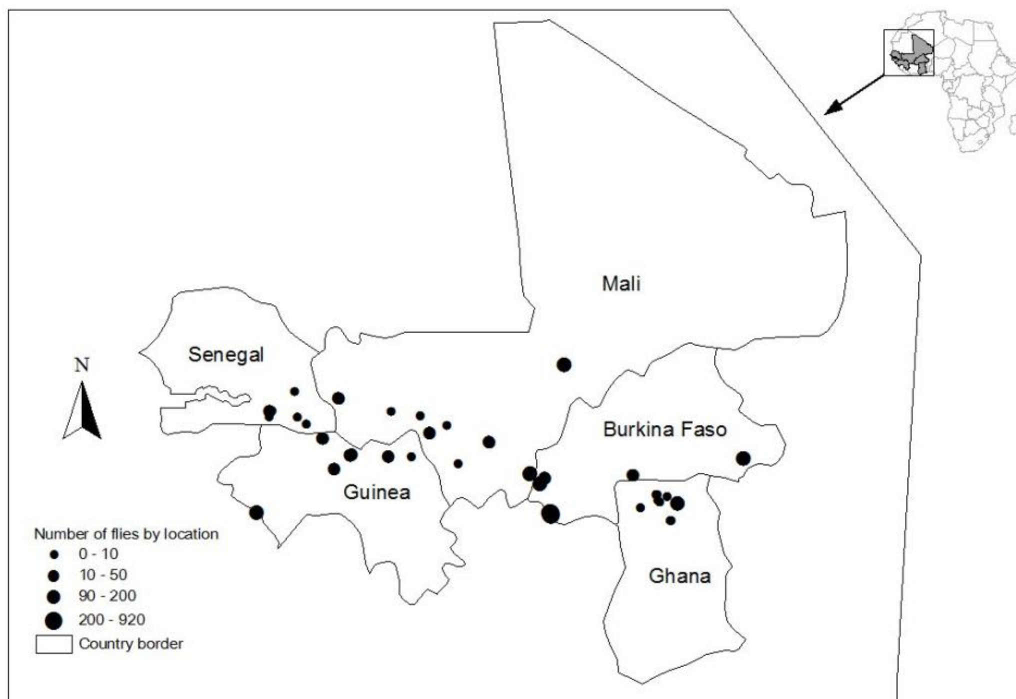

Figure 1. The geographical locations in the western African countries where tsetse samples were collected.

DNA extraction

The flies were removed from ethanol and rehydrated in distilled water. The wings and legs were removed for other studies. The total DNA was extracted from the remaining fly body using the DNeasy tissue kit (QIAGEN Inc, Valencia, CA) following the supplier's instructions and was eluted in 200 µl elution buffer. All the extracted DNA samples from these locations were tested for a tsetse-specific sequence to confirm the quality.

PCR amplification and prevalence analysis

SGHV prevalence

Polymerase chain reactions (PCR) were used to amplify the partial coding regions of two conserved putative ORFs, odv-e66 and dnapol (GenBank accession numbers: EF568108) using *Glossina pallidipes* Salivary Hypertrophy Virus (GpSGHV)-specific primers [47]. These primers were used in a multiplex PCR, and all the samples included a set of specific primers amplifying the *G. pallidipes* microsatellite GpCAG133 sequence to control the quality of the extracted DNA [48]. For all PCR amplifications, 22.5 µl of 1.1x Pre-Aliquoted PCR Master Mix (ABgene, UK) was used. A final volume of 25 µl of this mix contained:

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 the dNTPs. To the mix, 1.5 µl of template DNA plus forward and reverse primers were added to a final concentration of 0.2 mM per primer. Samples were considered virus-infected if any of the expected viral PCR product amplicons were detected. Data were accepted only if the control gene GpCAG133 sequence was amplified.

Trypanosome prevalence and genotyping

For trypanosome detection, PCR reaction was used according to Njiru et al., [8], using trypanosome specific primers to amplify the internal transcribed spacer 1 (ITS-1). PCR conditions were: 25 µl reaction volume contained 12.5 µl of Taq PCR Master Mix kit (Qiagen) (with 0.8 Units of Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 µM dNTP), 0.8 µM each of the ITS-1 forward (5'-CCGGAAGTTCACCGATATTG-3') and reverse (5'-TGCTGC GTTCTTCAACGAA- 3') primers (VBC, Biotech, Austria), 9 µl of sterile water and 2.5 µl of genomic DNA. Cycling conditions were: 94 °C for 15 min, 94 °C for 30 seconds, 60 °C for 30s, 72 °C for 30s, 40 cycles following by 72 °C for 5 min; PCR products were detected by Agarose (2 %) gel electrophoresis and ethidium bromide staining. The sample was considered infected with trypanosome by detecting single, double or triple bands ranging from 200 bp to 700 bp (see below). DNA from *T. congolense savanna* was used as positive control which gives a PCR amplicon of 650 bp.

To have better genotyping of the detected trypanosomes, positive samples from the first screen were amplified with ITS-1 forward (5'-TGTAGGTGAACCTGCAGCTGGATC-3') and reverse (5'-CCAAGTCATCCATCGCGACACGTT- 3') primers following Fikru et al. [49]. The detection of different trypanosomes was based on the length of the amplicon, i.e., *T. vivax* (200 bp), *T. equiperdum*, *T. evansi* and *T. brucei* (350 bp), *T. theileri* (450 bp) and *T. congolense savannah* (650 bp). DNA from *T. congolense savannah*, *T. vivax*, *T. theileri*, *T. brucei gambiense*, T. *brucei rhodesiense*, *T. brucei brucei*, *T. evansi* and *T. equiperdum* provided by Dr Stijn Deborggraeve were used as positive control.

Wolbachia prevalence and genotyping

PCR reaction with *Wolbachia* specific primers was used to screen the DNA of the wild tsetse flies for the presence of *Wolbachia*. The detection was based on the *Wolbachia* 16S *rRNA* gene and results in the amplification of a 438 base pair-long DNA fragment with the

Wolbachia specific primers wspecF and wspecR [29]. The PCR conditions used were as described above for the trypanosome detection and the cycling conditions were: 94 °C for 2 min, 94 °C for 30 seconds, 55 °C for 30s, 72 °C for 30s, 36 cycles following by 72 °C for 5 min. As a positive control for *Wolbachia*, DNA extracted from the Mediterranean fruit fly, *Ceratitis capitata* strain S 10.3 was used. This strain is transinfected with the *w*Cer4 *Wolbachia* strain of *Rhagoletis cerasi* [50].

Data Analysis

The data were analyzed with the software package R, using a generalized linear model (GLM) with the package stat [51]. Trypanosome, virus and *Wolbachia* prevalence in tsetse were respectively considered as response variables, while tsetse species, sex, countries and their interactions were used as explicative variables. The best model was selected on the basis of the lowest corrected Akaike information criterion (AICc), and the significance of fixed effects was tested using the likelihood test ratio [52, 53]. Then, for each country, GLM were used to assess differences in trypanosome, virus and *Wolbachia* prevalence between localities and species. Trypanosoma prevalence was compared between species by a pairwise comparison of proportions with a Bonferroni correction (package stats). Correlations between the prevalence of trypanosome species, salivary gland hypertrophy virus and *Wolbachia* were tested using the "rcorr" function of the Hmisc (Harrel miscellaneous package version 4.03, 2017).

Results

Global trypanosome prevalence

The trypanosome prevalence varied significantly from one country to another and from one species to another. Overall, 18.4% of the examined tsetse flies (n = 3102) were positive for trypanosomes, irrespective of tsetse species or country **(Table 3).** Trypanosomes were detected in *G. tachinoides* in Burkina Faso and Ghana; *G. p. gambiensis* in Burkina Faso, Guinea, Mali, and Senegal; *G. m. submorsitans* and *G. medicorum* in the Comoé forest in the south of Burkina Faso at the border with Côte d'Ivoire. The best model (lowest AICc) selected for the overall trypanosome prevalence retained the tsetse species and countries as variables that fitted well the data with no interaction. For tsetse species, *G. medicorum* (only caught in Folonzo village, and a protected area belonging to the village in Southern Burkina Faso) had the highest mean infection rate of 39.6% **(Figure 2A),** which was significantly higher than the mean infection rate in *G. p. gambiensis* ($P < 0.001$). The mean trypanosome

infection rate in *G. tachinoides* was also significantly higher as compared with *G. m. submorsitans* (P = 0.008; **Figure 2A**; **Supplementary file 1**).

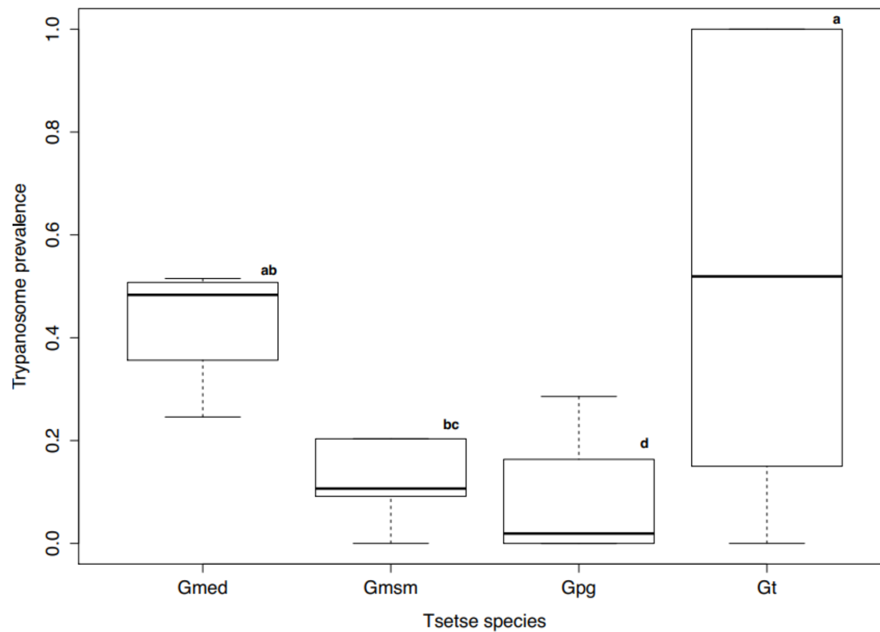

Figure 2A: Global prevalence of trypanosomes according to tsetse species (B). Boxes extend between the 25th and 75th percentile. A thick line denotes the median. The whiskers extend up to the most extreme values. Gmed: *Glossina medicorum*, Gmsm: *G. morsitans submositans*, Gpg: *G. palpalis gambiensis* and Gt: *G. tachinoides.*

Trypanosome prevalence by country was low in Guinea (2.2%) and Mali (6.9%) but high in Senegal (43.7%) and Ghana (61.1%) (**Table 3**). The Result showed no significant difference between the trypanosome prevalence in Burkina Faso, Guinea and Mali but the prevalence of these three countries was significantly different from that of Senegal and Ghana ($P < 0.05$) (**Figure 2B** and **Supplementary file 1**). The sex effect was not retained in the model highlighting no difference in the mean prevalence of male and female flies. All *G. tachinoides* flies collected from Fumbissi (n = 15), Grogro (n = 11), Kumpole (n = 7), Psikpé (n = 2) and Sissili Bridge (n = 6) in Ghana were infected with trypanosomes, and the overall prevalence in seven out of nine locations was relatively > 53% **(Table 4)**. Trypanosome prevalence in the other tsetse species fluctuated greatly with location, i.e., from 0% in the *G. p. gambiensis* flies collected in Comoé, Kenedougou and Bama to 34.5% in Moussodougou in Burkina Faso **(Table 5).** A similar trend was found in *G .p. gambiensis* flies collected in Mali and Guinea**.**

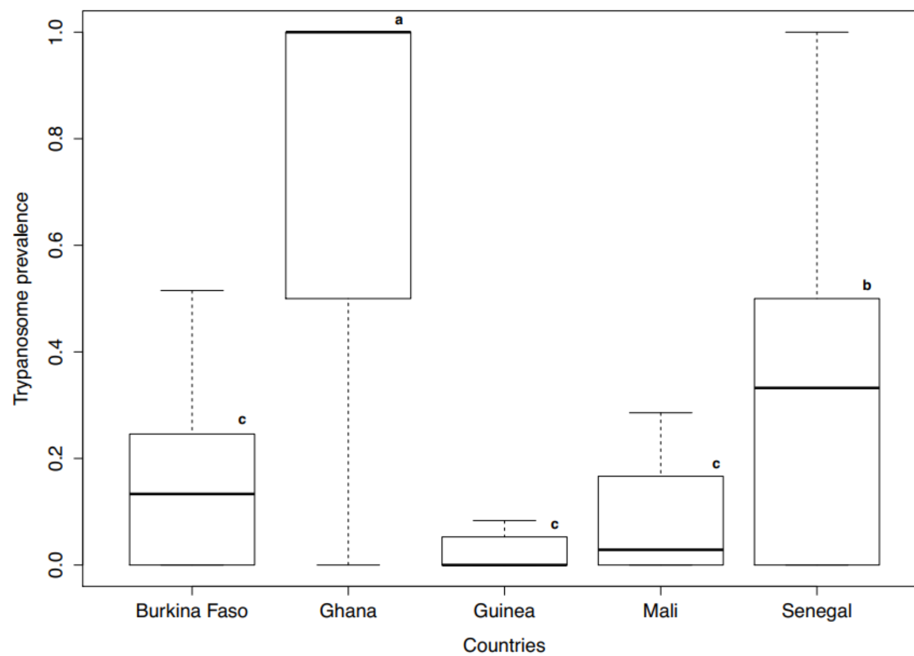

Figure 2B: Global prevalence of trypanosomes according to the country. Boxes extend between the 25th and 75th percentile. A thick line denotes the median. The whiskers extend up to the most extreme values. Gmed: *Glossina medicorum*, Gmsm: *G. morsitans submositans*, Gpg: *G. palpalis gambiensis* and Gt: *G. tachinoides.*

Table 3. Prevalence of trypanosomes, salivary gland hypertrophy virus and *Wolbachia* in tsetse tested samples

Species	**Country**	***Trypanosomes***	**Virus**	***Wolbachia***
G. tachinoides	Burkina Faso	(140/834) 16.79%	(25/834) 3%	(2/834) 0.24%
	Ghana	(143/234) 61.11%	(0/234) 0%	(0/234) 0%
G.p.gambiensis	Burkina Faso	(77/731) 10.53%	(14/731) 1.92%	(1/731) 0.14%
	Mali	(25/364) 6.87%	(15/364) 4.12%	(16/364) 4.40%
	Guinea	(7/314) 2.23%	(0/314) 0%	(13/314) 4.14%
	Senegal	(58/128) 43.75%	(0/128) 0%	(0/128) 0%
G. m. submorsitans	Burkina Faso	(62/343) 18.08%	(4/343) 1.17%	(1/343) 0.29%
G. medicorum	Burkina Faso	(61/154) 39.61%	(1/154) 0.65%	(1/154) 0.65%
Total		(570/3102) 18.38%	(54/3102) 1.74%	(30/3102) 0.96%

Table 4: Trypanosome prevalence in natural populations of *Glossina tachinoides* collected from Ghana

Location	Sample size	Prevalence
Bougouhiya	19	(3/19) 15.78%
Fumbissi	15	(15/15) 100%
Grogro	11	(11/11) 100%
Kumpole	7	(7/7) 100%
Mortani	41	(22/41) 53.65%
Nabogo	2	(0/2) 0%
Psikpé	2	(2/2) 100%
Sissili Bridge	6	(6/6) 100%
Walewale	131	(77/131) 58.77%
Total	234	(73/234) 31.19%

Table 5: Trypanosome prevalence in natural populations of *Glossina palpalis gambiensis* collected from Burkina Faso

Location	Sample size	Prevalence
Bama	77	(0/77) 0%
Comoé	123	(3/123) 2.43%
Folonzo	237	(27/237) 11.39%
Kartasso	136	(0/136) 0%
Kenedougou	41	(0/41) 0%
Moussodougou	142	(49/142) 34.50%
Total	731	(77/731) 10.51%

Prevalence of different trypanosome species in wild populations of tsetse in Western Africa

The results indicate that tsetse flies in West Africa could be infected with different species of trypanosomes in single or multiple infections. For *T. vivax* prevalence the best model retained countries as variable that fitted well the data indicating that the prevalence of *T. vivax* alone, did not differ significantly among tsetse fly species and sex (**Supplementary file 1**) but the mean infection rate of *T. vivax* in Senegal was significantly higher as compared to other countries ($P < 0.05$), also the prevalence in Ghana was significantly higher as compared to Guinea ($P = 0.030$; **Figure 3A**; **Supplementary file 1**).

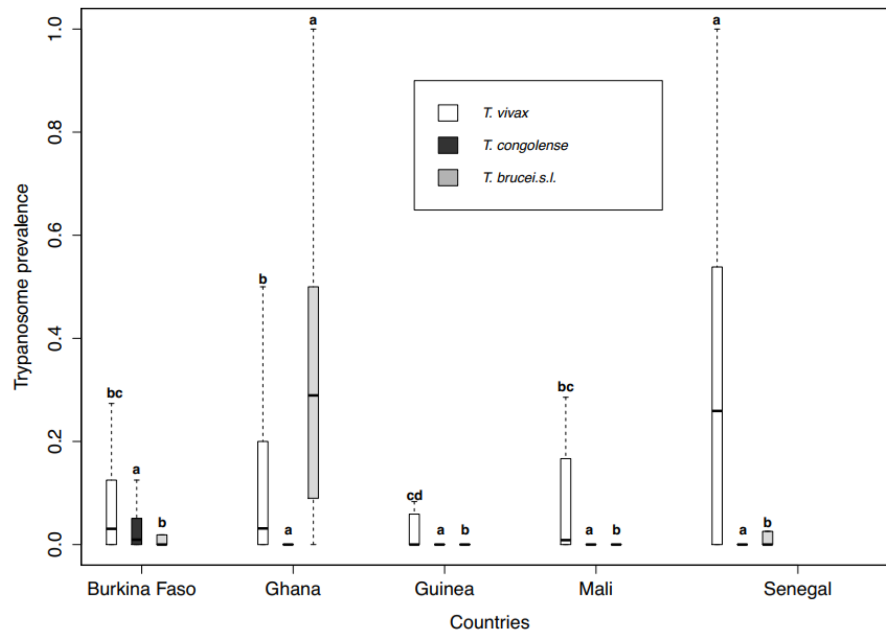

Figure 3A: Prevalence of *Trypanosoma vivax, Trypanosoma congolense* and *Trypanosoma spp* according to the country (A) and tsetse species (B). Boxes extend between the 25th and 75th percentile. A thick line denotes the median. The whiskers extend up to the most extreme values. Gmed: *Glossina medicorum*, Gmsm: *G. morsitans submositans*, Gpg: *G. palpalis gambiensis* and Gt: *G. tachinoides.*

GLM results for single infections with *T. congolense* selected for species as variable that fitted well the data indicated that the prevalence of *T. congolense* alone did not differ significantly among countries and sex **(Figure 3A)**. The *T. congolense* infection rate in *G. medicorum* was significantly higher as compared to *G. tachinoides*, *G. p. gambiensis* and *G. m. submorsitans* ($P < 0.05$; **Supplementary file 1)**. *T. congolense* infection rate in *G. p. gambiensis* was significantly lower as compared to *G. m. submorsitans* (**Figure 3B**; **Supplementary file 1**).

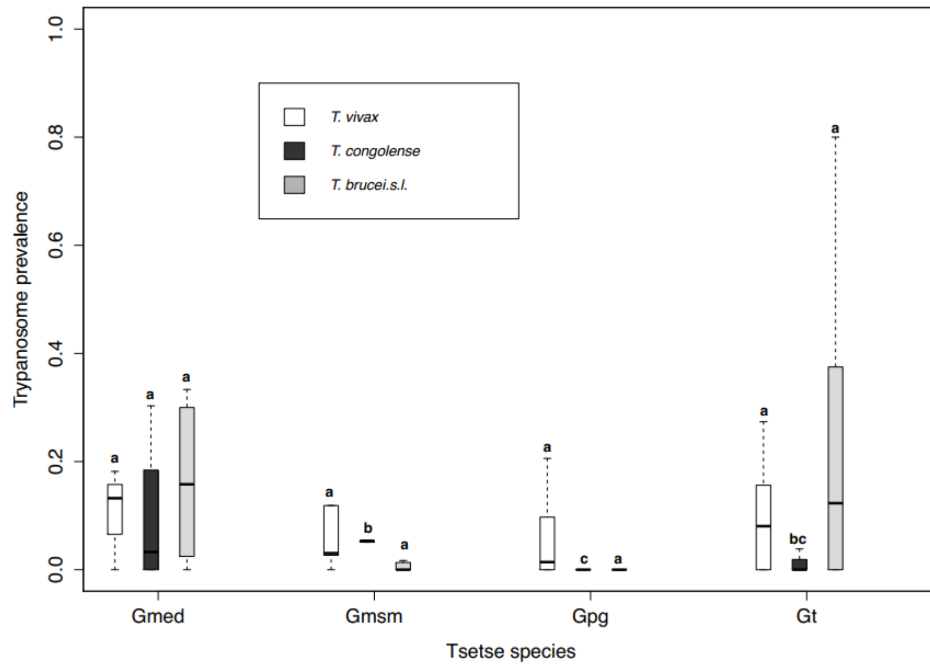

Figure 3B: Prevalence of *Trypanosoma vivax, Trypanosoma congolense* and *Trypanosoma spp* according to the tsetse species B. Boxes extend between the 25th and 75th percentile. A thick line denotes the median. The whiskers extend up to the most extreme values. Gmed: *Glossina medicorum*, Gmsm: *G. morsitans submositans*, Gpg: *G. palpalis gambiensis* and Gt: *G. tachinoides.*

Non-specific detection of *Trypanosoma spp.* (*Tz*) (including *T. brucei*, *T. evansi*, *T. equiperdum* and *T. theileri)* based on the primer detection was recorded in 19.4% of the samples (**Table 6**). Results model selected for countries as variable that fitted well the data indicating that the prevalence of *Trypanosoma spp*. did not differ significantly among countries and sex. The *Trypanosoma spp* prevalence in Ghana was significantly higher than the other countries ($P < 0.001$; **Figure 3A**; **Supplementary file 1**).

Table 6: Trypanosome species prevalence in natural populations of *Glossina* species from western Africa

	Tc	**Tv**	**Tz**	**Tc Tv**	**Tc Tz**	**Tv Tz**	**TvTcTz**
N° total positive				571			
N° positive	60	296	111	6	37	54	6
Percentage (%)	10.50	51.83	19.43	1.05	7.53	9.45	1.05

Tv: *Trypanosoma vivax* Tc:*Trypanosoma congolensis* and Tz: *Trypanosoma* Spp (*T. brucei, T. evansi, T. equiperdum* and *T. theileri*).

Analysis of the data with the well fitted model indicated that the coinfection of *T. congolense* with *T. vivax* did not differ between countries and sex. However *T. congolense* and *T. vivax* coinfection was significantly higher in *G. medicorum* (1.1%) as compared with the other tsetse species (*P* = 0.001; **Figure 4B**; **Supplementary file 1**). The coinfection rate of *T. vivax* and other *Trypanosoma spp* in Ghana was significantly higher than all other countries (*P* < 0.01, **Figure 4A**; **Supplementary file 1**). Analysis of coinfection of *T. congolense* and other *Trypanosoma spp* (7.5%) indicated that the infection rate in Ghana was significantly higher than Burkina Faso (*P* < 0.01, **Figure 4A**; **Supplementary file 1**).

Analysis of triple infection of *T. vivax*, *T. congolense* with other *Trypanosoma spp* selected for species as variable that fitted well the data indicating that the prevalence of *Trypanosoma spp* did not differ significantly among countries and sex. The infection rate in *G. medicorum* (1.1%) was significantly higher than in the other tsetse species (0%) (*P* < 0.001; **Supplementary file 1**).

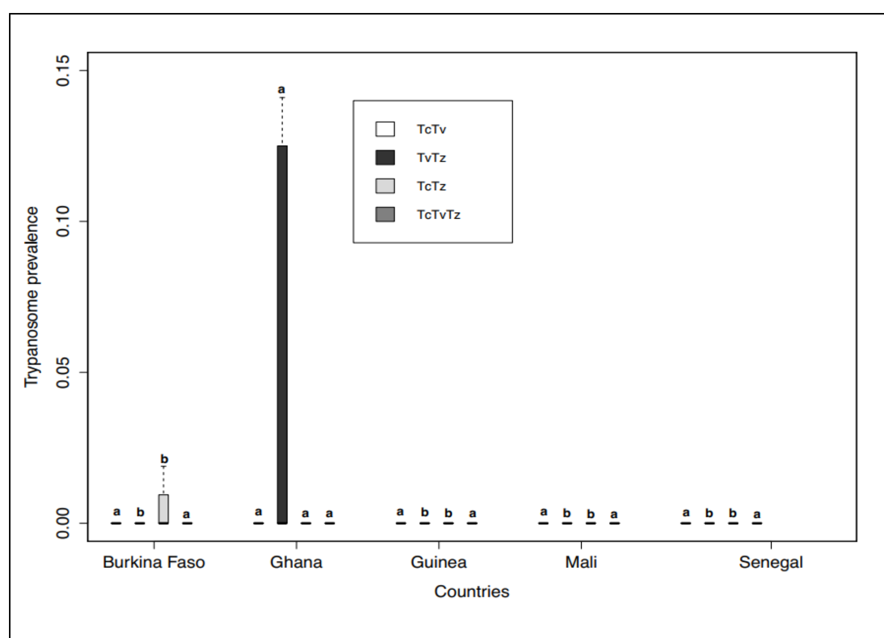

Figure 4A: Prevalence of Trypanosome coinfection according to the country. Boxes extend between the 25th and 75th percentile. A thick line denotes the median. The whiskers extend up to the most extreme values. Gmed: *Glossina medicorum*, Gmsm: *G. morsitans submositans*, Gpg: *G. palpalis gambiensis* and Gt: *G. tachinoides.* Tv*: Trypanosoma vivax*, Tc: *Trypanosoma congolensis* and Tz: *Trypanosoma spp (T. brucei*, *T. evansi*, *T. equiperdum* and *T. theileri).*

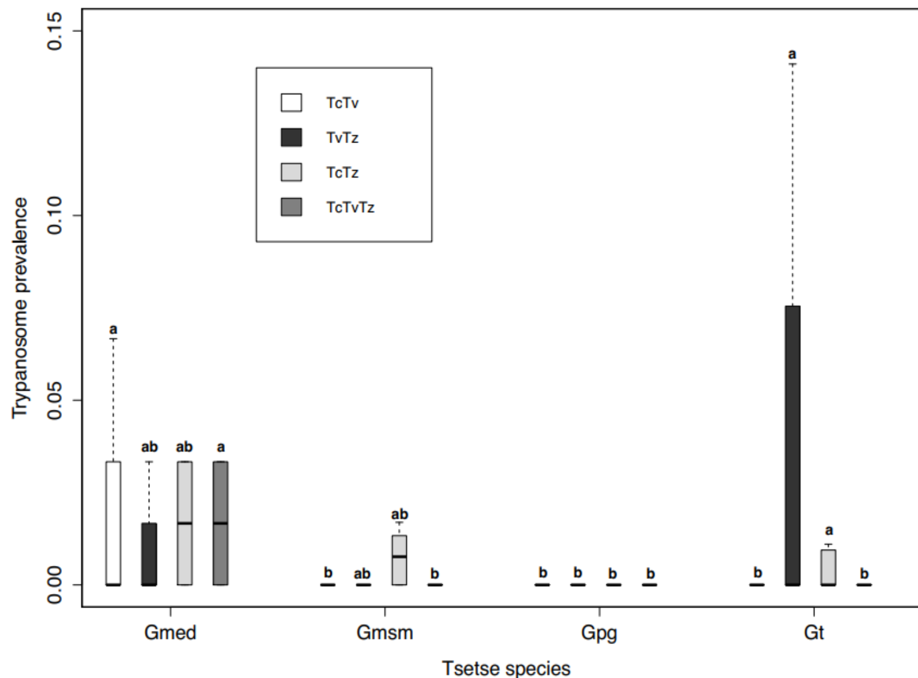

Figure 4B: Prevalence of Trypanosome coinfection according to the tsetse species. Boxes extend between the 25th and 75th percentile. A thick line denotes the median. The whiskers extend up to the most extreme values. Gmed: *Glossina medicorum*, Gmsm: *G. morsitans submositans*, Gpg: *G. palpalis gambiensis* and Gt: *G. tachinoides.* Tv*: Trypanosoma vivax*, Tc: *Trypanosoma congolensis* and Tz: *Trypanosoma spp (T. brucei*, *T. evansi*, *T. equiperdum* and *T. theileri).*

SGHV prevalence

Based on the PCR screen used in the present study, the average prevalence of SGHV in all collected flies was 1.7% (n = 54) **(Table 2).** The prevalence varied from 0% in *G. tachinoides* samples from Ghana and *G. p. gambiensis* samples from Senegal and Guinea to 4.1% in *G. p. gambiensis* flies from Mali **(Figure 5A and 5B)**. The result indicated that the SGHV prevalence of did not differ significantly among species and sex. However, the virus prevalence was significantly higher in Mali compared with the other countries (P = 0.001; **Supplementary file 1**).

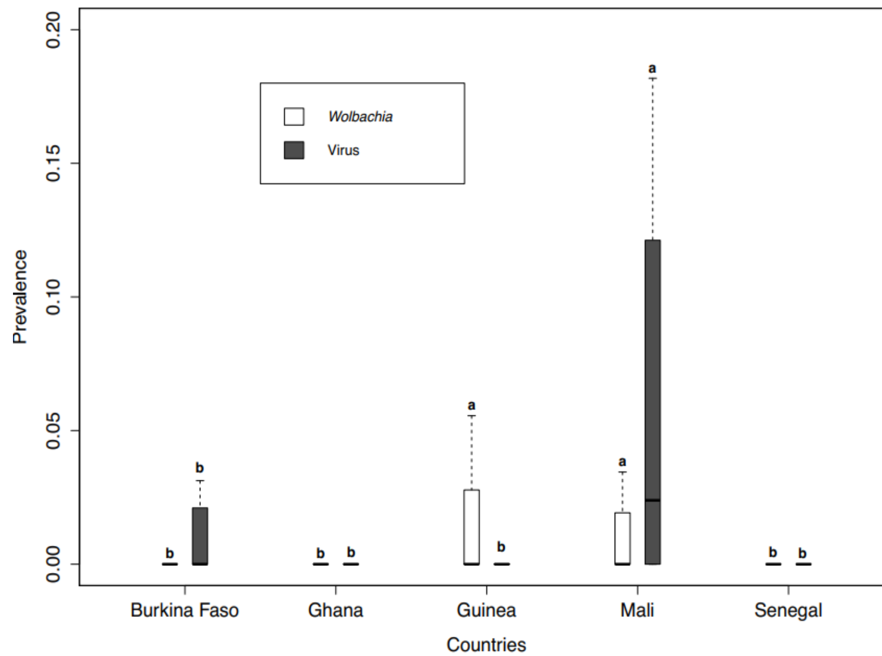

Figure 5A: Prevalence of Salivary gland hypertrophy virus (SGHV) and *Wolbachia* according to the country. Boxes extend between the 25th and 75th percentile. A thick line denotes the median. The whiskers extend up to the most extreme values. Gmed: *Glossina medicorum*, Gmsm: *G. morsitans submositans*, Gpg: *G. palpalis gambiensis* and Gt: *G. tachinoides.*

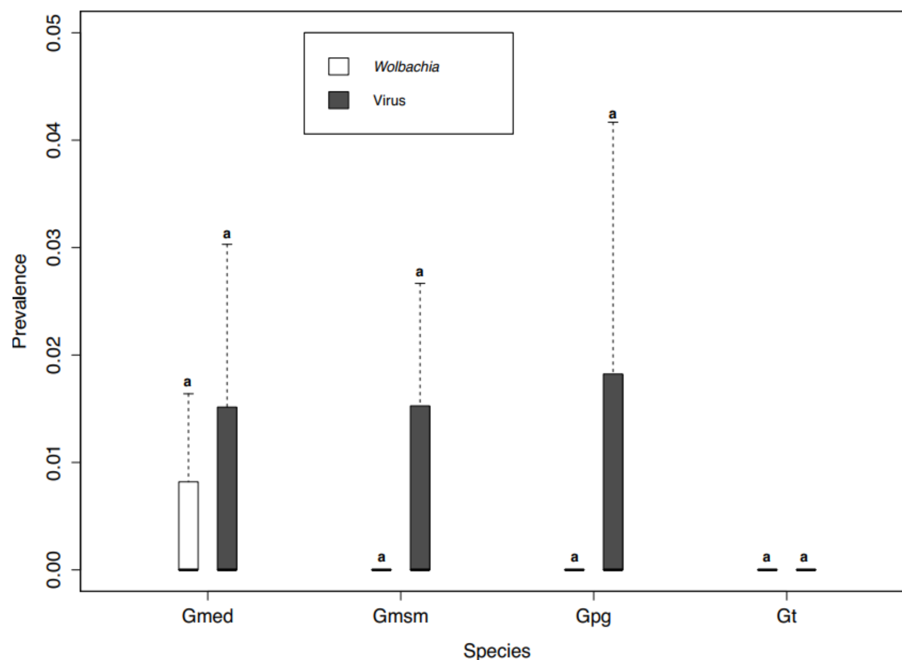

Figure 5B: Prevalence of Salivary gland hypertrophy virus (SGHV) and *Wolbachia* according to the tsetse species (B). Boxes extend between the 25th and 75th percentile. A thick line denotes the median. The whiskers extend up to the most extreme values. Gmed: *Glossina medicorum*, Gmsm: *G. morsitans submositans*, Gpg: *G. palpalis gambiensis* and Gt: *G. tachinoides.*

Prevalence of *Wolbachia*

The prevalence of *Wolbachia* was low in all tested species and averaged at 1.0 % (**Table 2**). The prevalence did not differ significantly among species and sex. The *Wolbachia* prevalence in Mali was significantly higher as compared to Senegal, Ghana and Burkina Faso ($P < 0.05$; **Supplementary file 1**). No other significant difference was observed **(Figure 5A and 5B)**.

Mixed infection of trypanosomes, SGHV and *Wolbachia*

The prevalence data indicate that the mean trypanosome infection rate was higher as compared with the prevalence of the SGHV and *Wolbachia*. Most of the flies that were infected with trypanosomes were negative for *Wolbachia*. In *G. tachinoides* and *G. m. submorsitans*, double infection with SGHV and trypanosomes was observed at a low prevalence, i. e. 0.5% and 0.4% respectively. No double infection of SGHV and trypanosome was detected in *G. p. gambiensis*. The *Trypansoma spp* infection rate was significantly positively correlated with that of the virus ($P < 0.001$), although the correlation was weak ($r = 0.45$). No significant correlation was observed between *Wolbachia* and SGHV.

Impact of tsetse fly gender on trypanosomes, SGHV and *Wolbachia* prevalence

There was no significant difference between male and female infection by trypanosomes ($P = 0.377$), SGHV ($P = 0.739$) or *Wolbachia* ($P = 0.362$).

Trypanosomes, SGHV and *Wolbachia* distribution per countries

Burkina Faso showed the highest species diversity with four tsetse species collected: *G. p. gambiensis*, *G. tachinoïdes*, *G. m. submorsitans* and *G. medicorum*. Among the ten localities sampled, these four species were found together in Folonzo and Comoe. *G. p. g. gambiensis* flies were found in four other localities: Bama, Kartasso, Kenedougou and Mousodougou. *G. tachinoïdes* and *G. m. submorsitans* flies were found together in Sissili, however, in Arly *G. tachinoïdes* only was found (**Table 2**). Flies infected with trypanosomes were found in five localities. *Trypanosoma vivax* prevalence was not different between localities and species (**Supplementary file 1**). For *T. congolense* no differences between localities were highlighted. However, significant differences were observed between tsetse species. *G. medicorum* was the most infected species (9%) and was different from all other species (*G .m. submorsitans* 5.2%; *G. tachinoïdes* 2.4% and *G. p. gambiensis* 0.4%; **Supplementary file 1**). For *Trypansoma spp*, significant differences were observed between tsetse species in Comoe and Folonzo. In both localities, *G. medicorum* (3.2% and 30% respectively) was significantly

more infected than *G. m. submorsitans*. (0.4% and 0.7% respectively) and *G. tachinoïdes* (0.2% and 1.5%) (**Supplementary file 1**). Flies infected with SGHV were found in four localities. No difference between tsetse species and localities was observed (**Supplementary file 1**). *Wolbachia* prevalence was not different between species. Tsetse flies (*G. tachinoïdes*, *G. p. gambiensis*, *G. medicorum* and *G. m. submorsitans*) from two localities were infected with *Wolbachia*. *Wolbachia* prevalence in tsetse flies from Kenedougou was significantly more important than Comoe (2.4% and 0.5% respectively).

In Mali, flies from only one tsetse species (*G .p. gambiensis)* were collected in the ten localities sampled. *T. vivax* infection was found in seven locality and the prevalence in Baoule (42.8%) was significantly higher than the others (Bagnuineda 16.6%, Banko 21.9%, Bani 1.4%, Kita 16.6%, Système Niger 1.1%, Système Sénégal 2%; **Supplementary file 1**). *T. congolense* was only found in Système Niger (1.1%) and *Trypansoma spp* in Sikasso (3.4%) and Système Niger (2.3%) with no differences. SGHV was found in the ten localities of Mali and Wolbachia in four without any differences (**Supplementary file 1**).

In Senegal, only *G .p. gambiensis* were found between the seven localities sampled. *T. congolense* infection was not found, however *T. vivax* infection was found in five localities (Mako, Fleuve G, Fleuve Gambie, Niokolo and Tambacounda) and *Trypansoma sp* in two (Diaguiri and Tambacounda) and no significant differences in trypanosome prevalence were found between different localities (**Supplementary file 1**). No SGHV and *Wolbachia* were found in tsetse flies analysed.

In Ghana, *G. tachinoides* was the only species caught among the eleven localities sampled and eight of them were found positive for trypanosomes. For *T. vivax,* significant differences in trypanosome prevalence were found between localities. The locality of Grogro showed the highest prevalence (36%) and was significantly different from all localities except Fumbissi. Contrary, the locality of Bougouhiya showed the lowest prevalence (0.05%) and was significantly different from Fumbissi, Grogro and Kumpole. Fumbissi was also different from Mortani, Sissili bridge and Walewale (**Supplementary file 1**). *T. congolense* was only found in one locality: Walewale. *Trypansoma spp* was found in the eight positive localities. Among these, flies collected at the localities of Kandiaga and Sissili bridge were the most infected (100% and 83% respectively) and were significantly different from all others but not between them. No virus and *Wolbachia* were found.

In Guinea, *G. tachinoides* was the only species caught among the eight localities sampled and six of them were found positive for trypanosomes. *T. congolense* and *Trypansoma spp* were not found and no significant difference in trypanosome prevalence for *T. vivax* was observed (**Supplementary file 1**). SGHV was absent and *Wolbachia* was found in three localities but no difference in prevalence was observed (**Supplementary file 1**).

Discussion

The results of this study indicate an overall low prevalence of SGHV and Wolbachia and a high prevalence of trypanosomes in the sampled wild tsetse populations. The prevalence of all three infections varied between species and between locations but there was no significant difference between male and female flies. All flies sampled in Kimpole (100%), Grogro (100%), Fumbissi (100%), Sissili Bridge (100%) and Psikpe (100%) of Ghana were infected with trypanosomes, an infection rate that was significantly higher as compared to other locations. In some cases, mixed infections with different trypanosome species were detected, as well as mixed infections of trypanosomes and SGHV. However, no mixed infection of trypanosomes or SGHV with *Wolbachia* was detected.

The method of detection and characterization of the type of trypanosome infection using the ribosomal internal transcribed spacer (ITS) is known to be sensitive and it provides quick information about the trypanosome type circulating in the infected area. However,, these identified trypanosomes may not be the only ones circulating within the different areas as was observed in Guinea. Other types of trypanosome species may also be circulating but due to the lack of PCR primers cannot be identified [54]. In addition, Pagabeleguem et al [55] noted that the trypanosome infection rate in tsetse flies was always higher by microscopy than PCR and suggested that almost half of the flies were infected by trypanosome species non-pathogenic for cattle.

The relatively high frequency of pathogenic trypanosomes in tsetse was previously linked to high AAT prevalence in cattle, especially in the locality of Folonzo in Burkina Faso [55]. It has therefore been suggested that the detection of trypanosome infection in tsetse flies might provide indirect information about the AAT prevalence in livestock in the selected area and hence the potential risk of clean animals to become infected. This may not be so relevant for HAT as the link between tsetse infection and disease prevalence is considered to be weak. In Guinea, for example, *T. brucei gambiense* is the pathogenic trypanosome identified in

humans, while no *T. brucei gambiense* infection has been found in tsetse confirming the usual very low (0.1%) mature infection rates of *T. brucei gambiense* in tsetse, even in active sleeping sickness foci [56].

The SGHV was reported in *G. p. palpalis* in Côte d'Ivoire in 1978 at a very low prevalence (0.3%) [57]. Although the prevalence of SGHV based on fly dissection was generaly low in wild tsetse populations (0.5-5%) [58] based on fly dissection, the prevalence detected by PCR can be very high (100%) [47]. These results clearly indicate that the SGHV prevalence in tsetse species in West Africa is significantly lower than the SGHV prevalence in *G. pallidipes* in eastern and southern Africa previously reported [47], where the virus prevalence varied from 2% to 100%, depending on the location. However the low virus prevalence in West African tsetse populations might be underestimated due to the primer specificity and the sensitivity of the PCR, as all primers were based on the nucleotide sequence of *G. pallidipes* SGHV. A different virus sequence in other tsetse species in West Africa would then result in a lower detection rate. To overcome this problem it is suggested to have the entire genome sequenced of each virus detected in each tsetse species to enable the selection of more specific and sensitive primers for virus detection.

Wolbachia is known to be present in wild tsetse populations [29, 59], and using standard PCR assays, it was detected in *G. m. morsitans*, *G. m. centralis* and *G. austeni* populations, but not in *G. tachinoides*. Using alternative assays *Wolbachia* was also detected at low infection rates in *G. fuscipes* and *G. morsitans* subspecies [59, 60]. The prevalence of *Wolbachia* in *G. p. gambiensis* from Burkina Faso was very low (~0.14%). In *G. m. morsitans* the prevalence of *Wolbachia* was higher and varied between 10 and 100% depending on the location [51]. In *G. f. fuscipes* collected from Uganda, the prevalence of *Wolbachia* varied between 26 and 55%, which is higher than the prevalence reported in this study [29]. It is important to note that in the study of Alam and colleagues the detection method used for screening the *Wolbachia* infection was the sequential PCR method (high sensitivity but low specificity). In this study and in the study of Doudoumis and colleagues, a traditional one step PCR was used for the detection [29, 59] to avoid any non-specific detection and to detect only high level *Wolbachia* infections that might interfere with the virus and trypanosome infection. We also tried to avoid detecting *Wolbachia* chromosomal insertions by using primers specific for active *Wolbachia* in the cytoplasmic [29, 61]. Presence of extensive *Wolbachia* insertions was discovered in the genome of its host *G. m morsitans* [61]. The low prevalence of *Wolbachia* detected in wild tsetse population in this study might be due to (i) the absence of *Wolbachia*

infection, (ii) the low titer of *Wolbachia* infection or (iii) the presence of another *Wolbachia* strain that cannot be detected with the primers used in this study.

Mixed infections of trypanosomes, SGHV and *Wolbachia* have been previously reported [59] and this was also the case in our study, although the correlation was low (r = 0.45; $P < 0.001$). In the study of Alam et al [59], the author mentioned the potential negative relationship between *Wolbachia* and SGHV infection, which was also observed in our study. Trypanosome infection was found in flies that were also infected with the SGHV but no flies that were infected with *Wolbachia* showed a trypanosome infection. This suggests that the presence of *Wolbachia* might mediate the presence of different pathogens and parasites, as previously described [36, 62, 63]. Due to the low prevalence of *Wolbachia*, no possible correlation between the *Wolbachia* infection and the trypanosomes and/or SGHV could be found. On the other hand a negative impact of trypanosome infection on *Wolbachia* presence cannot be excluded. However, these antagonistic relationships need further investigation and statistical analysis. If the assumption that *Wolbachia* might block trypanosome transmission is correct, these novel insights could be useful for the development and implementation of sterile insect technique-based population control strategies, e.g. releasing *Wolbachia*-infected males that both induce cytoplasmic incompatibility when mated with wild *Wolbachia*–free females and being refractory for trypanosome infection and transmission in a way similar to that recently developed for mosquitoes [64-67].

Conclusion

The results of this study indicate a high rate of trypanosome infection in tsetse wild populations but lower infection rate of both *Wolbachia* and SGHV. Mixed infections with different trypanosome species or trypanosome with SGHV were found. The high rate of trypanosome infection in tsetse populations might be used an indicator of the presence of trypanosomiosis in both human and animal by determining the different trypanosomes circulation in the targeted area. The low prevalence of *Wolbachia* in tsetse flies in West Africa and the lack of mixed infection of *Trypanosoma* spp., and *Wolbachia*, which might indicate an antagonistic relationship, require further investigation. The low prevalence of SGHV in the field population is encouraging for SIT programmes as it might exclude the SGHV outbreaks in tsetse mass-rearing established from such low infected populations;

however it encourages the implementation of the virus management strategies to control the virus infection to avoid such problem.

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Author Contributions

AMMA, AGP, BB, TA, SI, OAG: designed and supervised the research. OGMS, GDU, AA, AGP, AMMA: conducted the experiments. GG, OGMS, AGP, collected and analyzed data and prepared the figures. OGMS, ATC, PT, AGP, RJB: Provided live material for experiments. OGMS, GDU, AGP, MJBV, BK, RJB, GG, BB, TA, SI: participated in the writing of the manuscript. All authors have read and agreed to its content and that the manuscript conforms to the journal's policies.

Competing interests

The authors declare that they have no competing interests.

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Supplementary file 1.

Generalized linear model (GLM) fixed effect statistical results.

Test	Fixed effects	estimate	SE	T-value	P-value
Overall trypanosome prevalence	Intercept	0.37689	0.14872	2.534	0.01317
	Gpg	-0.35270	0.13576	-2.598	0.01112
	Gt	-0.27785	0.14400	-1.930	0.05712
	Gmsm	-0.27160	0.15774	-1.722	0.08888
	MLI	0.08008	0.08221	0.974	0.33285
	SN	0.31893	0.09600	3.322	0.00134
	GH	0.59835	0.13520	4.426	2.93e-05
	BKF	0.05504	0.09107	0.604	0.54728
Overall trypanosome prevalence	Intercept	0.424225	0.145590	2.914	0.00460
	Gmed	0.271598	0.157743	1.722	0.08888
	Gpg	-0.081106	0.125168	-0.648	0.51881
	Gt	-0.006252	0.134056	-0.047	0.96291
	GN	-0.318933	0.095999	-3.322	0.00134
	MLI	-0.238850	0.092744	-2.575	0.01181
	GH	0.279417	0.141850	1.970	0.05224
	BKF	-0.263893	0.100685	-2.621	0.01045
Trypanosoma vivax prevalence	Intercept	0.09770	0.04212	2.320	0.02277
	GH	0.06941	0.05957	1.165	0.24716
	SN	0.22951	0.07048	3.256	0.00162
	BKF	-0.02289	0.05362	-0.427	0.67055
	GN	-0.06796	0.06247	-1.088	0.27978
Trypanosoma vivax prevalence	Intercept	0.32720	0.05651	5.790	1.15e-07
	MLI	-0.22951	0.07048	-3.256	0.001622
	BKF	-0.25240	0.06553	-3.851	0.000227
	GH	-0.16009	0.07048	-2.271	0.025643
	GN	-0.29746	0.07295	-4.077	0.000102
Trypanosoma vivax prevalence	Intercept	0.02974	0.04614	0.645	0.520920
	SN	0.29746	0.07295	4.077	0.000102
	MLI	0.06796	0.06247	1.088	0.279784
	GH	0.13737	0.06247	2.199	0.030609
	BKF	0.04507	0.05683	0.793	0.430021
Trypanosoma congolensis prevalence	Intercept	0.012935	0.006171	2.096	0.0390
	Gmed	0.079216	0.016899	4.687	1.03e-05
	Gpg	-0.011808	0.007489	-1.577	0.1185
	Gmsm	0.029255	0.015365	1.904	0.0603
Trypanosoma congolensis prevalence	Intercept	0.04219	0.01407	2.998	0.00355
	Gt	-0.02925	0.01537	-1.904	0.06026
	Gmed	0.04996	0.02111	2.367	0.02018
	Gpg	-0.04106	0.01470	-2.794	0.00642
Trypanosoma Spp (Tz) prevalence	Intercept	0.34998	0.03820	9.162	2.54e-14
	BKF	-0.31235	0.04863	-6.423	7.36e-09
	GN	-0.34998	0.05666	-6.177	2.17e-08
	SN	-0.29010	0.06392	-4.538	1.85e-05
	MLI	-0.34447	0.05402	-6.377	9.03e-09
Tc-Tv prevalence	Intercept	0.016667	0.003287	5.070	2.25e-06
	Gt	-0.015658	0.003531	-4.435	2.71e-05
	Gmsm	-0.016667	0.004410	-3.779	0.000289
	Gpg	-0.016667	0.003405	-4.895	4.54e-06
Tv-Tz prevalence	Intercept	0.13394	0.02686	4.987	3.21e-06
	BKF	-0.12682	0.03419	-3.709	0.000370
	GN	-0.13394	0.03984	-3.362	0.001161

Test	Fixed effects	estimate	SE	T-value	P-value
	SN	-0.11124	0.04494	-2.475	0.015308
	MLI	-0.13394	0.03799	-3.526	0.000682
Tc-Tz prevalence	Intercept	0.037894	0.008111	4.672	1.11e-05
	BKF	-0.032569	0.010325	-3.154	0.00222
	GN	-0.037894	0.012030	-3.150	0.00225
	SN	-0.037894	0.013572	-2.792	0.00647
	MLI	-0.037894	0.011470	-3.304	0.00140
Tc-Tv-Tz prevalence	Intercept	0.016667	0.003533	4.717	9.17e-06
	Gt	-0.013997	0.003795	-3.688	0.000395
	Gmsm	-0.016667	0.004740	-3.516	0.000702
	Gpg	-0.016667	0.003659	-4.555	1.72e-05
SGHV virus prevalence	Intercept	0.034894	0.009162	3.808	0.000263
	GH	-0.034894	0.012957	-2.693	0.008530
	SN	-0.034894	0.015331	-2.276	0.025362
	BKF	-0.032345	0.011664	-2.773	0.006824
	GN	-0.011395	0.013590	-0.839	0.404099
Wolbachia prevalence	Intercept	0.034894	0.009162	3.808	0.000263
	GH	-0.034894	0.012957	-2.693	0.008530
	SN	-0.034894	0.015331	-2.276	0.025362
	BKF	-0.032345	0.011664	-2.773	0.006824
	GN	-0.011395	0.013590	-0.839	0.404099
the *T.v.* prevalence in Burkina Faso	Intercept	0.15648	0.05654	2.768	0.0244
	Folonzo	-0.02315	0.09793	-0.236	0.8191
	Moussodougou	0.11973	0.09793	1.223	0.2563
	Sissili	-0.02716	0.07996	-0.340	0.7428
	Gmsm	-0.12788	0.07996	-1.599	0.1484
	Gpg	-0.14240	0.09793	-1.454	0.1840
	Gt	-0.03349	0.07996	-0.419	0.6864
	Folonzo:Gmsm	0.22720	0.12642	1.797	0.1100
	Folonzo:Gpg	0.05152	0.13849	0.372	0.7196
	Folonzo:Gt	0.09832	0.12642	0.778	0.4591
T.c. prevalence in Burkina Faso	Intercept	0.18430	0.04133	4.459	0.000964
	Gmsm	-0.13156	0.05062	-2.599	0.024743
	Gpg	-0.17005	0.05336	-3.187	0.008656
	Gt	-0.13781	0.04773	-2.887	0.014777
T.z. prevalence in Burkina Faso	Intercept	0.030303	0.016419	1.846	0.124
	Folonzo	-0.025160	0.016419	- 1.532	0.186
	Kartasso	0.005298	0.016419	0.323	0.760
	Moussodougou	0.005378	0.016419	0.328	0.757
	Gmsm	-0.015036	0.023220	- 0.648	0.546
	Gpg	-0.013645	0.020109	- 0.679	0.528
	Gt	0.014817	0.020109	0.737	0.494
	Folonzo:Gmsm	0.036560	0.028439	1.286	0.255
	Folonzo:Gpg	0.065106	0.025961	2.508	0.054
Wolbachia prevalence in Burkina Faso	Intercept	0.008062	0.002904	2.776	0.0692
	Kenedougou	0.033604	0.006493	5.175	0.0140
T.v. prevalence in Mali	Intercept	0.42857	0.02822	15.184	0.00431
	Baguinega	-0.26190	0.04889	- 5.357	0.03312
	Banco	- 0.18277	0.03992	- 4.579	0.04453
	Bani	-0.41133	0.04889	- 8.414	0.01383
	Kita	- 0.26190	0.04889	- 5.357	0.03312
	SN	- 0.40934	0.04889	- 8.373	0.01397
	SS	- 0.38857	0.04889	- 7.948	0.01546
SGHV.	Intercept	0.16667	0.05889	2.830	0.0473

Test	Fixed effects	estimate	SE	T-value	P-value
prevalence in Mali	Banco	-0.13725	0.08328	- 1.648	0.1747
	Bani	0.0671	0.07212	- 0.931	0.4046
	Bougouni	-0.01190	0.07212	- 0.165	0.8769
	Sikasso	-0.04545	0.08328	- 0.546	0.6142
	SN	-0.14277	0.07212	- 1.979	0.1189
	SS	-0.12583	0.07212	- 1.745	0.1560
	Intercept	0.16667	0.05889	2.830	0.0473
Wolbachia prevalence in Mali	Intercept	0.14286	0.09085	1.572	0.361
	Bani	-0.10837	0.12848	- 0.843	0.554
	Sikasso	0.07290	0.11127	0.655	0.631
	SN	-0.12363	0.12848	- 0.962	0.512
T.v. prevalence in Senegal	Intercept	0.1111	0.1644	0.676	0.5689
	Fleuve gambie	0.2326	0.2014	1.155	0.3674
	Mako	0.8889	0.2325	3.823	0.0621
	Niokolo	0.5556	0.2325	2.389	0.1395
	Tambacounda	0.2923	0.2014	1.451	0.2838
*T.z.*prevalence in Senegal	Intercept	0.50000	0.03361	14.88	0.0427
	Tambacounda	0.45059	0.04116	- 10.95	0.0580
T.v. prevalence in Senegal	Intercept	0.1111	0.1644	0.676	0.5689
	Fleuve gambie	0.2326	0.2014	1.155	0.3674
	Mako	0.8889	0.2325	3.823	0.0621
	Niokolo	0.5556	0.2325	2.389	0.1395
	Tambacounda	0.2923	0.2014	1.451	0.2838
T.v. prevalence in Ghana	Intercept	0.80000	0.05279	15.155	0.00433
	Bougouhiya	- 0.73750	0.07466	-9.879	0.01009
	Fumbissi	- 0.13333	0.07466	-1.786	0.21602
	Kumpole	- 0.35000	0.06465	-5.413	0.03247
	Mortani	- 0.64375	0.07466	-8.623	0.01318
	Sissili bridge	- 0.60000	0.07466	-8.037	0.01513
	Walewale	- 0.68873	0.06465	-10.653	0.00870
T.z. prevalence in Ghana	Intercept	0.90000	0.06721	13.392	1.07e-05
	Kumopole	- 0.35000	0.09504	-3.683	0.010300
	Mortani	- 0.65521	0.09504	- 6.894	0.000460
	Fumbissi	- 0.54583	0.09504	- 5.743	0.001212
	Grogro	- 0.71667	0.09504	- 7.540	0.000282
	Kandiaga	- 0.10000	0.11640	0.859	0.423284
	Psikp	- 0.40000	0.11640	- 3.436	0.013863
	Walewale	-0.73249	0.09504	- 7.707	0.000250
T.v. prevalence in Guinea	Intercept	0.05000	0.01511	3.309	0.187
	Dekonkore	0.01250	0.02137	0.585	0.663
	Karifale	0.03333	0.02137	1.560	0.363
	Lemonako	0.03333	0.02137	1.560	0.363
	Mini	- 0.01552	0.02137	- 0.726	0.600
	Tinkisso	0.01624	0.01850	0.878	0.541
Wolbachia prevalence in Guinea	Intercept	0.08846	0.01154	7.667	0.0826
	Kifala	0.03154	0.01999	1.578	0.3596
	Tinkisso	- 0.03291	0.01999	- 1.647	0.3475

Chapter 4

Susceptibility of Tsetse Species to *Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV)

Susceptibility of Tsetse Species to *Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV)

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Abstract

Salivary gland hytrosaviruses (SGHVs, family *Hytrosaviridae*) are non-occluded dsDNA viruses that are pathogenic to some dipterans. SGHVs primarily replicate in salivary glands (SG), thereby inducing overt salivary gland hypertrophy (SGH) symptoms in their adult hosts. SGHV infection of non-SG tissues results in distinct pathobiologies, including reproductive dysfunctions in the tsetse fly, *Glossina pallidipes* (Dipteran; Glossinidae). Infection with SGHV in *G. pallidipes* resulted in the collapse of several laboratory colonies, which hindered the implementation of area wide integrated pest management (AW-IPM) programs that had a sterile insect technique (SIT) component. In the current study, we assessed the susceptibility of six *Glossina* species (*G. pallidipes*, *G. brevipalpis, G. m. morsitans, G. m. centralis*, *G. f. fuscipes* and *G. p. gambiensis*) to SGHV infections, and the impact of the viral infection on the fly pupation rate, adult emergence, and virus replication and transmission from the larval to adult stages. We also evaluated the ability of the virus to infect conspecific *Glossina* species through serial passages. The results indicate that the susceptibility of *Glossina* to GpSGHV varied widely amongst the tested species, with *G. pallidipes* and *G. brevipalpis* being the most susceptible and most refractory to the virus, respectively. Further, virus injection into the hemocoel of teneral flies led to increased viral titers over time, while virus injection into the third instar larvae delayed adult eclosion. Except in *G. pallidipes*, virus injection either into the larvae or teneral adults did not induce any detectable SGH symptoms. Further, virus infections were PCR-detectable in the fly carcasses that did not show infections of the SGs. Taken together, our results indicate that although GpSGHV may only cause minor damage in the mass-rearing of tsetse species other than *G. pallidipes*, preventive control measures are required to avoid viral contamination and transmission in the fly colonies, particularly in the facilities where multiple tsetse species are reared.

1 Introduction

The hematophagous tsetse flies (*Glossina* spp.) are responsible for transmission of African trypanosomoses, a group of anthropozoonotic neglected tropical diseases affecting humans and their livestock in most of sub-Sahara Africa (Steelman, 1976). The tsetse-infested countries are amongst the world's least developed where hunger and poverty have been partially attributed to the presence of tsetse and trypanosomosis (Vreysen, 2006). The Lack of effective vaccines and drugs against trypanosomoses makes tsetse vector control an attractive and sustainable disease management option (Leak, 1998). A promising vector control

approach is the sterile insect technique (SIT), particularly when it is applied within the frame of an AW-IPM approach (Klassen and Curtis, 2005; Vreysen et al., 2013). This control tactic depends heavily on large-scale production of sterile males, which upon release into the field, out-compete the wild males in mating wild virgin females; these matings result in no offspring, which will eventually lead to a decline and eventual elimination of the target insect populations (Vreysen et al., 2000). However, the *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV; *Hytrosaviridae* family) seriously hampers mass-production of *G. pallidipes,* a competent vector of several trypanosomes (Abd-Alla et al., 2010; Moloo et al., 1992). Although the GpSGHV has not been reported to cause any significant problems in the rearing of other tsetse species, earlier studies reported SGH symptoms in natural tsetse populations of *Glossina austeni*, *G. m. morsitans*, *G. nigrofusca nigrofusca*, and *G. pallicera pallicera* (Burtt, 1945; Ellis and Maudlin, 1987; Gouteux, 1987). It is unclear whether the viral strain or isolate found in *G. pallidipes* is the same strain reported in other tsetse species. Consequently, if adequate virus management strategies are not put in place, there is a risk of the spread of GpSGHV to tsetse species other than *G. pallidipes* that are mass-produced for vector control programs that have an SIT component. Therefore, it is important to investigate the impact of GpSGHV infection in other tsetse species that are of specific interest for SIT/AW-IPM campaigns against tsetse and trypanosomosis.

Members of the *Hytrosaviridae* family consist of a small group of enveloped, rod-shaped dsDNA viruses that infect some dipteran insects, in which they replicate in the salivary glands (SGs) that as a result become enlarged (SGH) (Abd-Alla et al., 2009b). So far, hosts for hytrosaviruses (SGHVs) include the hematophagous tsetse fly (infected with GpSGHV), the filth-feeder housefly *Musca domestica* (infected with MdSGHV), and the phytophagous syrphid fly *Merodon equestris* (Amargier et al., 1979). Unlike in the housefly where only one MdSGHV strain has been detected and sequenced, genomes of two GpSGHV strains/isolates have been fully sequenced; the two strains induce different pathobiologies in different tsetse rearing facilities based on the tsetse species, origin and domestication period (Abd-Alla et al., 2016). The observed differential GpSGHV pathologies might be attributed to genetic differences between the virus strains.

Due to the tsetse fly's adenotrophic viviparity, GpSGHV is readily transmitted via the milk gland secretions from the mother fly to the developing larvae (Boucias et al., 2013), and in most cases, the virus persists in an asymptomatic infection state. GpSGHV can induce cellular hypertrophy of the SG cells (i.e. enlarged SG cells capable of replication) (Kariithi et al.,

2013), which is associated with sterility in males and a reduction in the fecundity of female. Infection with GpSGHV in mass-rearing facilities occurs through feeding (*per os)* and via vertical transmission from mother to offspring (Abd-Alla et al., 2010). However, *G. pallidipes* is highly susceptible to intra-hemocoelic GpSGHV injection, which results in high viral titers ($\geq 10^9$ viral genome copies) but without either the onset of overt SGH symptoms or the release of detectable viral particles via fly saliva during *in vitro* membrane feeding (Boucias et al., 2013).

Due to the low number of SGHV strains/strains, and the limited studies conducted, little is known about the host range of these viruses. The virus (es) seems not to be restricted to *G. pallidipes* because earlier studies reported the occurrence of the SGH symptoms in several tsetse species (Burtt, 1945; Ellis and Maudlin, 1987; Gouteux, 1987). Additionally, injection of GpSGHV into third instar larvae of *G. m. morsitans* and *G. m. centralis* induced overt infection in both the male and female adults that emerge from the virus-injected larvae (Jura et al., 1993; Jura and Davies-Cole, 1992; Kariithi et al., 2013; Sang et al., 1996, 1997). However, these previous studies did not confirm any similarity between the viral genome injected in the larvae and the virus in the adults. Since latent GpSGHV infection state was confirmed, it is possible that the virus in the adults of *G. m. morsitans* and *G. m. centralis* is a different latent virus induced by the artificially-injected virus. This phenomenon was previously demonstrated the reactivation of latent *Mamestra brassicae* nuclear polyhedrosis virus (MabrNPV) infection by feeding the larvae of the cabbage moth (*M. brassicae*) larvae with the pine wood moth *Panolis flammea* NPV (PfNPV), which is related to MabrNPV (Hughes et al., 1993). Another study confirmed that serial passages of the virus in the insect host increase pathogenicity of the virus (Gani et al., 2014).

In the current study, in order to investigate the host range of the GpSGHV and its impact on other tsetse species, we assessed the susceptibilities six *Glossina* species to GpSGHV infection. We also evaluated the replication of the GpSGHV following intra-hemocoelic virus injection into larval and adult stages of tested species. Further, dissections and qPCR were used to assess the induction of latent infection, the development of overt SGH symptoms in adults emerging from virus-injected third-instar larvae and in the F_1 progeny produced by virus-infected mothers, and to assess the potential of enhancement of unapparent SGHV infection in conspecific larvae of the six *Glossina* species.

2 Materials and Methods

2.1 Tsetse species and experimental set-up

The six *Glossina* species (*G. pallidipes* (Uganda), *G. brevipalpis* (Kenya), *G. morsitans morsitans* (Zimbabwe), *G. morsitans centralis* (Tanzania), *G. fuscipes fuscipes* (Central African Republic) and *G. palpalis gambiensis* (Burkina Faso)) used in this study were obtained from colonies maintained at the Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria. Unless otherwise stated, all experimental flies were fed on warm, defibrinated bovine blood for 10-15 minutes, three times weekly, using an *in vitro* membrane feeding technique (Feldmann, 1994). Tsetse adults, all deposited third instar larvae, and pupae were incubated at 24±0.5°C until adult eclosion. Fly productivity and adult emergence were assessed using standard procedures (Feldmann, 1994).

2.2 Virus source and inoculations

Salivary glands (SGs) with overt SGH symptoms were dissected from *G. pallidipes* males **(Figure 1)** and used to prepare the virus inoculum as described by Boucias et al. (2013) with slight modifications that included aseptic SG dissections and use of non-filtered virus inoculum. For conspecific virus injections, other than in *G. pallidipes*, SGs were dissected from 10-day old flies (males and females) that emerged from the larvae produced by mothers initially injected with the virus suspension derived from virus-infected *G. pallidipes*. Infection in the SGs was verified by the PCR protocol described by Abd-Alla et al. (2007). After the PCR diagnostics, the PCR-positive SG homogenates were used to prepare the virus inoculum as described above.

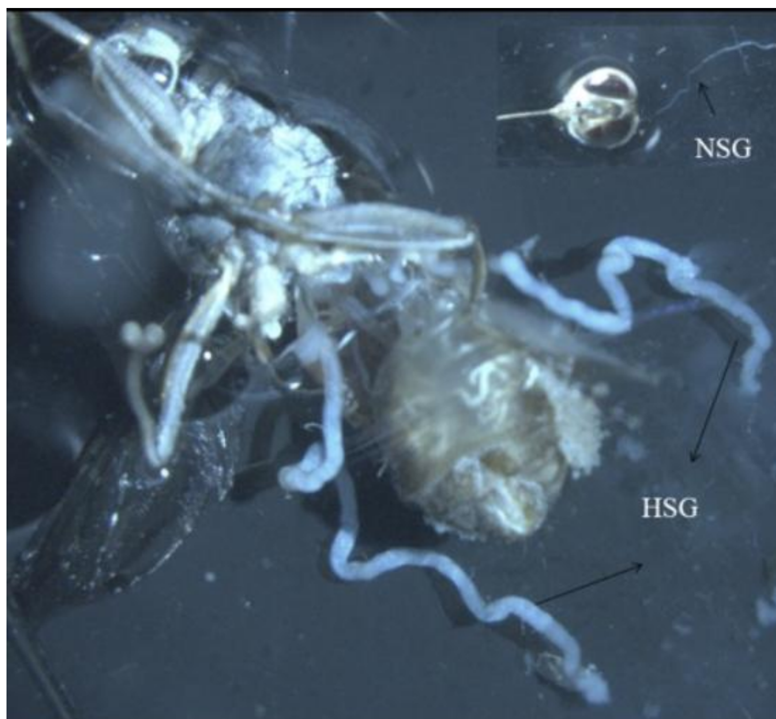

Figure 1: Symptoms of *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) in tsetse fly *G. pallidipes* males with Normal (NSG) and Hypertrophied (HSG) salivary gland.

2.3 Virus replication in adults and transmission to F1 progenies

To monitor GpSGHV replication in adults, and the transmission of the virus from infected parents to their F_1 progeny, teneral (non-fed) flies were immobilized (2-6°C for 5 min) 24 h post emergence, then maintained in a plastic Petri-dish on ice and inoculated in the thoracic cavity with either 2 µl of filter-sterilized phosphate buffered saline (PBS, control) or 2 µl of the virus suspension using a 1ml Myjector U-40 Insulin type syringe (Teruma, Leuven, Belgium). For each tsetse species, 160 male and female flies at a 1:3 mating ratio were injected and placed in standard tsetse holding cages (20 cm diameter x 5 cm height) with a density of 80 flies per cage; 2-3 replications were carried out for each species. After the injections, 8 flies (6 females and 2 males) were randomly sampled from each treatment at 0-, 1-, 3-, 5-, 7- and 9-days post injection (dpi), and subsequently frozen at -20°C until further analyses.

2.4 Extraction of total DNA and PCR amplifications

After each sampling time-point, the remaining flies (n=112) were maintained under standard rearing conditions for 120 days and collected pupae were incubated until adult emergence of the F_1 progeny. All flies that remained alive at the end of the 120-day experimental period were dissected to assess for SGH symptoms. The F_1 flies that emerged from the collected

pupae were reared until they were 10 days old, after which 8 flies from each treatment were randomly selected and frozen at -20°C until further analyses. Total DNA of individual flies was extracted from the samples collected in the parental and F_1 generations using the DNeasy tissue kit (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. Viral titers were estimated using pooled genomic DNA (6 females and 2 males). For each DNA pool, the DNA concentration in each individual fly was determined using a spectrophotometer (Nanodrop-Synergy H1 Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA), followed by dilutions to ensure that all individual samples contained equal final DNA concentrations. Then, 30 µl of each diluted DNA sample was pooled and used to quantify viral titers by quantitative PCR (qPCR) (Abd-Alla et al., 2009a) at 0-, 1-, 5, and 9- dpi; the tsetse *β-tubulin* gene was used as a housekeeping gene to normalize the qPCR reactions. The primers and the PCR condition are given in **Supplementary Table 1**.

2.5 Impact of GpSGHV on survival and productivity of injected adults

To assess the impact of virus infection on fly survival and productivity, PBS- and virus-injected teneral females and males were kept together for mating (Gooding et al., 1997). Pupal production was monitored weekly for 12 weeks, and the fly productivity was expressed as number of pupae per female per 10 days (pf10d). The total percentage weekly mortality of the adult flies was recorded and pupae that did not emerge by day 35 of incubation were considered dead, and were therefore discarded. The emerged F_1 flies were sexed and reared for 10 days post emergence, after which the prevalence of SGH symptoms and viral titers were assessed as described above.

2.6 Virus replication in the pupal stage and transmission to the adult stage

To monitor virus replication during the transition of larvae into adult stages, freshly deposited third instar larvae were injected with either PBS or virus suspension using a modified protocol previously described by Jura et al. (1993). Briefly, larvae were briefly immobilized (1 min at 4°C), and then injected with 1 µl of PBS or virus suspension using a 100-µl NanoFil syringe (World Precision Instruments, Inc., Sarasota, USA) equipped with a 35-gauge beveled needle. The needle was accurately placed 1 mm away from the two larval polypneustic lobes. Correct injection was verified by observing blanching of the larva. Larvae were then placed in plastic dishes over ice for 1 min to allow wound-healing and subsequently allowed to pupate (in this manuscript pupate and pupation refer to pupariate and pupariation) for 2 h at room temperature. Successful pupation rates were assessed 24 h post larval-injection (hpi); pupae were incubated at 25 ± 0.5°C until adult emergence. Ten days post adult eclosion, all flies

were assessed for the occurrence of SGH symptoms by microscopy during SG dissections. The total DNA was extracted from the fly carcasses, and the SGs were homogenized in PBS (1 pair of SG/100 µl PBS) and assayed for virus presence by PCR (Abd-Alla et al., 2007).

2.7 Impact of GpSGHV infection on the induction of SGHV latent virus in tsetse conspecific

To assess the impact of GpSGHV infection on the possible induction of latent SGHV infections in other tsetse species, PCR-positive SG were collected from 10-day old adults (other than *G. pallidipes*) that had emerged from larvae that were injected with GpSGHV and a virus suspension was prepared as described above. The virus suspension collected from each tsetse species was injected into third instar larvae of the same species. Larvae that pupated were maintained until adult emergence and adults were maintained for 10 days and then dissected to assess the SGH status and SG was tested with PCR as described above. The observed emergence rate in the GpSGHV or viremic SG homogenate infected larvae was corrected with Abbott’s formula (Abbott, 1925).

2.8 Statistical analysis

The significance of the overall differences of the virus titers obtained from the various treatments were assessed by ANOVA (Sokal and Rohlf, 2012), and the significance of differences between the group's means (PBS vs. virus injections, and the six *Glossina* species) was determined by Tukey's honestly significant difference (HSD) test. The analyses were done in R (R Development Core Team, 2008) using RStudio (Allaire, 2012; Baier and Neuwirth, 2007).

3 Results

3.1 Susceptibility of different Glossina species to GpSGHV infections

The intra-hemocoelic virus injections into teneral female and male adults showed that all the six tsetse species were susceptible to GpSGHV infection (**Figure 2A**). The baseline viral density of the PBS-injected flies remained relatively stable over the 0-9 dpi period. Except in *G. f. fuscipes*, the viral density increased significantly in virus injected flies over the 0-9 dpi period for all tested *Glossina* species (df = 3, 36; F = 63.2; $P \ll 0.001$) (**Figure 2A, Supplementary Figure 1**), but virus density varied significantly between the different tsetse species (df = 5, 330; F = 3.92; $P < 0.05$). There was no significant difference in susceptibility to virus infection between female and male flies for all tsetse species (df = 1, 35; F = 0.95; $P > 0.05$)) (**Figure 2B**). However, on day 5 post injection, males of four (*G. m. centralis*, *G. m.*

morsitans, *G. pallidipes* and *G. p. gambiensis*) out of the six species had higher viral density than the females (df = 1, 68; F = 1.24; *P* > 0.05). Unlike in the other species, the viral densities in *G. brevipalpis* increased exponentially 5 dpi (df = 1, 6; F = 9.14; *P* <0.01) (**Figure 2B**). The virus densities in *G. pallidipes* increased as of 5 dpi, (**Figure 2A**), but in the case of *G. m. centralis* and *G. m. morsitans*, viral titers only increased after 9 dpi. Comparing the overall increase in the viral density during the 0-9 dpi period of the six species, *G. p. gambiensis* males showed the highest increase in viral densities, followed by *G. m. morsitans* males and *G. p. gambiensis* females. Despite this temporal increase in viral titers, dissection of the SGs at the end of the experimental period (120 dpi) showed no evidence of overt SGH symptoms in any of the six *Glossina* species

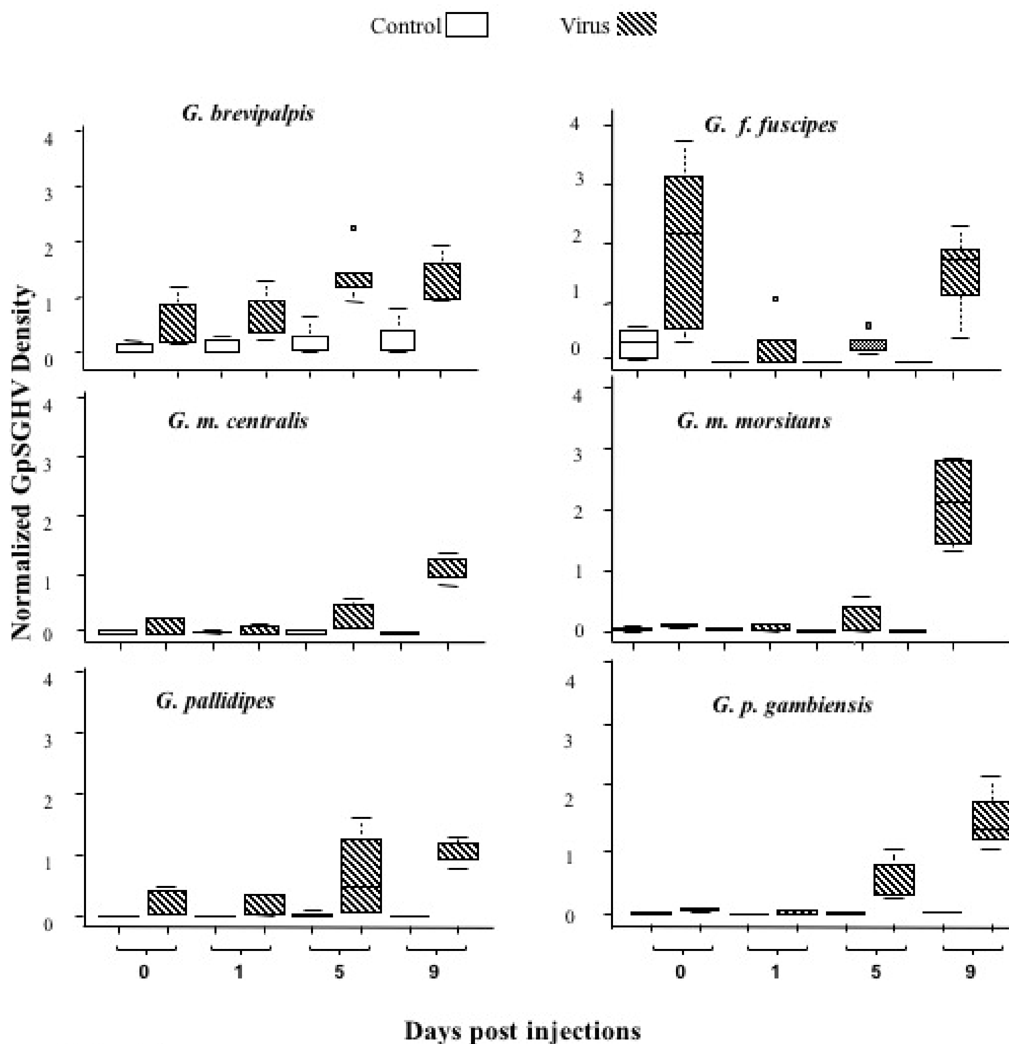

Figure 2A: Susceptibilities of *Glossina* species to GpSGHV. Teneral adults from the six *Glossina* species were injected (intra-hemocoelic) with GpSGHV suspension and PBS, Viral titers in were quantified by qPCR from flies sampled from each species at 0, 1, 5 and 9 days post adult emergence. The qPCR data were normalized against the tsetse housekeeping gene, β-tubulin gene.

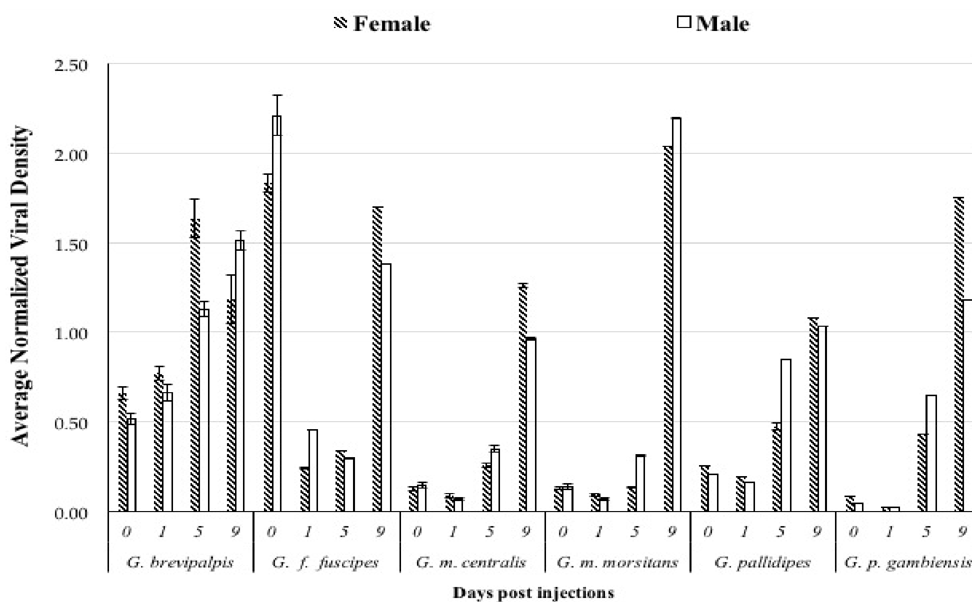

Figure 2B: Susceptibility of *Glossina* species of GpSGHV density were determined only for Virus injected females and males by qPCR from flies sampled from each species at 0, 1, 5 and 9 days post adult emergence.The qPCR data were normalized against the tsetse housekeeping gene, β-tubulin gene.

3.2 Impact of GpSGHV on the survival and productivity of female flies and their F1 progeny

3.2.1 Survival and productivity of injected female flies

Cumulative data over the 120-day experimental period revealed that injecting the virus in adults significantly increased mortalities in both females and males compared with the untreated controls for all six *Glossina* species (df = 1, 20; F = 73.50; $P << 0.001$) (**Figure 3A**). The highest and lowest virus-induced mortality was recorded in *G. pallidipes* (62% compared to 23% in the controls) (df = 1, 4; F = 10136; $P << 0.001$), and in *G. brevipalpis* (23.8% compared to 4% in the controls (df = 1,4; F = 102.5; $P <<0.001$), respectively (**Figure 3A**). The virus-induced mortality was similar in *G. f. fuscipes*, *G. p. gambiensis* and *G. m. centralis* (i.e. 53.7%, 51.2 and 48.3%, respectively). Injecting the virus in adult (females and males) flies significantly reduced (df = 1, 4; F = 37.2; $P < 0.05$) pupal production in *G. f. fuscipes* (pf10d) compared to non-injected control flies (**Figure 3B**), but not in *G. pallidipes* (df = 1,2; F = 0.5378; $P > 0.05$), *G. brevipalpis* (df = 1,2; F = 0.0612; $P > 0.05$), *G. p. gambiensis* (df = 1,2; F = 0.616; $P > 0.05$). Virus injection significantly increased pupal production in *G. m. morsitans* (df = 1, 2; F = 676; $P < 0.05$), or insignificantly increased in *G. m. centralis* (df = 1,2; F = 0.5378; $P > 0.05$), but this aberrant result was most likely an experimental error (**Figure 3B**).

3.2.2 Emergence and virus prevalence F1 progeny

In general, a significant difference was observed in the adult emergence rate among different virus-injected tsetse species (df = 5, 12; F = 3028.7; $P < 0.001$). Emergence of F_1 descendants from pupae produced by virus-injected female parents was reduced significantly as compared with the PBS injected control flies across all six species (df = 5, 24; F = 278; $P << 0.001$) (**Figure 3C**). The control groups showed high emergence rates (>75%) for all tested species, except for *G. p. gambiensis* (50%), which had lower emergence rates than the F_1 adult emergence from virus-injected mothers of other species. Although virus injection in adult *G. brevipalpis* induced the lowest parental mortality rates as compared with other species (**Figure 3A**), the injected virus had more impact on the emergence rate of the F_1 progeny as compared with the untreated controls, i.e. 64.2% versus 95.2% in the control groups (**Figure 3C**). Viral density was high enough to be measurable by qPCR in the F_1 adults produced by the virus-injected *G. pallidipes* and *G. m. morsitans* flies, whereas only background viral densities were detected in the control flies and in the progenies produced by virus-injected mothers of the other four tsetse species (**Figure 3D**). There were no detectable SGH symptoms in any of the F_1 progeny across all the species except *G. pallidipes* (41.9% in virus injected flies versus 4.0% in the control flies).

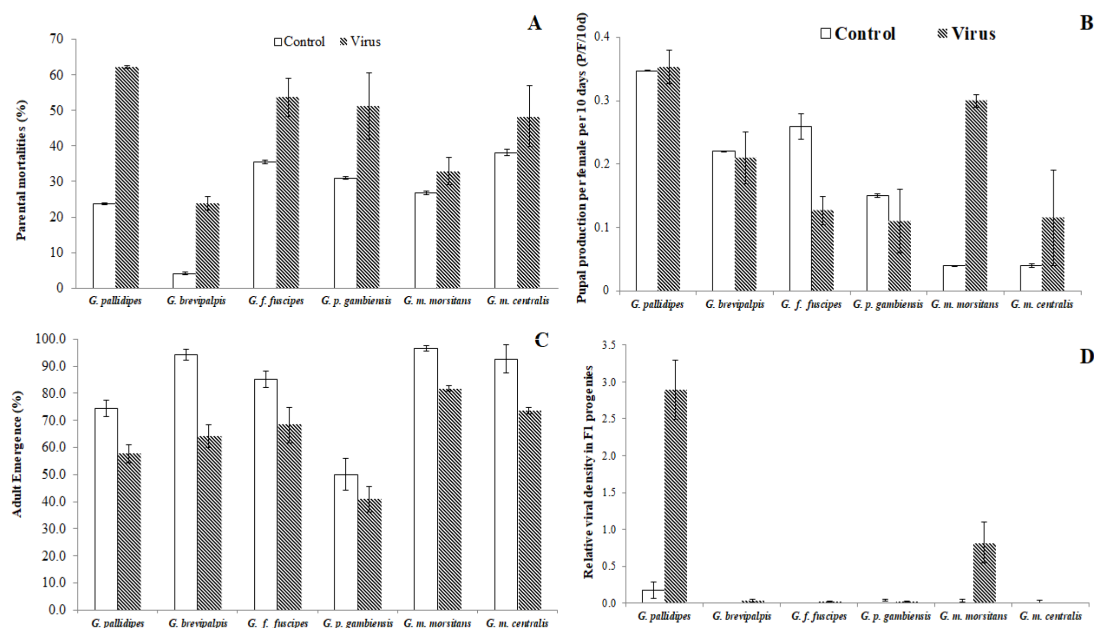

Figure 3: Impacts of GpSGHV infection on fly survival and productivity. Teneral females were injected with GpSGHV suspension and PBS and mated with healthy males. Mortalities (Panel A) and pupal production per female per 10 days (pf10d) (Panel B) were monitored weekly for 12 weeks. The rates of adult emergence and the prevalence of virus infections of the F1 progenies are shown in Panel C and Panel D, respectively. The qPCR quantification data on viral titters were normalized against the tsetse housekeeping gene, β-tubulin.

3.3 GpSGHV injection in larvae and adult emergence, virus transmission and replication in adult flies

3.3.1 Adult emergence of injected larvae

The third instar larvae that successfully pupated after injection were incubated for adult emergence. Compared to the expected normal pupal period for *Glossina* spp., adult emergence was delayed by 3-5 days in all the six species irrespective of whether the larvae had been injected with PBS or virus (data not shown). The impact of virus injection on adult eclosion is presented in **Table 1**. Generally, adult emergence rates between the PBS and the virus injected larvae or between the different species injected with the virus were not significantly different (df =1, 24; F = 2.72; $P > 0.05$ and df = 5, 12; F =1.711; $P > 0.05$, respectively). However, *G. brevipalpis* showed the largest difference in the average adult emergence rate between the PBS and virus-injected flies (74.7% and 24.4% respectively), whereas the differences in *G. m. centralis* and *G. m. morsitans* were smaller (26.54% (virus infected) versus 39.23% (control) and 48.47% versus 60.97%, respectively) (**Table 1**).

3.3.2 Virus replication and occurrence of SGH symptoms in adult flies emerged from GpSGHV injected larvae

Salivary glands were dissected on day 10 post-emergence from males and females that had developed from PBS- and virus-injected larvae to assess the occurrence of the SGH symptoms (**Table 1**). SGH symptoms were observed in 6.2% and 60.1% of adult *G. pallidipes* that developed from the PBS-injected and the virus-injected larvae, respectively. Dissection results for the other five species were negative for SGH symptoms. In addition to the fly dissections, PCR analyses were carried out on all dissected SGs and their corresponding carcasses to assess GpSGHV infections (**Table 1**). The frequency of virus infections detected by PCR of the SG homogenates of all species showed large variations. The frequency of virus infection was significantly different (df = 5, 12; F = 99.3; $P << 0.001$) among different species with the highest virus infection rate was in *G. f. fuscipes* and no virus detected in *G. brevipalpis* (**Table 1**). Virus was detectable by PCR in the fly carcasses of all the flies that emerged from the virus-injected larvae, but without overt SGH symptoms. The infections were generally higher in the carcasses than in the SG homogenates. For instance, whereas 100% of *G. pallidipes* carcasses were PCR- positive, only 60.1% of the dissected SGs homogenates were PCR positives for virus infection. The only exception was in *G. f. fuscipes*, where the infection rate was significantly in the carcasses (17%) than in the SGs (27%) (df = 1,1; F = 13, 4; $P > 0.05$). Further, 37.5% of the *G. f. fuscipes* carcasses of the adults that

emerged from the PBS-injected larvae had detectable virus infections. Whether this result is a case of virus reactivation from a latent state requires further investigations. Only *G. brevipalpis* was not diagnosed with the virus infection in the SG homogenate (**Table 1**).

Table 1. Rate of adult emergence and prevalence of SGHV symptoms in adult flies that emerged from PBS- and virus injected larvae: Tukey multiple comparisons of means denoted by a different alphabetical letter indicate a significantly difference for six species. Testing was done at the 95% family-wise confidence level.

Species	Treatment	N	Adult emergence (%)	Prevalence of the SGH symptoms in the F_1 progeny		
				By dissection	PCR analysis	
					SGs	Carcass (negative SGs)†
G. p. gambiensis	PBS	366	41.5	0/32 (0.0)	0/32 (0.0)	0/16 (0.0)
	GpSGHV	357	42.8	0/153 (0.0)	25/153 (16.3) [b, c]	11/16 (68.7) [b]
G. m. centralis	PBS	209	39.2	0/32 (0.0)	0/32 (0.0)	0/16 (0.0)
	GpSGHV	358	26.5	0/95 (0.0)	7/95 (7.3) [a, b]	9/45 (20.0) [a]
G. pallidipes	PBS	172	51.7	2/32 (6.2)	2/32 (6.25)	0/16 (0.0)
	GpSGHV	290	54.4	95/158 (60.1)	95/158 (60.12) [d]	32/32(100.0) [c]
G. m. morsitans	PBS	269	60.9	0/32 (0.0)	0/32 (0.0)	0/16 (0.0)
	GpSGHV	262	48.4	0/127 (0.0)	9/127(7.08) [a, b]	39/41 (95.1) [c]
G. f. fuscipes	PBS	294	40.8	0/120 (0.0)	0/32 (0.0)	0/24 (0.0)
	GpSGHV	207	41.0	0/85 (0.0)	21/85 (27.1) [c]	3/17 (17.6) [a]
G. brevipalpis	PBS	87	74.7	0/65 (0.0)	0/32 (0.0)	0/16 (0.0)
	GpSGHV	250	24.4	0/61 (0.0)	0/61 (0.0) [a]	12/12 (100.0) [c]

† The numbers within one column followed by the same lower-case letters do not differ significantly at the p=0.05 level. N= Number of successfully pupated larvae.

3.4 Impact of GpSGHV induction of SGHV latent infection in conspecific Glossina species

We investigated the impact of GpSGHV infection on the induction of SGHV latent infection or the possibility to enhance the GpSGHV infection through passaging the virus in conspecific tsetse species. For this, we prepared SG homogenates dissected from 10-days-old adults that emerged from virus-injected larvae and proved as virus infected by PCR, and then re-injected the virus suspensions into the conspecifics third-instar larvae (see materials and methods). The viral density in 10-days old adults emerged from virus injected larvae were significantly different (df = 4, 10; F = 94,4; $P << 0.001$) across all tested species, with the highest viral genome copy number recorded in *G. pallidipes* (~$10^{5.9}$ copies) and the lowest in *G. p. gambiensis* (~$10^{3.7}$ copies) (**Figure 4**). There were no detectable viral infections in the SGs dissected from 10-day-old *G. brevipalpis* that were used for the conspecific injections; *G. brevipalpis* was therefore not included in the conspecifics bioassays.

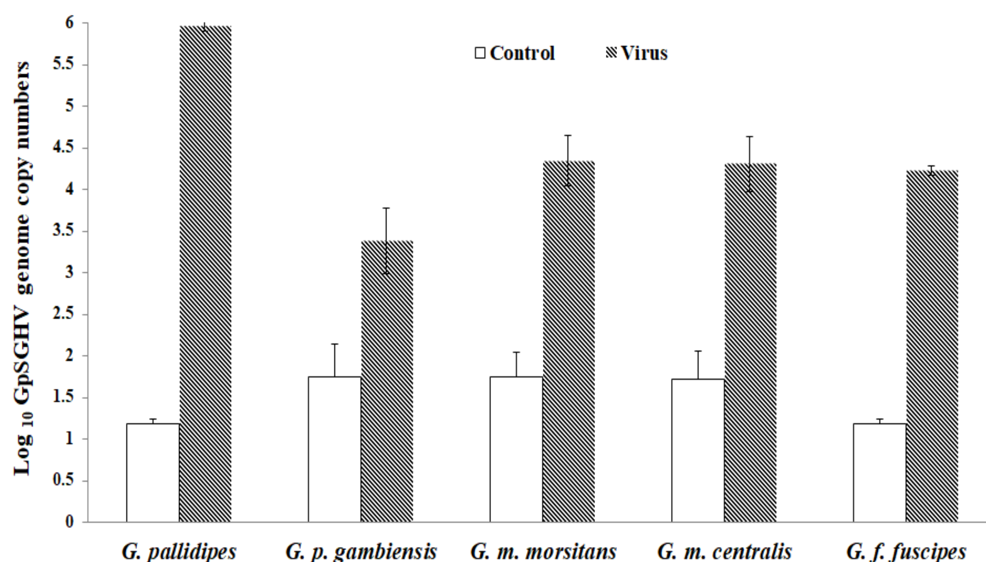

Figure 4: Viral copy numbers in conspecific Glossina species. Absolute viral copy numbers in the homogenates of the SGs dissected from 10-day old flies. These SG homogenates from the viremic flies were used to inject newly larviposited third instar larvae in conspecific species.

3.4.1 Adult emergence of flies developed from larvae injected with SG homogenates derived from conspecifics

When the SG homogenates were re-injected into conspecific third instar larvae, the rates of pupation and of adult emergence (**Table 2**) varied widely amongst the *Glossina* species. The

results indicate that the virus suspensions prepared from the GpSGHV (as a positive control) or viremic conspecifics had a variable effect on the rate of adult emergence compared to the controls (PBS-injections) with the emergence rate decreased in *G. m. centralis* and *G. m. morsitans*, but slightly increased in both *G. p. gambiensis* and *G. f. fuscipes*. No significant differences were observed among different species (df = 4, 10; F = 1.031; $P > 0.05$), or between the emergence rate of the GpSGHV and conspecific injected groups (df = 5, 24; F = 1.68; $P > 0.05$) (**Table 2**).

3.4.2 Virus infections in adults that developed from larvae injected with SG homogenates derived from conspecific species

When SGs were dissected from the 10-day old flies, overt SGH symptoms were only detectable in 59.5% of *G. pallidipes* flies that developed from larvae injected with virus homogenates prepared from hypertrophied SGs of *G. pallidipes* (**Table 2**). When the dissected SG homogenates were subjected to conventional PCR, virus infections were detected in the glands dissected from flies injected with virus suspensions prepared from the hypertrophied SGs of *G. pallidipes*. *G. pallidipes* had the highest viral prevalence (93.7%), while the prevalence in *G. f. fuscipes* and *G. p. gambiensis* were 24.3% and 18.8%, respectively. The viral prevalence was lowest in *G. m. morsitans* (9.8%) and *G. m. centralis* (9.2%) (**Table 2**). The difference in the viral prevalence amongst the tested species was statistically significant (df = 4,10; F = 124,77; $P << 0.001$) (**Table 2**). Of the conspecifics, virus infection was detectable only in the SGs dissected from 2.3% of the *G. m. centralis* flies. As it is possible that injected GpSGHV does not reach and replicate in fully differentiated tsetse SGs (Boucias et al., 2013), we analyzed the carcasses of the flies that did not show virus infections in the dissected SGs. Virus infections were detectable in all the species in the flies that emerged from larvae injected with virus suspensions prepared from hypertrophied SGs of *G. pallidipes* (**Table 2).** The prevalence of virus infections varied significantly among the tested species (df= 4,10; F= 11.366; $P << 0.001$) and was highest in *G. pallidipes* (93.7% of the analyzed individuals), but virus infections in the other species were also common; 66.6%, 63.6%, 50% and 28.5% in *G. m. morsitans*, *G. m. centralis*, *G. p. gambiensis* and *G. f. fuscipes*, respectively. Of the conspecific injections, virus infections were detectable in the carcasses of *G. f. fuscipes* (56.2%), *G. m. morsitans* (50%) and *G. p. gambiensis* (6.25%) (**Table 2**).

Table 2. Rate of adult emergence and prevalence of SGHV symptoms in conspecific *Glossina* species that emerged from PBS-, GpSGHV- and Virus-injected larvae of different tsetse species: Tukey multiple comparisons of means denoted by a different alphabetical letter indicate a significantly difference for six species. Testing was done at the 95% family-wise confidence level.

Species	Treatment	N	Adult emergence (%)	Prevalence of the SGH symptoms in the F_1 progeny		
				By dissection	PCR analysis	
					SGs	Carcass (PCR-negative SGs)†
G. pallidipes	PBS	139; (53.6)	50.3	0/70 (0.0)	0/70 (0.0)	0/16 (0.0)
	GpSGHV	184; (63.0)	22.8	25/42 (59.5)	25/42 (59.5) [a]	15/16 (93.7) [a]
G. p. gambiensis	PBS	155; (49.8)	60.0	0/93 (0.0)	0/93 (0.0)	0/16 (0.0)
	GpSGHV	250; (63.9)	52.8	0/132 (0.0)	24/132 (18.8) [c]	4/8(50.0) [bc]
	Conspecific)	313; (68.4)	66.4	0/208 (0.0)	0/208 (0.0)	1/16 (6.25) [b]
G. m. centralis	PBS	67; (50.0)	56.7	0/38 (0.0)	0/38 (0.0)	0/16 (0.0)
	GpSGHV	92; (52.8)	58.7	0/54 (0.0)	5/54 (9.2) [d]	7/11 (63.6) [ac]
	Conspecific	183; (75.3)	46.4	0/85 (0.0)	2/85 (2.3)	0/14 (0.0) [b]
G. m. morsitans	PBS	82; (50.9)	84.1	0/69 (0.0)	0/69 (0.0)	0/16 (0.0)
	GpSGHV	131; (62.3)	54.2	0/71 (0.0)	7/71 (9.8) [d]	6/9 (66.6) [a]
	Conspecific	124; (80.0)	33.8	0/42 (0.0)	0/42 (0.0)	8/16 (50.0) [a]
G. f. fuscipes	PBS	130; (50.9)	46.1	0/60 (0.0)	0/60 (0.0)	0/16 (0.0)
	GpSGHV	259; (68.7)	47.4	0/123 (0.0)	30/123 (24.3) [b]	4/14 (28.5) [b]
	Conspecific	114; (62.9)	52.6	0/60 (0.0)	0/60 (0.0)	9/16 (56.2) [a]

† The numbers within one column followed by the same lower-case letters do not differ significantly at the p=0.05 level. N= Number of successfully pupated larvae. For carcasses, letter a to b indicate significant

4 Discussion

The successful rearing of sterile male tsetse flies in adequate numbers is a fundamental prerequisite for the successful implementation of the SIT as part of operational AW-IPM programs against tsetse populations. The presence of symptomatic GpSGHV infections in colonies of *G. pallidipes* and its detrimental effect on the flies' productivity that jeopardize the entire mass-rearing process are well documented (Abd-Alla et al., 2010). However, these negative effects can be mitigated through the development and implementation of effective virus management strategies that have eliminated the risk associated with virus presence in these colonies (Abd-Alla et al., 2012, 2013, 2014). In many tsetse-rearing facilities, multiple tsetse species are usually reared, thus risking virus transmission from one colony to the other and from one species to the other, especially when the same feeding equipment (e.g. feeding trays and membranes) is used for the different colonies. With the exception of *G. pallidipes*, GpSGHV infections do not cause significant problems in the rearing and productivity of most other tsetse species. However, intra-hemocoelic injection of GpSGHV into the third instar larvae of *G. m. morsitans* lead to the development of SGH symptoms in the emerged adults associated with a shortened adult life span (Jura et al., 1993; Sang et al., 1997). These earlier reports indicate a wide host range of GpSGHV and a potential ability to infect colonies of these tsetse species. As an example, some production problems were recently reported with the maintenance of *G. f. fuscipes* colonies in Bratislava, Slovakia and Addis Ababa, Ethiopia, and, in both facilities, colonies of different tsetse species are maintained together with *G. pallidipes*. In Bratislava, the problem was very severe and resulted in the complete loss of the *G. f. fuscipes* colony, whereas in Addis Ababa, the colony size was drastically reduced from 1.3 million females to less than 100,000 flies over a period of 52 weeks. Although the reasons of the production problems with the *G. f. fuscipes* colonies are yet to be elucidated, it was deemed important to evaluate the risk of colonies of species other than *G. pallidipes* becoming infected with GpSGHV, and to clarify whether appropriate control measures will be needed to control the virus in facilities rearing multiple tsetse species.

The results of this study demonstrate that all tested tsetse species can become infected, although at different levels, with the GpSGHV by injecting the adult flies or the third instar larvae, and the virus can replicate itself in these flies. However, SGH symptoms were only observed in the virus-infected *G. pallidipes*, but not in the other tested species Similar observations were made with the *Musca domestica* MdSGHV, which can infect stable flies

(*Stomoxys calcitrans*) and black dump flies (*Hydrotaea aenescens*), but does not induce SGH symptoms in the heterologous hosts (Geden et al., 2011).

Our data indicate GpSGHV-induced increase in fly mortality, and reductions in pupae productivity and emergence rate in all species tested, despite the absence of SGH symptoms. These negative effects of virus presence on colony performance of all tsetse species tested, may affect the efficiency and cost efficacy of SIT applications. Although it is unlikely that virus presence in colonies of these species jeopardizes colony stability as it does in *G. pallidipes*, it will make the mass-rearing process more tedious and expensive, i.e. a slower rate of increase in colony size and the need to maintain more females in the colony to produce the same number of sterile males.

The failure to detect overt SGH symptoms in any of the adult flies or third instar larvae (except in *G. pallidipes*) that were virus-injected is in agreement with previous reports (Abd-Alla et al., 2007; Boucias et al., 2013) but contradicts previous results with *G. m. centralis* and *G. m. morsitans* (Jura et al., 1993; Sang et al., 1997). The observed difference between our and previous results may be attributed to differences in the virulence of the viral strain. It is possible that the GpSGHV strain used in our study was less pathogenic than the strain(s) used in the earlier studies. Alternatively, the tsetse colonies used in our study have been cultured for more than 2 decades and it is possible that the flies derived from these colonies have become more adapted to the virus compared with the tsetse strains used in the studies in the 1990's. As an example, the GpSGHV strain used in our study was derived from a *G. pallidipes* colony that has been cultured under artificial laboratory rearing since 1967 (Rogers and Kenyanjui, 1972). Although this colony was reported to be GpSGHV-infected with 10% of the flies showing overt SGH and with 100% of the flies carrying asymptomatic infection (Abd-Alla et al., 2007), no reports are available of the loss of the colony. The relative long domestication period with the virus infection provide an ideal opportunity for this species to adapt to the virus infection, most probably through selecting processes that provided an advantage for more moderate or less pathogenic virus strains. This is probably because flies infected with a more pathogenic virus strain developed SGH and hence, became less productive and died earlier and were selected out from the colony. The tolerance to the GpSGHV viral strain of the IPCL colony was contrasted by the rapid collapse of two other *G. pallidipes* colonies also maintained at the IPCL. The first colony was established from material collected in Lambwe valley, Kenya in November 1983 and which was lost in 1987 (IAEA, 1987, 1988). The second colony was established with material collected in 1999 from

Arba-Minch, Ethiopia, but collapsed in 2002 after observing SGH in > 85% of the flies (Abd-Alla et al., 2007). The common issue in both colonies is the short domestication period in the laboratory, which possibly did not provide sufficient time for the host-virus co-adaption. Alternatively, it may be also due to the higher virulence of the virus strain infecting these colonies did not allow opportunity for the host adaptation to the virus infection. Most likely, the virus strain currently available in the IPCL facility seems to be less pathogenic to the adapted *G. pallidipes* colony and might be different from the virus strains used in previous studies.

It was previously demonstrated that intra-hemocoelic injection of GpSGHV in adult flies did not cause the development of SGH symptoms while it leads to SGH development in the F_1 offspring. It was therefore concluded that the virus infection requires element(s) from undifferentiated tissues to induce SGH symptoms (Boucias et al., 2013). In view of this, it was anticipated that intra-hemocoelic injection of GpSGHV into the third instar larvae might lead to the development of SGH in adult flies of the tested species. SGH was however only observed in adult *G. pallidipes* flies that emerged from virus-injected larvae and the absence of SGH in other species seem to indicate the existence of additional barriers that hampers the development of SGH. Perhaps a delay in virus replication rate prevented a threshold virus copy number of 10^6 being reached that is required to cause the SGH. Although a lower virus copy number might be a cause for the absence of SGH, in the virus-injected adults of *G. pallidipes*, more than 10^6 virus copy numbers were obtained but without occurrence of overt SGH symptoms. This might indicate that virus replication and transmission from infected pupae to adult play a major role in the development of SGH; the detection of SGH in one-day old *G. pallidipes* adults that emerged from pupae produced by virus injected mothers is clear evidence of virus replication in the pupal stage (Boucias et al., 2013). The absence of SGH in the injected third instar larvae of other tsetse species than *G. pallidipes* might be due to unknown challenges that block virus replication in the pupae which reduce the virus copy numbers needed to induce SGH in emerged adults. Viral density in the surviving pupae was not assessed after injecting larvae to investigate this point and this will be analyzed in a further study. Differences in virus copy number, mortality rate, and productivity in adults injected with the GpSGHV or in the adult emergence rate, virus density or virus infection rate in both virus-injected adults and larvae of the different tsetse species may be due to the species biology and associated microbiota.

Although the GpSGHV-induced mortality and the reduced productivity observed in the injected adult flies of different *Glossina* species was not surprising, it is unknown how viral infection result in host mortalities. However, our results agree with previous reports on the increased mortality rate of wild *G. pallidipes* infected with the GpSGHV (Jaenson, 1986). In general, virus infections in insects are often associated with various biological costs, such as reduced growth/development rates and productivity (Cabodevilla et al., 2011). Host insects generally respond to pathogen infection by reduction of cellular metabolism (cessation of the synthesis and turnover of macromolecules), cellular signaling, amongst other processes (e.g. transcription and translation) (Hand and Hardewig, 1996). In the event that the virus pathogenesis progresses, this metabolic depression could lead to programmed cell death (apoptosis) (Sparks et al., 2008), which in turn severely affects the viral gene expression, DNA replication and production of progeny virus. Consequently, it is possible that the range of hosts in which a certain virus can replicate is influenced by the ability of host insect cells to commit suicide during virus infection. Together, these facts could partially explain the differential virus-induced mortalities observed amongst the six *Glossina* species in the current study. It is difficult to explain why this high mortality was not observed in virus-injected *G. brevipalpis* but it might be related to its larger body size; the same amount of virus inoculum was injected in all flies, which resulted in a relative lower virus concentration per unit of weight in *G. brevipalpis* compared to other species.

The observed significant reduction in adult emergence of the F_1 progeny produced by the virus-infected mothers compared to their PBS-injected counterparts across all the six *Glossina* species is most probably due to the biological cost of the virus infection. It is however noteworthy that although virus injection did not show high parental mortalities in *G. brevipalpis* (unlike in the other species) compared to the controls, the virus caused the highest reduction in adult emergence of the F_1 progenies produced by this species. Virus infection could interfere with larvae/pupae metamorphosis in several ways, including neuroendocrine regulation of hormonal synthesis, or transcriptional disruption of the expression of enzymes that are critical for metamorphosis. This has been demonstrated during virus infection in various dipterans such as the fruit fly, *Drosophila melanogaster* and the tobacco hornworm, *Manduca sexta* (Uhlirova et al., 2003).

The effects of GpSGHV injections in third instar larvae on pupation rate, pupal period, and adult emergence varied widely amongst the *Glossina* species. GpSGHV caused the lowest pupation rate in *G. pallidipes*, which is not surprising in view that this species seems to be the

most susceptible to the virus. As the injection process was conducted in the late third instar larvae few minutes before pupation, it is possible that the failure of the larvae to pupate was caused by the mechanical damage during injection and handling and not by the presence of the virus. The results indicate slight delays and reduction in adult emergence of *G. brevipalpis*, *G. m. morsitans* and *G. m. centralis* from pupae that developed from GpSGHV-injected larvae. Given that there were no variations in the pupae incubation conditions such as temperature and humidity, these results imply that the delayed adult emergence, at least in the above-mentioned *Glossina* spp., was due to the virus infection. Our results are in agreement with results obtained by Jura *et al*., (1993) who reported an adult emergence rate of *G. m. morsitans* after virus injections into larvae (76 %), compared to 85.8 % in the controls. It is possible that the reduction in the emergence rate is hormonally-mediated. Our study however, did not include investigations into the ecdysteroid titers in the treated larvae.

In tsetse species other than *G. pallidipes*, the absence of the SGH in the 10-day old adults emerged from virus injected larvae together with the detection of the virus in some SGs and most of the fly' carcasses, leaves some room for speculation on the nature of the virus detected in each species. First, we hypothesize that the infection with GpSGHV might just induce a latent virus infection in each species; in such case the induced virus might be more pathogenic to the conspecific as has been reported in baculoviruses (Hughes et al., 1993). Secondly, we hypothesize that, although GpSGHV is the virus transmitted in our study in each tsetse species, the virus might not yet be adapted to these different tsetse species. The GpSGHV infection in these tsetse species might be improved through serial passages as was demonstrated also for baculoviruses (Gani et al., 2014). Taken together, the absence of enhanced virus infection in other species might indicate absence of species-specific latent virus. Instead, it is more likely that the injected GpSGHV strain was transmitted from pupae to adult in each species. The reduced pathogenicity observed in the conspecific injection is most probably due to injecting a lower viral copy number in the conspecific compared to the virus collected from SGs. In addition, a single virus passage through heterogeneous host may be insufficient to improve GpSGHV pathogenesis (Gani et al., 2014).

In tsetse mass-rearing facilities where several tsetse species are maintained and fed on the *in vitro* membrane feeding system, due to economic reasons (i.e. feeding trays, heating mats etc.), there is a tendency to use the same membrane for several successive feeding rounds of the same or even several species (Feldmann, 1994). It is important to note that rearing of *G. pallidipes* is in general more challenging compared with other tsetse species due to the

GpSGHV infection, therefore in case of feeding more than one species on the same membrane, it is often to feed the *G. pallidipes* flies first followed by flies of the other species. This may also be also due to the fact that so far, no other species have been reported to be affected with the GpSGHV. However, this feeding protocol might have contributed to the loss of the *G. f. fuscipes* colony in Bratislava and the reduction in size of the colony maintained in Addis Ababa, Ethiopia. Additional research will be required to elucidate the reasons of the bad performance of these colonies.

Conclusions

Finally, it should be noted that the data from our study was based on intra-hemocoelic virus injections, which is not the natural infection route for the virus. Oral infection is the primary GpSGHV infection route, which consists of several barriers (e.g. peritrophic membrane). Compared to the injections, the oral infection route may significantly reduce chances of productive virus infection. The implications of these facts are that since the intra-hemocoelic injection did not induce development of overt SGH in most of the *Glossina* species analyzed in this study, it is much less likely that the natural route (via blood meal feeding) will induce the expression of overt disease symptoms. This notwithstanding, the finding that all the *Glossina* species are susceptible to GpSGHV infections and reduce colony performances points to the need for the implementation of strict protocol to protect the colonies from GpSGHV infection. We have already developed, and implemented GpSGHV management protocols that are effective in the control of the virus in *G. pallidipes* colonies (Abd-Alla et al., 2012, 2013, 2014).

Conflict of Interest

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

Author Contributions

Conceived and designed experiments: GDU, AGP, AMMA. Performed experiments: GDU. Analyzed data: GDU, AGP, HMK, AMMA. Wrote paper: HMK, GDU, AMMA, AGP, MJBV. All authors read and approved final manuscript.

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Supplementary Material

The data for this paper are available at the Harvard Dataverse through the following link (https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/LZS5EB).

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Supplementary Material

Supplementary Table 1. List of primers used for quantitative PCR (qPCR) analyses of GpSGHV and microbiome in *Glossina* species.

Target Gene	Primer Name	Primer Sequence (Listed 5- to -3)	Annealing Temperature (°C)	Amplicon Size (bp)	References
odv-e66 (GpSGHV ORF5)	qPCRFwda	CAAATGATCCGTCGTGGTAGAA	60	51	(Abd-Alla etal.,2009;Abd-Alla et al., 2011)
	qPCRRev	AAGCCGATTATGTCATGGAAGG			
β-tubilin (Tsetse Fly)	Tsetse-tubulinF	GAT GGT CAA GTG CGA TCC T	55	355	(Caljon et al., 2009)
	Tsetse-tubulinR	TGA GAA CTC GCC TTC TTC C			

Reference List for Supplementary Material

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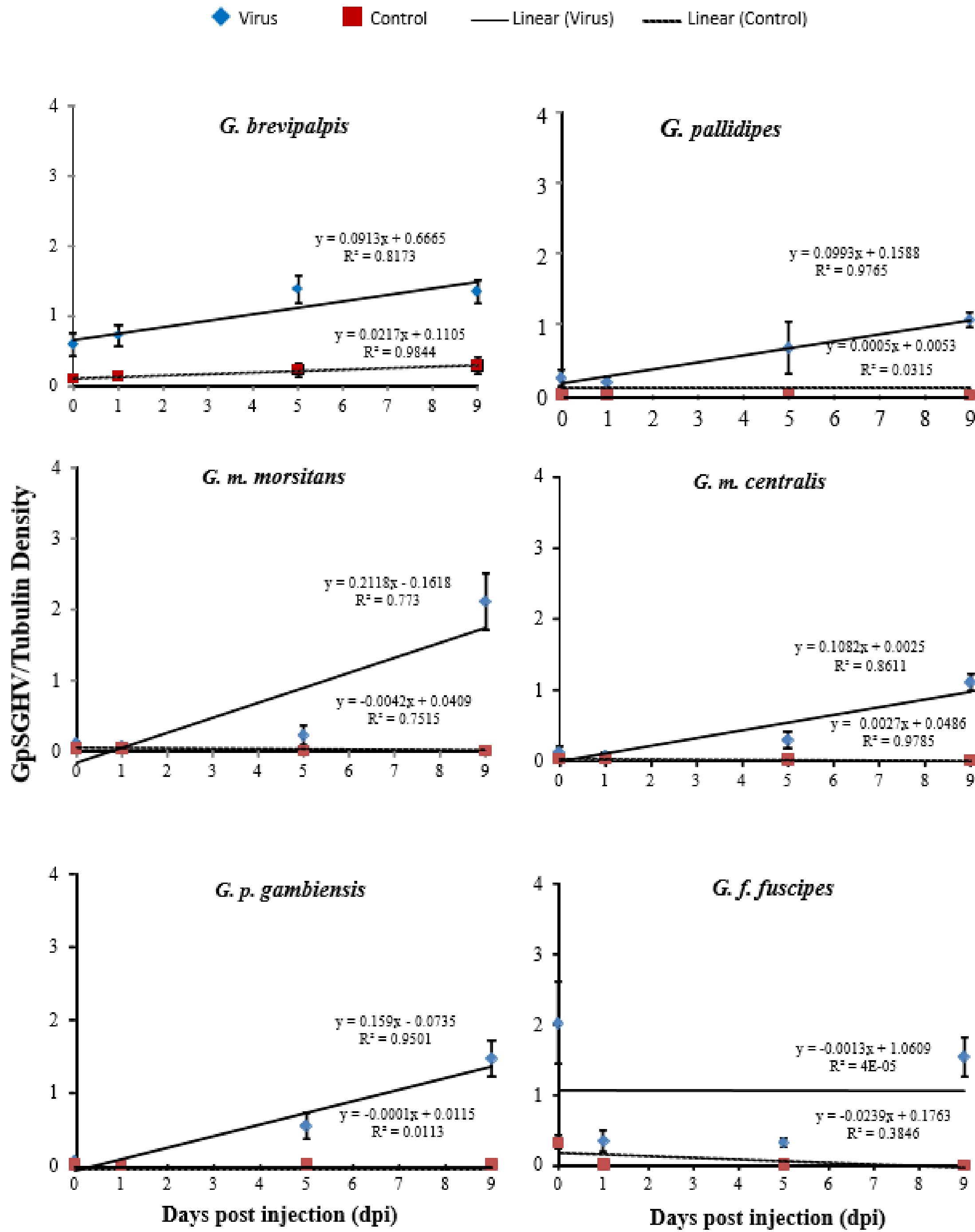

Figure S1: Regression line of the increased virus density of injected flies.

Chapter 5

Impact of *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) on a heterologous tsetse fly host, *Glossina fuscipes fuscipes*

Impact of Glossina pallidipes salivary gland hypertrophy virus (GpSGHV) on a heterologous tsetse fly host, *Glossina fuscipes fuscipes*

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Abstract

Tsetse flies (Diptera: Glossinidae) are the vectors of African trypanosomosis, the causal agent of sleeping sickness in humans and nagana in animals. *Glossina fuscipes fuscipes* is one of the most important tsetse vectors of sleeping sickness, particularly in Central Africa. Due to the development of resistance of the trypanosomes to the commonly used trypanocidal drugs and the lack of effective vaccines, vector control approaches remain the most effective strategies for sustainable management of those diseases. The Sterile Insect Technique (SIT) is an effective, environment-friendly method for the management of tsetse flies in the context of area-wide integrated pest management programs (AW-IPM). This technique relies on the mass-production of the target insect, its sterilization with ionizing radiation and the release of sterile males in the target area where they will mate with wild females and induce sterility in the native population. It has been shown that *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) infection causes a decrease in fecundity and fertility hampering the maintenance of colonies of the tsetse fly *G. pallidipes.* This virus has also been detected in different species of tsetse lies. In this study, we evaluated the impact of GpSGHV on the performance of a colony of the heterologous host *G. f. fuscipes*, including the flies' productivity, mortality, survival, flight propensity and mating ability and insemination rates. Even though GpSGHV infection did not induce SGH symptoms, it significantly reduced all examined parameters, except adult flight propensity and insemination rate. These results emphasize the important role of GpSGHV management strategy in the maintenance of *G. f. fuscipes* colonies and the urgent need to implement measures to avoid virus infection, to ensure the optimal mass production of this tsetse species for use in AW-IPM programs with an SIT component.

Background

Tsetse flies (Diptera: Glossinidae) are the only cyclical vectors of the pathogenic African trypanosomes that cause human African trypanosomosis (HAT) or sleeping sickness and African animal trypanosomosis (AAT) or nagana in sub-Saharan Africa [1]. There are 33 species and subspecies of tsetse flies that all belong to the genus *Glossina*, divided into the Morsitans, Fusca, and Palpalis groups [2]. Although all tsetse species can transfer pathogenic trypanosomes, members of the Palpalis and Morsitans groups are the primary trypanosome vectors [3]. For instance, *G. f. fuscipes* is a significant vector of trypanosomes in central Africa [4], particularly in Uganda and Western Kenya [5]. In the absence of effective vaccines and drugs against HAT and AAT [6] vector control represents the most efficient strategy to manage these diseases in mainly rural areas [7, 8]. Currently accepted tsetse control tactics are the sequential aerosol technique [9], stationary bait methods (traps and targets) [10], the live bait technology [11] and the sterile insect technique (SIT). The SIT is based on the mass-rearing and sterilization of males with ionizing radiation (e.g., gamma irradiation), and the sequential release of adequate numbers of sterile male insects in the target area [12]. Mating between sterile males and wild females will result in non-viable embryos, leading to the gradual reduction of the target insect population [13]. The SIT has proven to be a powerful control tactic for use against tsetse flies and other Diptera as part of area-wide integrated pest management (AW-IPM) approaches [14]

The implementation of AW-IPM programs with an SIT component against tsetse flies poses significant challenges with respect to colonization and mass-rearing of the target species. Many factors, such as infections with pathogens when the insects are reared continuously or under suboptimal rearing conditions [15], might lead to failures in establishing and maintaining large tsetse colonies and, as a consequence, fail to produce insects of adequate quality.

Infections of tsetse flies derived from natural populations and laboratory colonies with the pathogenic salivary gland hypertrophy virus (SGHV) [16-18], a member of the *Glossina* Hytrosavirus genus and *Hytrosaviridae* family have been frequently observed [19]. SGHV is a rod-shaped enveloped virus (100 x 700-1000 nm) containing a large double-stranded DNA genome of 190 kb [19]. The virus infection is mostly asymptomatic in tsetse flies, but in some cases it can lead to the development of salivary gland hypertrophy (SGH) symptoms, which has been associated with a reduction in the flies' productivity and eventually loss of the

colony [20-22]. SGH prevalence of this virus in natural tsetse populations vary across tsetse species and their locations, but are usually low (prevalence of 0.3 to 7 %) [23] However, under mass-rearing conditions of *Glossina pallidipes*, high prevalence rates have been observed that were associated with the use of the *in vitro* membrane feeding technique that favors horizontal transmission of the virus. In *G. pallidipes*, a species that is considered an efficient vector of trypanosomes [24], SGH symptoms were associated with abnormalities of the ovaries and testicular degeneration, leading to reduced productivity in both male and female flies [15, 23, 25, 26]. Data available on prevalence rates of the virus in colonies of *G. pallidipes* showed that colony decline and eventual collapse could not be averted when the SGH infection rate in the colony reached 70 % (Abd-Alla et al., 2016). To mitigate the negative effects of the virus on colony performance, several virus management strategies were developed that have proven to be effective [27-29].

Although SGH symptoms have been detected in natural populations of other tsetse species such as *Glossina austeni, G. morsitans morsitans, G. nigrofusca nigrofusca* and *G. pallicera pallicera* [16, 30, 31], no SGH symptoms have been observed in *G. f.* fuscipes but asymptomatic infection was detected [32]. However, in laboratory colony, intra-hemocoelic injections of GpSGHV into five heterologous tsetse species (*G. brevipalpis, G. m. morsitans, G. m. centralis, G. f. fuscipes* and *G. p. gambiensis*) showed a significant increase in the titer of viral DNA, demonstrating the ability to replicate in these heterologous species [33].

The Government of Ethiopia has embarked on an AW-IPM program with an SIT component to eradicate a *G. f. fuscipes* population in the Deme river valley of Southern Ethiopia [34-36]. The campaign required the establishment and expansion of a colony of the target species in the mass-rearing facility in Kality on the outskirts of Addis Ababa. The colony was initiated with seed material from a colony maintained at the Slovak Academy of Sciences (SAS), Slovakia. Although colony growth was acceptable in the initial stages subsequent low productivity and high mortality resulted in a drastic reduction in colony size. Similar observations were made at the SAS, where the colony was lost. It is worth noting that more than one tsetse species is being maintained in both facilities, including *G. pallidipes,* a species well known to be infected with the GpSGHV, a situation that may facilitate the transmission of GpSGHV from one tsetse species to another especially if both species were fed using the same membrane as was the case in the SAS colonies. It is important to note that SGHV was detected by PCR in natural populations of *G. f. fuscipes* with a prevalence of 25-40% [32] and an increase in virus titer in virus injected flies has recently been demonstrated [33]

This study was undertaken as part of efforts to understand the possible causes of the poor colony performance. In this study, we report on the impact of GpSGHV on the performance of *G. f. fuscipes* flies using standard quality control parameters, such as adult longevity, female productivity and mortality, flight propensity, mating ability, and insemination rate.

Methods

Tsetse Flies

The *G. f. fuscipes* flies used in this study originated from a colony that was established from wild collected material in the Central African Republic (CAR) and maintained since 1986 at the Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria. Experimental flies were fed for 15-20 minutes, three times per week with defibrinated bovine blood using an artificial (*in vitro*) membrane feeding system [37]. The adult flies were held in medium size cages (11 cm diameter x 5 cm high) at a ratio of 1:3 male to female under standard tsetse colony rearing conditions (24 ± 0.5°C and 75 ± 5% relative humidity (RH)) [38]. The SGHV is not detectable in this colony by PCR.

Preparation of virus inoculum and intra-hemocoelic injection

The GpSGHV inoculum was prepared from intact hypertrophied salivary glands dissected from a 10-day-old male *G. pallidipes* showing overt SGH symptoms [39]. Briefly, the hypertrophied glands were homogenized in phosphate buffered saline (PBS) at a concentration of one pair of glands/ml and the homogenate was centrifuged at 400 x *g* for 2 min at room temperature. The supernatant was transferred to a new sterile tube and used immediately after preparation of the inoculum.

Using a 1ml Myjector U-40 Insulin type syringe (Teruma, Leuven, Belgium) either 2µl of filter-sterilized PBS (control) or the virus suspension was injected into the thoracic cavity of prechilled adult flies. For each treatment, newly emerged teneral (male and female) flies were injected and placed into standard holding cages (20 cm diameter x 5 cm high) at the required mating ratio and each experiment was replicated 3 times. Non-injected and PBS-injected flies were used as non-injected controls to evaluate the impact of injection and the virus infection on the flies' performance.

Prevalence of GpSGHV infection in *G. f. fuscipes* injected flies

The tsetse genomic DNA was extracted from individual non-injected, PBS- and virus-injected flies using the DNeasy Blood & Tissue kit (QIAGEN Inc., Valencia, CA) following the manufacturer`s instructions. The titer of GpSGHV was determined in *G. f. fuscipes* injected males and females on 0, 9 and 18 days post injection by polymerase chain reaction (PCR) using the method previously described by Abd-Alla et al. [20]. Equal volume of individual DNA sample was pooled (n = 6 for females and n=2 for males) and measured to determine the DNA concentration by spectrophotometry (Nanodrop-Synergy H1 Multi-Mode Reader, BioTek, Instruments, Inc., USA), DNA samples were diluted to final concentration of 4 ng/µl and 5 µl was used as template for the qPCR reaction. The qPCR was performed with odv-e66 (GpSGHV ORF5) gene using the method previously described [20, 39, 40] and the tsetse *β-tubulin* gene was used as a housekeeping gene to normalize the qPCR reactions.

Impact of GpSGHV infection on survival and productivity of *G. f. fuscipes*

To evaluate the impact of GpSGHV challenge on *G. f. fuscipes,* their productivity and longevity under both normal feeding (blood meal offered three times per week) and starvation stress (no blood feeding) conditions was monitored in non-, PBS- and virus-injected flies. For each treatment, seven males and twenty-one females were kept in standard holding cages and each treatment was replicated 3 times. The productivity data is presented as total pupae over the experimental period per initial female (PPIF).

Impact of GpSGHV infection on the flight propensity of *G. f. fuscipes*

The flight propensity of virus injected flies, non-injected and PBS-injected flies (the latter two as negative controls) was assessed at 7, 14, 21, 28, 35 and 42 days post injection under normal feeding conditions. Flight tests were carried out in netted cubic mating cages (45 x 45 x 45 cm) that contain a black Polyvinyl Chloride (PVC) tube (8.9 cm diameter, 3 mm thick wall, 10 cm high). The PVC tube allowed light entering only from the top and the walls were coated with unscented talcum powder to prevent the flies from walking out the tube [41]. Standard FAO/IAEA/USDA protocols (FAO/IAEA/USDA, 2014 http://www-naweb.iaea.org/nafa/ipc/public/QualityControl.pdf) were used with a few modifications i.e. rather than using pupae, the adult flies were chilled at 4° C for 5 min prior to the test, to enable the transfer to the tube. For each test, seven chilled males and twenty-one chilled females were put in a plastic Petri dish (90 mm diameter) with the base covered by black porous paper, and the number of flies that had escaped from the tube "flier" was recorded

during one hour [42]. The light intensity at the top of the tubes was 500 lux. Six replicates were conducted for each treatment.

Impact of GpSGHV infection on the mating ability and insemination rate of *G. f. fuscipes*

The mating ability and the insemination rate of untreated (normal colony) *G. f. fuscipes* males of different ages (3-, 6-, 9- and 12-days post emergence) were assessed to determine the optimal mating age [43]. Forty (40) teneral males were released in mating cages mating cages (45 x 45 x 45 cm), followed 15 minutes later by an equal number of 9 - day old virgin females for mating. Mating events were observed under standard tsetse rearing conditions from 9:30 to 12:30 h to cover the morning mating activity peak [44]. The optimal mating age test was replicated 3 times and mating tests of virus-challenged flies were repeated 9 times. All flies were offered a blood meal 24h before mating to increase the mating rate, and non-fed flies were removed and replaced. The propensity for mating ratio was calculated according to the proportion of females that mated for each treatment [44]. After determining the optimal male mating age, 6 to 9-day old non-injected, PBS-injected, and virus-injected virgin males (40 males) were tested as described above to determine mating ability and insemination rate of experimental flies.

Mating pairs were transferred to small cages (4 cm diameter x 6 cm high) and kept for 24h, after which the males were removed and the females dissected under a binocular microscope to determine insemination rate. Mated female flies were dissected in PBS under a binocular microscope and the insemination rate and spermathecal contents were assessed subjectively at ×100 magnification using a Carl Zeiss compound microscope [45]. The spermathecal fill and insemination rate were obtained by assessing the content of the spermathecae pairs. Spermathecal fill was scored to the nearest quarter for each spermathecae separately as empty (0), quarter full (0.25), half-full (0.50), three-quarter-full (0.75) and full (1.0), For the statistical analysis, quarter full (0.25), half-full (0.50), three-quarter-full (0.75) were considered as partial fill. The amount of sperm transferred was then computed as the mean spermathecal filling values of the spermathecae pairs [46].

Statistical Analysis

The significance of the virus injections on the various parameters was assessed by an ANOVA test [47]. Pairwise comparisons between group means (PBS vs. virus injections,

non-injected vs. virus injections and non-injected vs. PBS injections effect on flies) was than determined by Tukey`s honestly significant difference (HSD) test. The analyses were performed in R [48, 49] using RStudio version 3.4.1. [50] The data was transformed using the Box-Cox procedure from the packages. ggplot2[51], lattice v0.20-35 [52] and MASS v7.3.[53].

All survival analyses were performed using Graph Pad Prism version 5.0 for Windows (GraphPad Software, San Diego California, USA; graphpad.com). The effect of the treatments on fly longevity was analysed using a Log-rank (Mantel-Cox) test. Differences between treatments pairs were tested using the Bonferroni method. Mean longevity (or age in days at death) was calculated from the sum of the number of live flies on each day until the death of the last fly, divided by the number of flies in the group at the start of the experiment. The level of significance was 0.05 for all statistical analyses.

Results

Detection of GpSGHV infection in injected flies

The GpSGHV titer in virus-injected flies was assessed by qPCR at various times post injection to investigate whether the virus could infect and replicate in injected flies. The qPCR results indicate a significant increase in the virus titer over time (F= 1.34, df= 1, 34, $P < 0.001$). The results indicate that the virus replication was rather slow as no significant increase in the virus titer between 0 time and 9 dpi was observed, but later the virus titer increase by 5. 22 fold change at 18 dpi **(Figure 1)**. In addition, results demonstrated a significant difference in the virus titer between the virus injected flies and negative controls (PBS- injected flies (F= 21.51, df = 1, 68, $P < 0.001$).

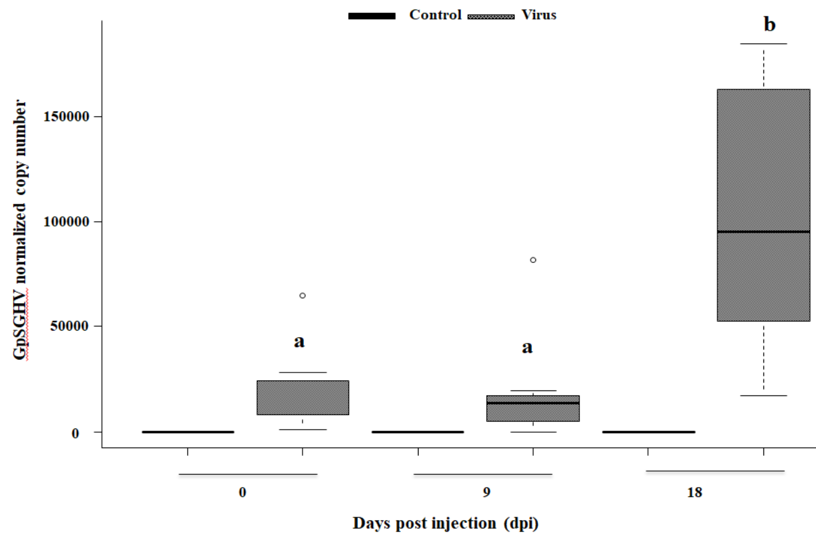

Figure 1. Detection of GpSGHV infection in injected *G. f. fuscipes*. Quantification of GpSGHV titer in virus and PBS- injected flies over 18 day post injection.

Impact of GpSGHV infection on *G. f. fuscipes* productivity and survival

Virus challenge reduced the productivity of the flies significantly (F= 52.05, df= 2,6, P < 0.0001) (**Figure 2**). This reduction was significant when compared with PBS-injected (P < 0.001) and non-injected (P < 0.0005) flies. The injection process had no impact on their productivity as no significant difference (P = 0.079) was observed between non-injected and PBS-injected flies.

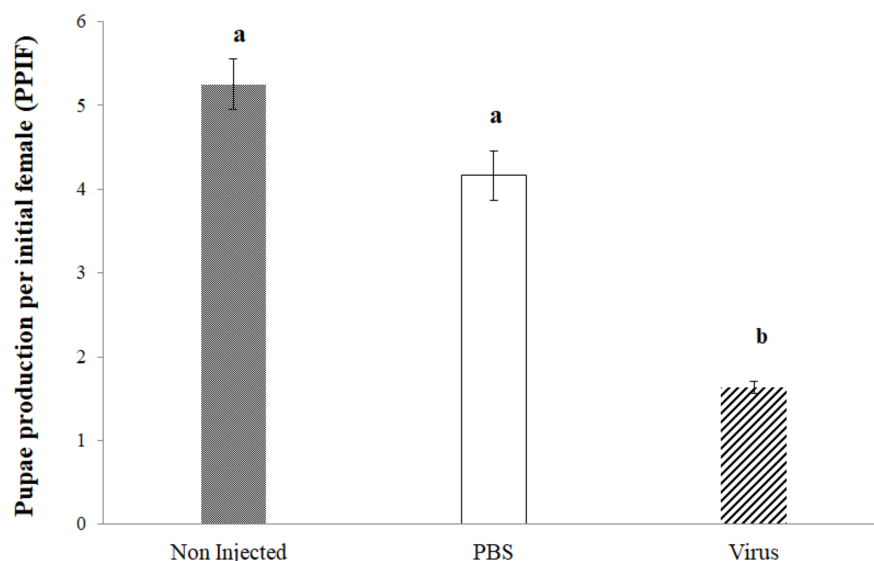

Figure 2. Impact of GpSGHV infection on *G. f. fuscipes* fly productivity and survival. Teneral females were injected with GpSGHV suspension or PBS with non-injected controls. Pupal production per initial female (PPIF) were monitored weekly for 110 days.

Adult survival was evaluated under normal feeding and starvation stress conditions. The daily survival rate of the fed virus-injected flies (males and females) was significantly lower than the non-injected and PBS-injected fed flies (Log-rank X^2 = 61.31, df = 2, P < 0.0001) (**Figure 3**). The mortality rate of the virus-injected flies was higher (100%) than PBS- (75%) and non-injected flies (70%) when measure at 80 days post injection. The survival rate of injected flies varied significantly between males and females (Log-rank X2 = 86.26, df = 3, P < 0.0001) **(Figure 3)**. Under normal colony conditions, the survival of GpSGHV-infected females was significantly reduced as compared with PBS-injected females (Log-rank X^2 = 48.3; df =1, $P <$ 0.0001) and non-injected females (Log-rank X^2 = 58.3, df = 1, $P < 0.0001$) (**Figure 3A**), however, no significant difference (Log-rank X^2=0.50; df = 2, $P > 0.05$) in survival was observed between virus-injected and non-injected males (**Figure 3C**).

Under starvation stress, the survival rate of the flies was significantly lower than the survival under normal condition regardless of treatment. However, the virus- and PBS-injected females showed a lower survival (Log-rank X^2 = 87.02, df = 2, $P < 0.001$, less than 10 days) as compared with the non-injected females (**Figure 3B**). Similar to female flies, the virus- and PBS-injected males lived a significantly shorter time (Log-rank X^2= 8.741; df = 2, $P < 0.001$) (less than 10 days, similar to female survival) as compared with the non-injected males (**Figure 3D**).

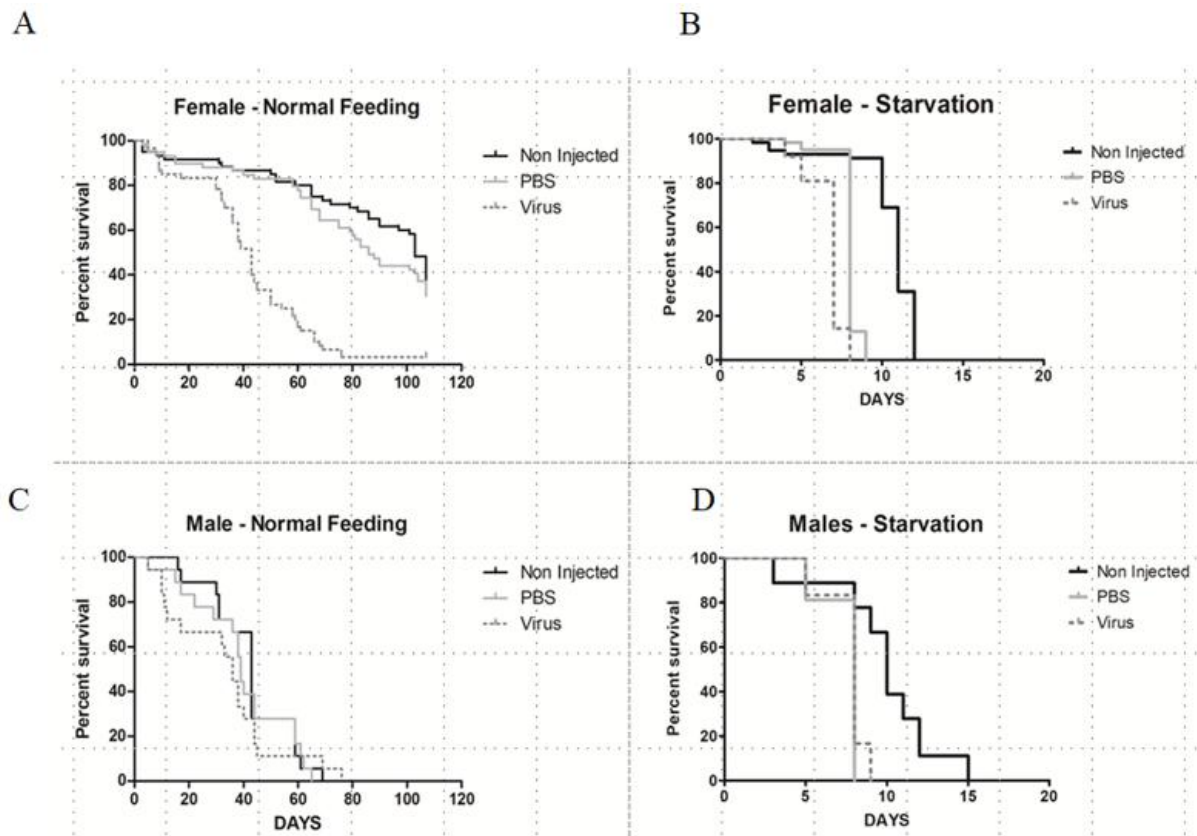

Figure 3. Survival of *G. f. fuscipes* species infected with GpSGHV. **A** and **C**: adult survival under normal feeding condition for females and males respectively. **B** and **D** adult survival under starvation stress conditions for females and males respectively.

Flight propensity of GpSGHV injected *G. f. fuscipes*

The results of the flight propensity tests indicated that GpSGHV infection had no significant impact (F = 1.4; df = 2, 42; P = 0.25) on the flight propensity of *G. f. fuscipes* males and females as compared with the PBS-injected and non-injected flies (**Figure 4**). The average percentage of fliers for different treatments was evaluated at different times post emergence (7, 14, 21, 28, 25, 42 days). No significant difference in flight propensity was recorded at different times regardless of treatment (F = 0.08; df = 1, 52; P = 0.91).

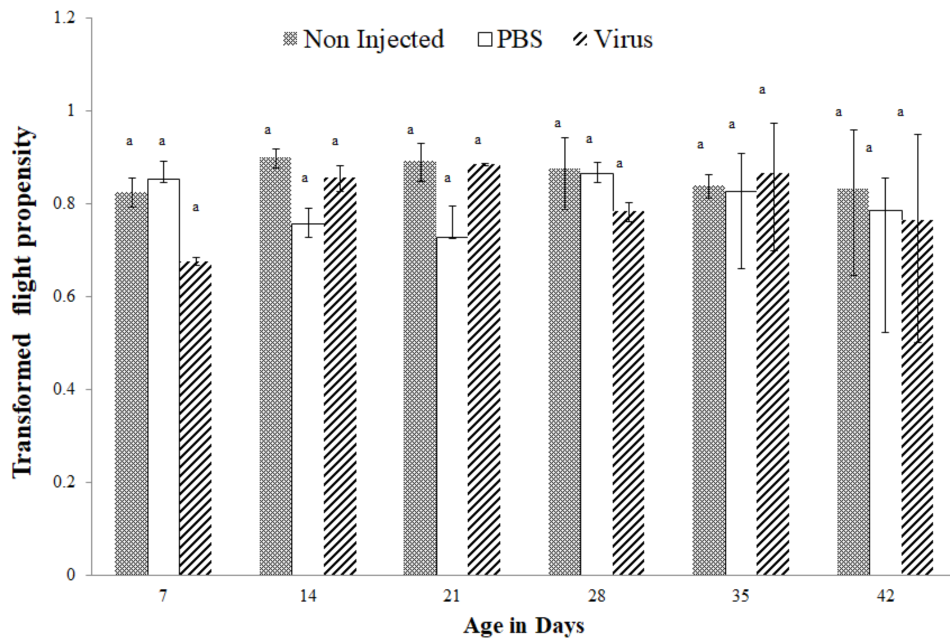

Figure 4. Impact of GpSGHV injection on *G. f. fuscipes* flight propensity at 7, 14, 28, 35 and 42 days post injections (dpi). The data was angular transformed for normality and detransformed for presentation. Mean ± SE.

Impact of GpSGHV infection on *G. f. fuscipes* flies mating ability

In order to assess the impact of the GpSGHV infection of the flies' mating ability, it was essential to determine the optimal mating age of untreated flies. Mating propensity of 3, 6, 9 and 12 day-old males differed significantly (F= 3.07, df= 3, 8, P < 0.001) with 3 day-old males having a significantly lower mating success as compared with older males (P < 0.001). However, no significant difference in the mating propensity of 6, 9 and 12 day-old males (P > 0.05) was observed **(Figure 5A)**.

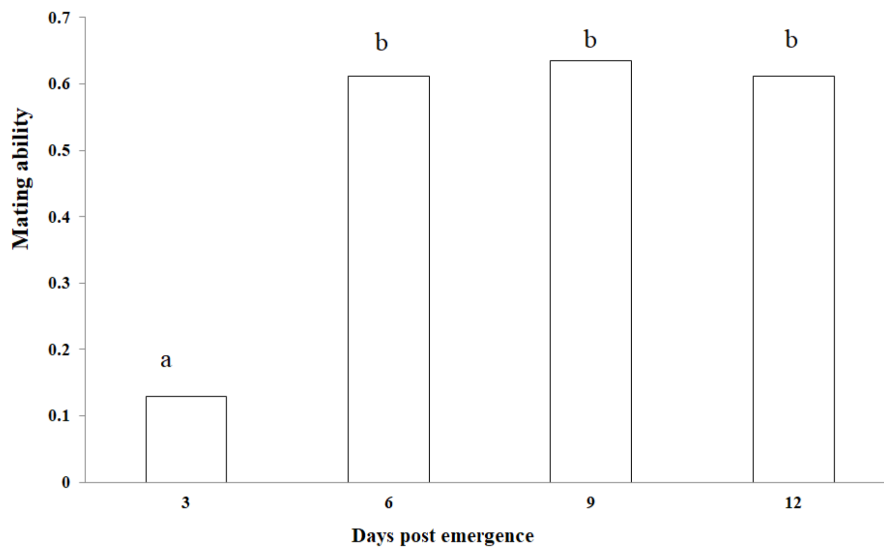

Figure 5A. Impact of GpSGHV injection on *G. f. fuscipes* mating ability. **A:** mating propensity of 3, 6, 9 and 12 day old untreated *G. f. fuscipes* males;

Therefore, 6-9 day-old males were used to assess the impact of GpSGHV infection on the mating ability of male flies. Most of the mating pairs were formed in the first hour after introduction of the females into the mating cages, and mating gradually reduced during the remaining 2 hours of the test. In general, mating propensity of non-, PBS- and virus-injection flies was significantly different (F= 4.89, df = 2, 24, P = 0.016). The mating propensity of virus-injected males was significantly reduced as compared with non-injected males (P = 0.014) (**Figure 5B**), while, no significant difference was observed between PBS-injected and non-injected males (P = 0. 59) or between the PBS and virus-injected males (P = 0.11).

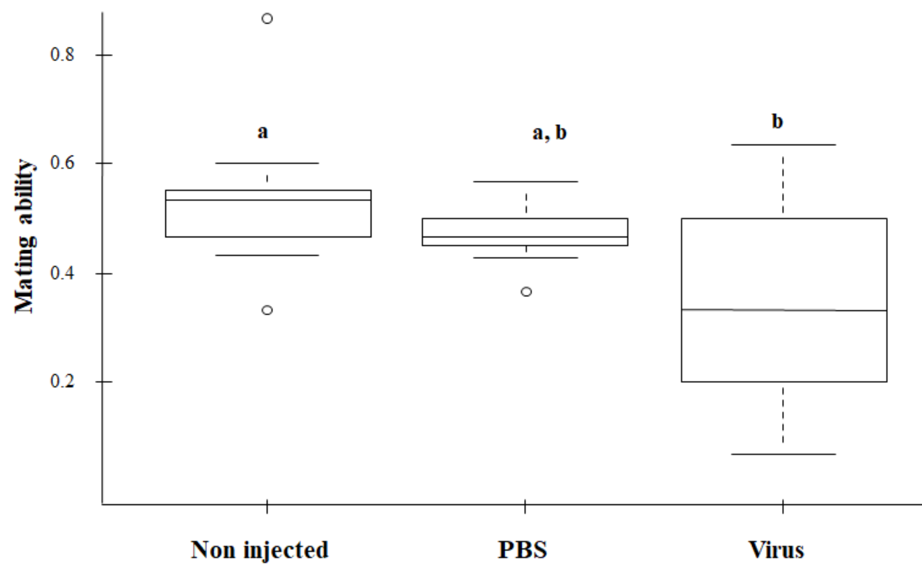

Figure 5B. Impact of GpSGHV injection on *G. f. fuscipes* mating ability. Nine day old virgin males from different treatments mated with 9 day old females.

Impact of GpSGHV infection on insemination rate

Females mated with untreated males of different ages showed variable insemination rates (**Figure 6A**). The proportion of females with empty spermathecae decreased as male age increased (F = 17.89, df = 1, 6, *P* = 0.005). The percentage of females with partially and fully filled spermathecae increased slightly but not significantly (F = 2.6, df = 1, 6, *P* = 0.15) while the percentage of females with fully filled spermathecae increased significantly (F = 6. 74, df. = 1, 6; *P* = 0.04) with increasing age of the males (**Figure 6A**).

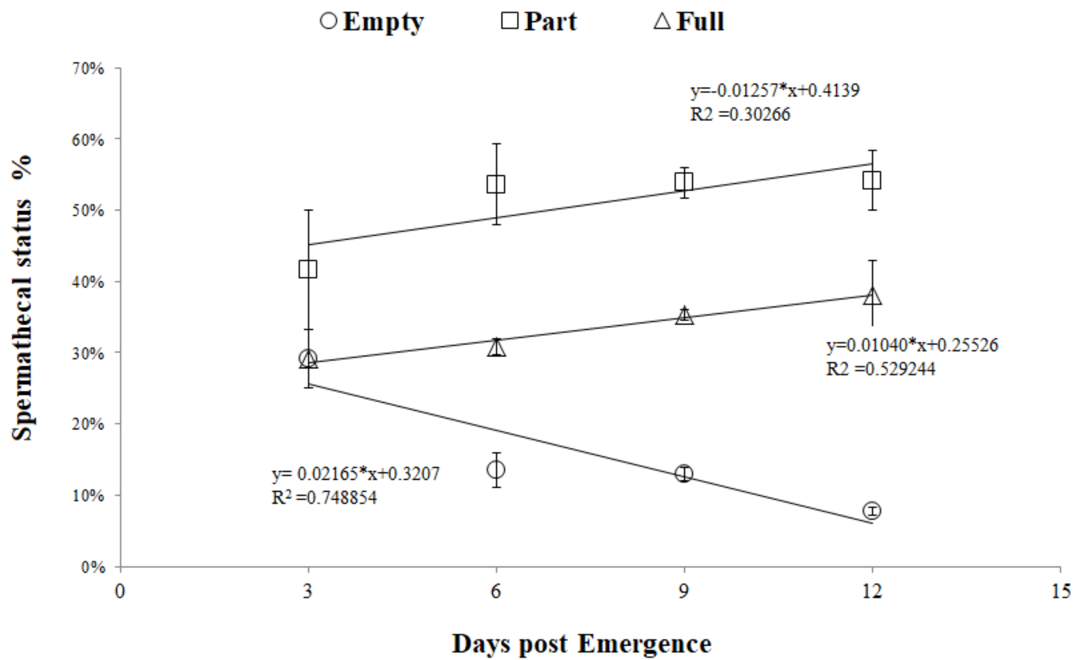

Figure 6A. Impact of GpSGHV infection on *G. f. fuscipes* female insemination rate when mated with 3, 6, 9 or 12 day old untreated males; Proportion of empty spermathecae reduced significantly with male age (y=-0.02165*x+0.3207, *P*= 0.005); proportion partially filled (y=-0.01257*x+0.4139, *P*= 0.1577) and completely filled increased (y = 0.01040 * x + 0.25526, *P*= 0.04 between 3 and 12 days post emergence.

The GpSGHV injection of the males did not affect the insemination rate for empty, partially and fully filled spermathecae values or empty spermathecal values (F = 0.19, df = 2, 24, *P* = 0.8261) (**Figure 6B**).

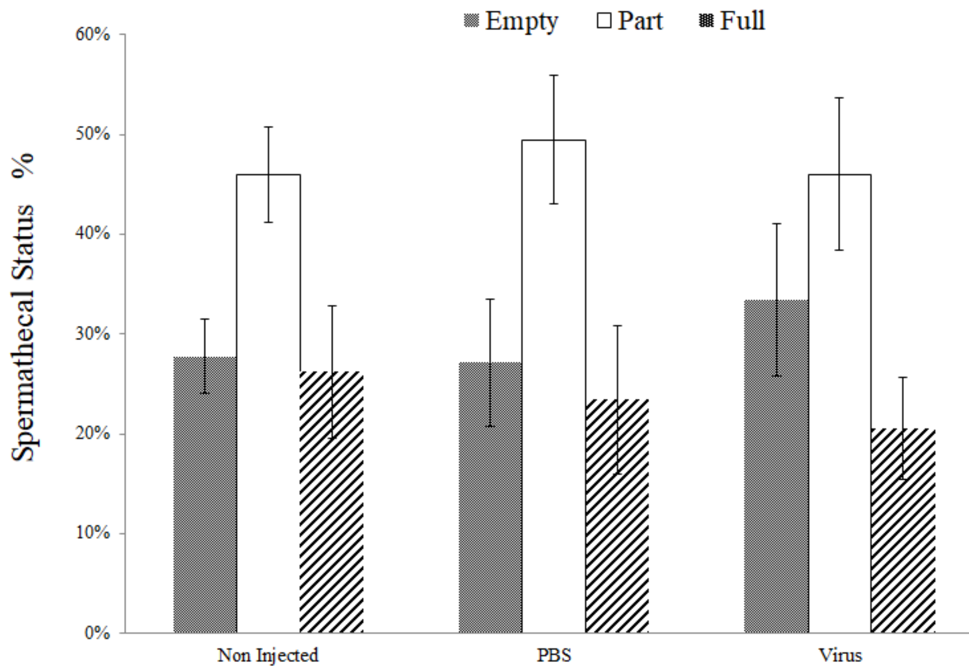

Figure 6 B. Impact of GpSGHV infection on *G. f. fuscipes* female insemination rate when mated with 9-days old virgin males from different treatments.

Discussion

The challenge in establishing large colonies of tsetse flies in mass-rearing facilities for the implementation of the SIT component in AW-IPM programs has always been a strong driver to explore and identify the key factor(s) affecting tsetse biology. The collapse of colonies of *G. pallidipes* at the IPCL and in Ethiopia prompted a decade of research on the productivity problems in these colonies and its association with the GpSGHV. As a result, virus management strategies have been developed to mitigate the instability in production of these colonies [20, 27-29]. In view of the similarity of low productivity of the *G. pallidipes* and *G. f. fuscipes* colonies maintained at the SAS in Bratislava, Slovakia and Addis Ababa, Ethiopia, this study was conducted to investigate whether a potential GpSGHV infection might contribute to the low performance of the *G. f. fuscipes* colony. Our data indicates that the presence of the virus indeed reduced various important quality parameters such as adult longevity, female productivity and male mating ability and, in addition, increased the mortality rate. Conversely, flight ability and insemination rate of virus-challenged flies was not affected as compared with uninfected ones.

Despite the negative impact of virus challenge on the flies' performance, no SGH symptoms were observed in injected *G. f. fuscipes* flies and no virus transmission to the F_1 progeny was detected (data not shown). These findings are in agreement with recent data demonstrating that the GpSGHV can replicate in five heterologous tsetse species without inducing SGH or being vertically transmitted to the F_1 offspring [33]. The results also agree with previously reported data on the significant reduction in the lifespan of *G. pallidipes* challenged with GpSGHV [54]. In addition, similar results were obtained by injecting the house fly *Musca domestica* salivary gland hypertrophy virus in a heterologous host, *Stomoxys calcitrans*, where the infection had a negative impact on survival and fecundity of the heterologous host without the development of SGH symptoms [55].

The GpSGHV infection in *G. f. fuscipes* affected fitness parameters such as increased mortality and reduced fecundity which are the key parameters for colony stability and growth. Moreover, the effects of virus infection affected more deeply females than males. This is especially relevant for tsetse flies whose productivity is lower–as compared with to that of most insects [56]. This negative impact on female mortality and productivity under normal colony conditions may explain the problems in maintaining the colonies (both in Slovakia and in Ethiopia) and its ultimate reduction in colony numbers. The lower female survival due to the presence of the virus agrees with previous reports on *G. pallidipes*, showing that females with apparent viral infection as indicated by their enlarged salivary glands had a significantly shorter lifespan than females with normal salivary glands [57]. Likewise, the longevity of virus-infected *G. m.centralis* flies was significantly reduced as compared with uninfected control flies [26].

Our observed positive correlation between male age and mating success was in agreement with previously reported data.that in field cage conditions, males younger than 8 days showed a significant lower mating ability [43]. Our results showed that 3 day-old males were less successful in mating than older males, but no further significant difference was observed between 6-day old or older males. Similar observations were reported with other species, i.e. 3-day old male *G. brevipalpis* and *G. austeni* were less successful in mating as compared with older males [58]. In other studies, 6-8 day old-males *G. p. gambiensis* were used for mating studies [59] and older *G. pallidipes* males copulated more often than young males [44, 60].

The significant reduction of the mating ability of GpSGHV-challenged male *G. f. fuscipes* flies is an additional negative impact of the presence of the virus. The observed reduction in

mating success as measured in small mating cages that mimic well the situation in standard tsetse holding cages, might partly explain the reduction in the females' fecundity as almost half of the females were not inseminated when offered a mating opportunity with virus-injected males. These results are in agreement with previous studies on the mating performance of *G. pallidipes* in small laboratory cages [57] or in walk-in field cages [61]. Our data are also in agreement with results of *Helicoverpa zea* males infected with the Hz-V2 virus, that were slower in approaching healthy females for mating as compared with non-infected males [62, 63]. This reduction in mating propensity might be a result of reduced flying and searching activity for females or possibly a negative selection by females against infected males [64].

Our data ~~to~~ imply a different outcome when compared with the results of Odindo [65] who reported no significant difference in mating performance between symptomatically infected and asymptomatic *G. pallidipes* flies. In addition, in contrast to our study, Jura and Davies-Cole (1992) speculated that SGHV-infected, and hence sterile, *G. pallidipes* males showed increased mating competitiveness and concluded that these males could be used for SIT applications [66]. Although our and the experiments of Odindo [65] and Jura and Davies-Cole [66] were conducted in similar settings (small laboratory cage), the different results are most likely due to the different tsetse species (*G. pallidipes* versus *G. f. fuscipes*) populations or strains used in the study. However, no data are so far available on the impact of the virus in males on the potential selection of females for mating partners. Further studies on the presence of the virus and its impact on the biological mechanisms of mating are necessary.

The virus injection has no significant impact on flight propensity and insemination rate of infected flies. The absence of a negative impact on the adult flight propensity (males and females) observed in this study contradicts the finding of Odindo [64] who speculated that the presence of the virus resulted in reduced physical male activity in *G. pallidipes*. It also contradicts the observation of Burand and Tan [63] who observed that the Hz-1 virus makes the *H. zea* male lazier and slower to move. The reduction in the mating propensity of virus-infected males might be due to reduced physical male activity. This might indicate that the physical activity required for the flight propensity test is much less than that required for successful mating and therefore the infected males had the propensity to fly but lost the ability to conduct normal mating activity.

The absence of any significant impact of the virus infection on insemination rate might be due to the completion of sperm development during the pupal stage in *Glossina* species.i.e. before the virus infection performed at adult stage. The results contradict earlier data indicating that virus infected *G. pallidipes* males with SGH were unable to successfully inseminate females after mating [21, 61]. The difference between our current data and these published earlier might be due to a different level of virus infection (virus infected *G. f. fuscipes* showed no sign of SGH versus *G. pallidipes* males with SGH indicating a higher density of virus particle per flies (> 10^6) and a different tsetse species.

Conclusions

The data presented in this paper directly demonstrates the negative impact of GpSGHV infection on the establishment and maintenance of *G. f. fuscipes* colonies, which will be crucial for the production of sufficient male flies of adequate biological quality for the application of the SIT programmes. The combination of increased female fly mortality and the reduction in mating propensity of the virus-infected males will shorten the production period and therefore will necessitate an increase in colony size to compensate for the loss in production. Finally, virus-infected males might have a lower competitiveness under field conditions, which will require increased release rates. These combined effects of the presence of the virus in *G. f. fuscipes* colonies will impose serious challenges to mass-rear and produce sufficient sterile males of adequate biological quality and will make the SIT component more expensive and less competitive with other control tactics [67]. Management strategies to mitigate the negative effects of virus presence that were based on the use of a clean feeding system (each fly receives a clean blood meal) and the mixing of the blood meals with the antiviral drug valacyclovir were recently developed for *G. pallidipes* colonies. However, the implementation of these strategies has so far been restricted to *G. pallidipes* colonies where flies showed clear SGH symptoms [27-29]. So far, the absence of SGH symptoms in many tsetse species including *G. f. fuscipes* has excluded the virus-infection as a possible cause for the poor performance of certain colonies and consequently no virus management strategies were implemented. The data presented in this manuscript strongly indicates that colonies that perform poorly should be screened for the presence of the virus with PCR and in confirmed cases, virus management strategies should be implemented even when no SGH symptoms are observed. Special caution is required in those tsetse mass-rearing facilities where *G. pallidipes* colonies are maintained with colonies of other tsetse species.

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Author Contributions

AMMA, AGP, RLM: designed, supervised the research and writing of the manuscript. GDU, AGP, AMMA: conducted the experiments, collected and analyzed data and prepared the figures. PT, AGP: Provided live material for experiments. GDU, AGP, MJBV, JB, PT, RLM: participated in the writing of the manuscript. All authors have read and agreed to its content and that the manuscript conforms to the journal's policies.

Competing interests

The authors declare that they have no competing interests.

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Chapter 6

Combining paratransgenesis with SIT: Impact of ionizing radiation on the density of *Sodalis glossinidius*

Combining paratransgenesis with SIT: Impact of ionizing radiation on the density of *Sodalis glossinidius*

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Short Title: Impact of irradiation on *Sodalis glossinidius* density in *G. m. morsitans*

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Keywords: Symbiont, Glossinidae, African trypanosomosis, African trypanosomiasis

Abstract

Tsetse flies (Diptera: Glossinidae) are the cyclical vectors of African Trypanosomosis, which has been identified as a neglected tropical disease in both humans and animals in many regions of sub-Saharan Africa. The sterile insect technique (SIT) has shown to be a powerful method to manage tsetse fly populations when used in the frame of an area-wide integrated pest management (AW-IPM) program. To date, the release of sterile males to manage tsetse fly populations has only been implemented in areas to reduce transmission of animal African Trypanosomosis (AAT). The implementation of the SIT in areas with Human African Trypanosomosis (HAT) would require additional measures to eliminate the potential risk associated with the release of sterile males that require blood meals to survive and hence, might contribute to disease transmission. Paratransgenesis offers the potential to develop tsetse flies that are refractory to trypanosome infection by modifying their associated bacteria (*Sodalis glossinidius*). Here we assessed the feasibility of combining the paratransgenesis approach with SIT by analyzing the impact of ionizing radiation on the density of *Sodalis* and the vectorial capacity of sterilized tsetse males. Adult *Glossina morsitans morsitans* that emerged from puparia irradiated on day 22 post larviposition did not show a significant decline in *Sodalis* density as compared with non-irradiated flies. Conversely, the *Sodalis* density was significantly reduced in adults that emerged from puparia irradiated on day 29 post larviposition and in adults irradiated on day 7 post emergence. Moreover, irradiating 22 day-old puparia reduced the density of *Wolbachia* and *Wigglesworthia* in emerged adults as compared with non-irradiated control, but the irradiation treatment had no significant impact on the vector competence of the flies. Although the irradiation treatment significantly reduced the density of some tsetse fly symbionts, the density of *Sodalis* recovered with time in flies irradiated as 22 day-old puparia. This recovery offers the opportunity to combine a paratransgenesis approach using modified *Sodalis* to produce males refarctory to trypanosome infection with the release of sterile males to minimize the risk of disease transmission, especially in HAT endemic areas. Moreover irradiation did not increase the vector competence of the flies for trypanosomes.

Background

Tsetse flies (*Glossina* spp., Diptera: *Glossinidae*) are the cyclical vectors of African trypanosomes, which cause a devastating and economically important infectious disease; sleeping sickness or Human African Trypanosomosis (HAT) in humans and nagana or Animal African Trypanosomosis (AAT) in livestock. Nagana causes high mortality in livestock and HAT is a serious health burden and risk to 60 million people in endemic regions of 36 countries in sub-Saharan Africa [1, 2]. *Trypanosoma vivax*, *T. congolense* and *T. brucei brucei* are the major tsetse transmitted pathogens in livestock [3] while *T. brucei rhodesiense* and *T. brucei gambiense* cause sleeping sickness in humans [4]. Members of the *morsitans* and *palpalis* groups of *Glossina* are efficient vectors of HAT and AAT [5]. In the absence of vaccines and efficient, safe and inexpensive drugs [6, 7], combined with increasing resistance against the current trypanocidal drugs [6, 8], control of the insect vector remains an essential part of managing disease transmission [9-11]. Most of the vector control strategies are insecticide-based [12, 13].

The sterile insect technique (SIT) is a species specific, safe, efficient, environment friendly, biological-based control tactic to manage populations (suppression or/and elimination) of insect pests and disease vectors [14]. The SIT entails mass-rearing the target insects, sterilization of the males using ionizing radiation and sequential area-wide release of a large number of sterile males into the target area. The sterile flies compete for mating with the female wild population, interrupting their reproductive potential ultimately resulting in population reduction or elimination [15, 16].

It is crucial that when large numbers of male vectors are released their ability to transmit pathogens should be curtailed to the maximum possible extent. In past and current tsetse fly programmes that had an SIT component, sterile males received two blood meals supplemented with the trypanocidal drug isometamidum chloride (10 µg/ml) before their release to minimize the risk of disease transmission. This treatment reduces the transmission ability of flies for *T. b. brucei* (no transmission) and *T. congolense* (5-fold transmission decrease) under laboratory conditions [17]. However, a field study demonstrated that the use of this treatment was not entirely sufficient to prevent sterile males of *Glossina palpalis gambiensis* from transmitting the trypanosomes *T. congolense* and *T. vivax* trypanosomes [18]. Therefore, the development of tsetse fly strains refractory to trypanosome transmission

would significantly contribute to the applicability of the SIT for the management of tsetse flies, especially in HAT endemic areas (**Figure 1**).

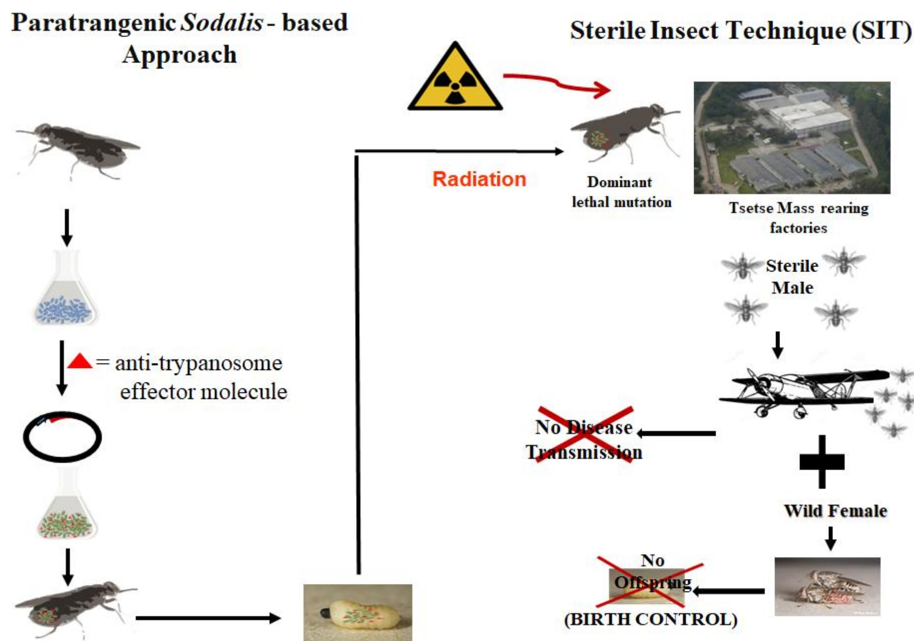

Figure 1. Schematic diagram of the combination between paratransgenesis and sterile insect technique (SIT).

Tsetse flies harbor four main symbiotic microbes; *Wigglesworthia, Sodalis, Wolbachia* [19-21] and the recently found *Spiroplasma* [22-24]. The primary mutualist *Wigglesworthia* resides intracellularly in bacteriocytes forming a specialized organ called 'the bacteriome' as well as extracellularly within maternal milk gland secretions. It provides dietary supplements that are necessary for host fecundity and is involved in the maturation process of the adult immune system. In the absence of *Wigglesworthia* in the larvae, subsequent adults are characterized by an underdeveloped cellular immune system and exhibit unusual susceptibility for trypanosome infections and are sterile [25-29]. The facultative symbiont *Sodalis* displays a wide tissue tropism and is present both intra- and extracellularly in the tsetse fly midgut, muscle, fat body, milk glands, and salivary glands The functional role of *Sodalis* in tsetse flies is relatively unknown although its influence on host longevity and modulation of susceptibility to trypanosome infection has been reported [30-32]. While all individuals in laboratory colonies harbor *Sodalis*, infection density in natural populations

varies from 0 to 85% in the different species analyzed [33-35]. The third symbiont, *Wolbachia* is an alpha-proteobacterium, located intracellularly in tsetse germ line tissues and is involved in cytoplasmic incompatibility. *Wolbachia* can be found in natural populations of tsetse flies with a prevalence varying between 0 and 100 % depending on the species [36, 36, 37, 37]. Finally, *Spiroplasma* is a genus of wall-free motile, gram-positive [22, 23] and associated both endocellularly and extracellularly in a variety of arthropods. It was recently identified as a novel symbiont of *G. f. fuscipes* and *G. tachinoides* [24].

Symbiotic microbes in tsetse flies have a vital role due to their significant influence on the biology of the fly, its reproduction, immunity, elicitation of phenotypes and potential effects on their vector competence for trypanosomes [35, 38-40]. Understanding the interactions of the symbionts and parasites occuring in tsetse hosts might facilitate the development of tsetse flies refractory to trypanosome infection by modifying their symbionts. Paratransgenesis is a new genetic method based on modifying symbiotic organisms of insect vectors using recombinant technologies to express effector molecules, including ones that can potentially block pathogen development [41, 42]. As trypanosomes develop in the midgut, proventriculus and salivary glands of tsetse flies, foreign gene products need to be expressed in at least one of those tissues. [26, 43]. *Sodalis* is an ideal candidate for paratransgenesis due to its presence in the midgut and the fact that it is one of the few insect bacterial symbionts that can be cultured and genetically modified *in vitro* [5, 43-46]. *Sodalis* has been genetically engineered to express and release significant amounts of functional anti-trypanosome nanobodies in different tissues of the tsetse fly [47]. A crucial step in implementing paratransgenic in tsetse flies for use in SIT programmes is the stable colonization of sterile male flies with recombinant *Sodalis* strains expressing trypanosome-interfering proteins. However, the impact of ionizing radiation on tsetse symbionts, especially *Sodalis*, is unknown.

The recent demonstration of tsetse pupae sex separation using near infrared imaging several days before adult emergence from the puparium [48] opens the possibility of irradiating males in the puparial stage. We investigated the impact of ionizing radiation on *Sodalis* density in adult *G. m. morsitans* flies after irradiation at three life stages after sex separation first becomes possible. Although the tsetse fly males are the sex of interest for SIT programmes, the impact of radiation on *Sodalis* density in females was also investigated as this effect has not been analyzed previously. After determining the optimum development phase for irradiation, i.e. having the least effect on *Sodalis* density, we tested the impact of irradiation on the male’s vector competence for trypanosomes as well as the impact on *Wigglesworthia,*

and *Wolbachia*. We discuss the significance of our findings in the context of improving the application of SIT and paratransgenesis to manage tsetse fly populations and hence African trypanosomosis.

Material and Methods

Tsetse Fly

The colony of the tsetse fly *G. m. morsitans* used in this study originated from Zimbabwe and has been maintained at the Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria since 1997. The colony and experimental flies were maintained at 24±0.5°C and 75 – 80 % rH and were fed on defibrinated bovine blood using the artificial (*in vitro*) membrane feeding system for 15-20 minutes three times per week [49, 50].

Analysis of the dynamics of Sodalis density in a G. m. morsitans colony

To assess the dynamics of *Sodalis* density in the *G. m. morsitans* colony established under laboratory rearing condition, samples of 4 males and 4 females were taken on day 0, 1, 2, 3, 4, 5, 6, 7 14, 21 and 30 post emergence. Samples were placed at -20°C until DNA extraction.

Experimental design

The impact of gamma irradiation was determined on both females and males. They were irradiated at three developmental stages to assess the effect on the density of *Sodalis*, *Wigglesworthia* and *Wolbachia*: (a) 7-day old adults, (b) 29-day old puparia (36 ± 12 h before emergence), (c) 22-day old puparia. Adult tsetse males that emerged from puparia irradiated with 110 Gy on day 22 post larviposition were tested for vector competence for trypanosomes.

Irradiation procedures

The tsetse puparia and adults were irradiated in air at the IPCL, Seibersdorf, Austria using a 60Co Gammacell® 220 (MDS Nordion Ltd., Ottawa, Canada). The dose rate was measured by alanine dosimetry as 2.144 Gy·sec-1 on 2015-03-03 with an expanded uncertainty (k=2) of 3.2%. The radiation field was mapped using Gafchromic HD-V2 film and the dose uniformity ratio in the volume used for the experiments was < 1.2 for adult exposures and < 1.1 for pupal

exposures. The desired radiation doses were given by varying the time of exposure of the samples to give minimum doses of 20, 50 and 110 Gy. Untreated puparia or flies were used as control (0 Gy) and handled in the same manner. For adults, 7-day old flies (males and females) were placed in small cages (11 cm diameter x 4.5 cm height) and placed in the center of the chamber for treatment. The handled were placed in plastic Petri-dishes (diameter 5.5 cm, height 1.5 cm) that allowed irradiation in the center of the chamber.

In the first part, 7-day old males and females that had already been offered three normal blood meals were irradiated with 20, 50 and 110 Gy at a density of 72 flies per cage with two replicates. After irradiation, all emerged flies of the treatment and the control goups were held under standard insect rearing conditions and were offered normal blood meals every other day of the week during the experiment. Four females and 4 males were frozen for each dose on day 0, 1, 7 and 14 post-irradiation. For the day 0 group, both females and males were frozen approximately 20 minutes after irradiation. All frozen samples were kept at -20°C untill being used for further analysis. The experiment was replicated twice.

In the second and third part of the study, batches of puparia were collected on the same day on day 22- and 29 post larviposition. Collected puparia for each radiation dose were kept in Petri dishes and exposed to 20, 50 and 110 Gy. The experiment was replicated two and three times for 22- and 29-day old puparia, respectively. Irradiated and non-irradiated pupae were kept under standard colony conditions. Daily examinations were made for fly emergence, and non-emerged puparia were recorded for each treatment. Emerged flies were collected daily and transferred to standard fly holding cages (20 cm diameter x 5 cm height) at a density of 72 flies per cage. Emerged female and male flies were held in separate holding cages during the experiment. Four females and 4 males were frozen on day 0, 1, 3, 5, 7 and 14 post emergence separately for each dose and kept at -20°C until further analysis.

DNA Extraction and Quantitative PCR

The total DNA of each individual fly was extracted from the collected flies using the DNeasy tissue kit (QIAGEN Inc, Valencia, CA) following the manufacturer's instructions. The extracted DNA was eluted in 200 µl elution buffer and DNA extracts from individual samples were pooled (4 females and 4 males, separately). The pooled DNA concentration was measured by spectrophotometry (Nanodrop-Synergy H1 Multi-Mode Reader, BioTek, Instruments, Inc., USA). All DNA samples were diluted to a final concentration of 4 ng/µl

and 5 µl of the diluted DNA was used for qPCR to determine symbiont density as previously described [51, 52]. The tsetse reference gene *β-tubulin* was used to normalize the qPCR reactions [52]. *Sodalis, Wigglesworthia* and *Wolbachia* densities were quantified for both sexes at different days post irradiation/emergence of all treatments by qPCR using primers that target the *fliC, codhoc* and *Wolbachia 16S rRNA* genes, respectively. The primers and the PCR conditions are given in **Supplementary Table 1**.

Tsetse fly infection with trypanosomes, maintenance, and dissection

For the infection experiment, teneral flies emerged from 22-day old irradiated (110 Gy) and non-irradiated puparia (collected and irradiated at the IPCL and shipped to the Unit of Veterinary Protozoology, Institute of Tropical Medicine (ITM), Antwerp, Belgium) were offered a blood meal containing a highly transmissible pleiomorphic *T. brucei brucei* (*Tbb*) AnTAR1 strain, 24 hours after emergence. *Tbb* AnTAR1 is a post-tsetse fly strain derived from the EATRO 1125 stabilate that was originally isolated from a bushbuck in Uganda in 1966 [53]. Parasitized blood was harvested with heparin from cyclophosphamide-immune suppressed mice (Endoxan®, Baxter) 6 days post-infection and mixed with defibrinated horse blood (E&O Laboratories) to obtain > 10^6 bloodstream form (BSF) trypanosomes/ml with 80% intermediate/stumpy forms in the infectious blood meal. This tsetse-trypanosome infection model has given good infection rates in the midgut and salivary glands of tsetse flies [54]. Flies that did not take the infectious blood meal were excluded from the experiment. Subsequently, the remaining flies were maintained for four weeks at 26 ± 0.5°C and 65 ± 5% relative humidity and offered uninfected defibrinated horse blood three times per week using an artificial membrane feeding system. Twenty eight days after the infective blood meal, individual flies were analyzed for the presence of procyclic and metacyclic trypanosomes by microscopical examination of their midguts and salivary glands, respectively. Differences in infection rates between irradiated and control flies were compared using Fisher's exact test (two-sided) and considered significant if *P*-values were lower than 0.05.

Statistical analysis

The statistical analysis and graphics were executed in R (R Core Team, 2017) using RStudio version 3.4.1. [55] with the packages ggplot2 v2.2.1 [56], lattice v0.20-35 [57] and MASS v7.3.47 [58]. Data was checked for normality and transformed where necessary using the

Box-Cox routine. The data was log transformed where the 95% confidence interval of lambda includes 0 and transformed with $(x^{\lambda}-1)/\lambda$ in other cases. The significance of the overall differences between the different doses obtained from the various treatments was assessed by ANOVA [59]. The significance of differences between the group's means (different radiation doses vs. non - irradiation individually analyzed for each day post emergence and irradiation time) was determined by Tukey's honestly significant difference (HSD) test. The *P*-values were calculated from the data with the significance threshold selected as 0.05. All regression analyses were conducted using the linear model for different doses and different times and coefficient factors (slope), t and *P* values are presented for females and males in **Supplementary Table 3 and 4, respectively**.

Results

Dynamics of Sodalis density in non-irradiated G. m. morsitans adults

Our experiments carried out under laboratory conditions indicated that *Sodalis* density was correlated with fly age and sex. For both males and females, after fly emergence from the puparia, an exponential increase in *Sodalis* density was observed, reaching a stable high density plateau when flies are aged beyond 3 weeks. In addition, *Sodalis* density was significantly higher in female than male flies ($P < 0.001$, regardless of fly age (**Figure 2, Supplementary Table 2**).

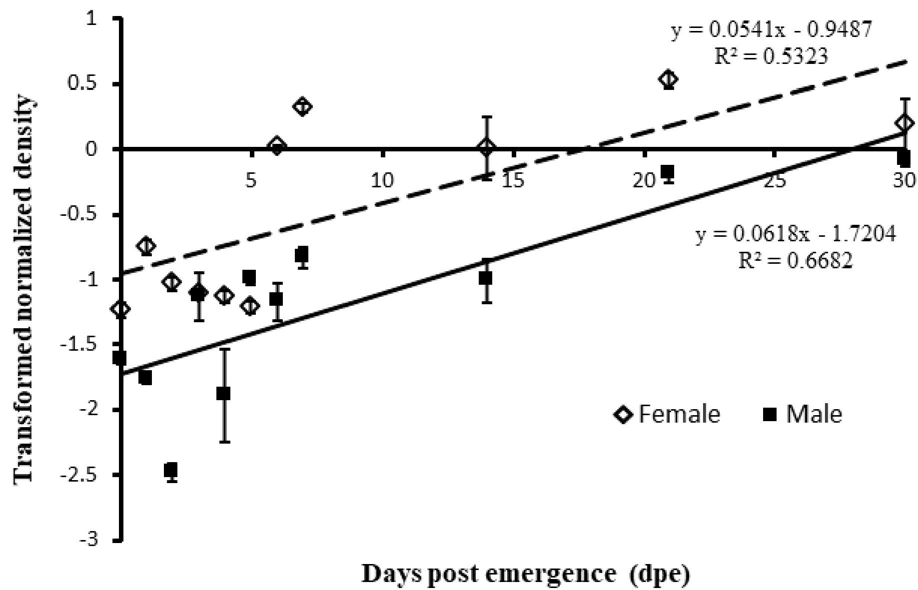

Figure 2. Dynamic of *Sodalis* density in *G. m. morsitans* adult flies maintained under laboratory colony conditions.

Impact of irradiation on Sodalis density in *G. m. morsitans*

We evaluated the impact of irradiation on the density of *Sodalis* in adult flies after treatment at three different life stages i.e., (i) as 7-day old adults, (ii) 29-day old puparia and (iii) as 22-day old puparia.

Adults irradiated at 7-days

In male flies, the radiation dose and time after irradiation significantly influenced the *Sodalis* density. *Sodalis* density decreased significantly with increasing radiation dose ($P < 0.001$), but increased significantly ($P < 0.001$, **Supplementary Table 2**) with time after irradiation. The negative correlation between radiation dose and *Sodalis* density was most obvious on day 1 and 7 post irradiation. On the day of emergence, no significant impact was observed between the different doses and *Sodalis* density. On day 14 post irradiation, the difference in *Sodalis* density among the different doses was lower than that observed on day 1 and 7 post irradiation but it remained significant **(Supplementary Table 3).** For the irradiated samples and regardless of the dose, the density of *Sodalis* on day 14 post irradiation was relatively higher than the density observed on day 0, 1 and 7 day post irradiation (**Figure 3, Supplementary Table 3**). The rate of increase of *Sodalis* density was higher in irradiated

samples than non-irradiated controls. In non-irradiated flies, there was no significant regression between *Sodalis* density and time **(Supplementary Table 2, Supplementary Figure 2B).**

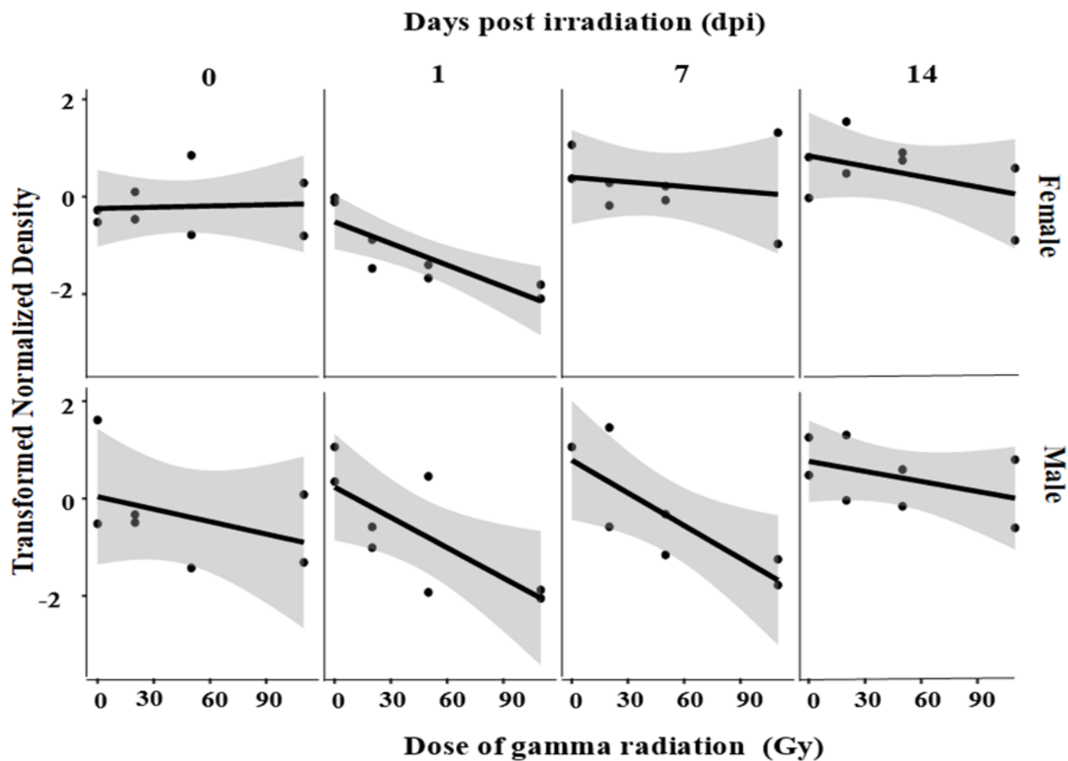

Figure 3. Impact of different ionizing radiation doses on *Sodalis* density in adult flies irradiated at 7-day post emergence at different time post irradiation.

The negative impact of radiation dose on *Sodalis* density was lower in females than males; this was mainly obvious on day 7 post irradiation (**Figure 3, Supplementary Table 2**). The impact on the *Sodalis* population following the irradiation treatment was most obvious on day 1 post irradiation. This decrease in *Sodalis* density was less obvious but significant on day 14 post irradiation. No significant decrease in *Sodalis* density due to the increase in the dose was observed on day 7 post irradiation. On the day of irradiation, no significant regression between dose and *Sodalis* density was observed (**Supplementary Table 3**). Over time, there was an increase in *Sodalis* density regardless of the dose. The increase in *Sodalis* density was greater in samples irradiated with 20 and 50 Gy than in 110 Gy and non-irradiated samples **Supplementary Table 4, Supplementary Figure 2A,)**.

Adults emerged from 29-day old irradiated puparia

While for the irradiated adult treatment the analysis was done on different days after irradiation, the adults emerged from irradiated puparia were analyzed on different days post emergence. The irradiation significantly reduced *Sodalis* density in males ($P < 0.001$) **(Supplementary Table 3)** irrespective of the day after emergence; however, *Sodalis* density significantly increased with time after emergence during the test period regardless of the dose ($P < 0.001$) (**Figure 4**, **Supplementary Table 2**). *Sodalis* density was inversely correlated with the radiation dose and was most obvious on days 1, 3 and 5 post emergence (**Supplementary Table 3**). Although *Sodalis* density was lower in irradiated males than control flies regardless of time the increase in *Sodalis* density over time was higher in irradiated samples compared to control. The rate of increase in *Sodalis* density was higher in samples treated with 50 and 110 Gy as compared with 20 Gy. In non-irradiated samples, *Sodalis* density did not increase with time **(Supplementary Table 4, Supplementary Figure 3B).**

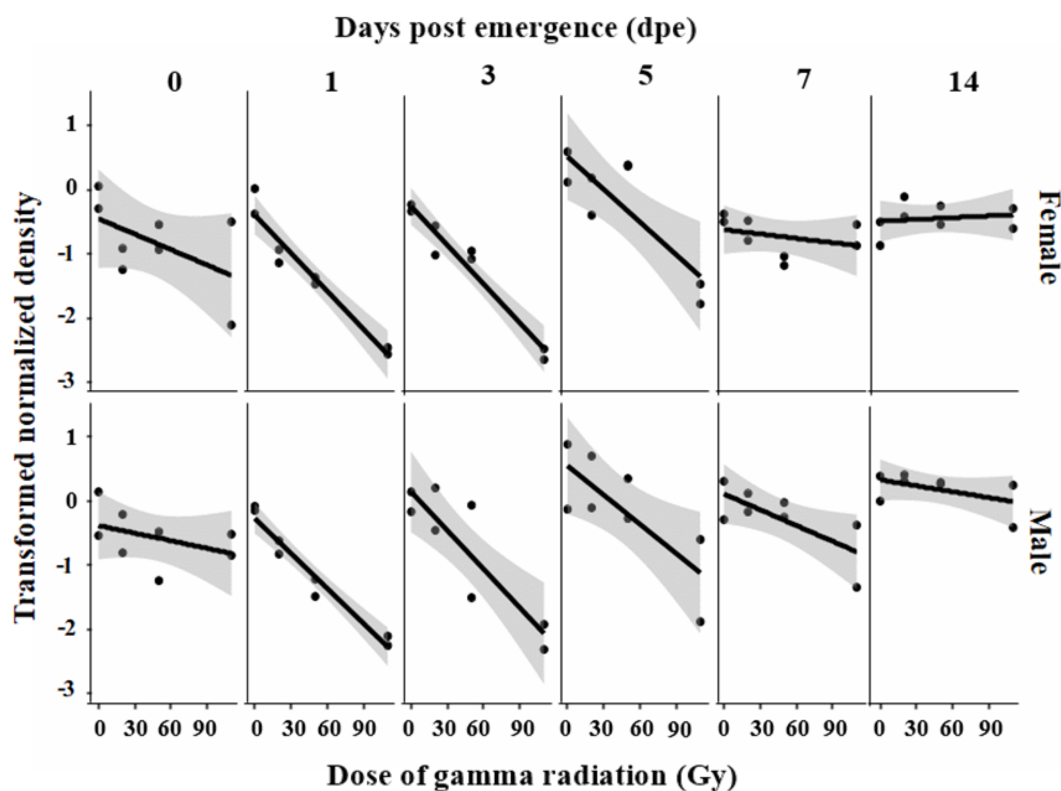

Figure 4. Impact of different ionizing radiation doses on *Sodalis* density in adult flies emerged from irradiated 29-day old puparia at different time post emergence.

In females, *Sodalis* density decreased significantly with increasing irradiation dose, on days 1, 3, and 5 post emergence. *Sodalis* density was negatively correlated with radiation dose on days on days 0, 1, 3, 5, 7 and 14 post emergence **(Figure 4, Supplementary Table 3)**. Although the density of *Sodalis* in irradiated treatments was in general lower than the non-irradiated control as observed in males, an exception was found at day 14 post emergence, where *Sodalis* density was slightly higher than the control. In the irradiated samples, the lowest *Sodalis* density was found in the samples treated with 110 Gy except on day 7 post emergence, where the lowest density was observed in 50 Gy-irradiated samples (**Supplementary Table 3**). Similar to males, there was significant positive regression between *Sodalis* density and time post emergence ($P < 0.01$) **(Supplementary Table 4)** in the female samples irradiated at 110 Gy (**Figure 4**). The rate of increase in *Sodalis* density was higher in the 110 Gy-irradiated samples as compared with that in 20 and 50 Gy irradiated samples. Surprisingly a significant decrease in *Sodalis* density over time was observed in non-irradiated samples ($P = 0.011$) **(Supplementary Table 4, Supplementary Figure 3A)**.

Adults emerged from 22-day old irradiated pupae

The quantification of *Sodalis* density in adult flies (males and females) emerged from puparia irradiated at 22-day old showed a different profile from that observed in flies irradiated as adults or as 29-day old puparia. However, day post emergence and sex significantly affected *Sodalis* density whilst *Sodalis* density in general was independent of radiation dose (**Figure 5**). As there was a significant interaction between time and treatment ($P = 0.017$) and between sex and time ($P < 0.01$) and treatment, the data for each time were analyzed separately for males and females (**Supplementary Table 2)**

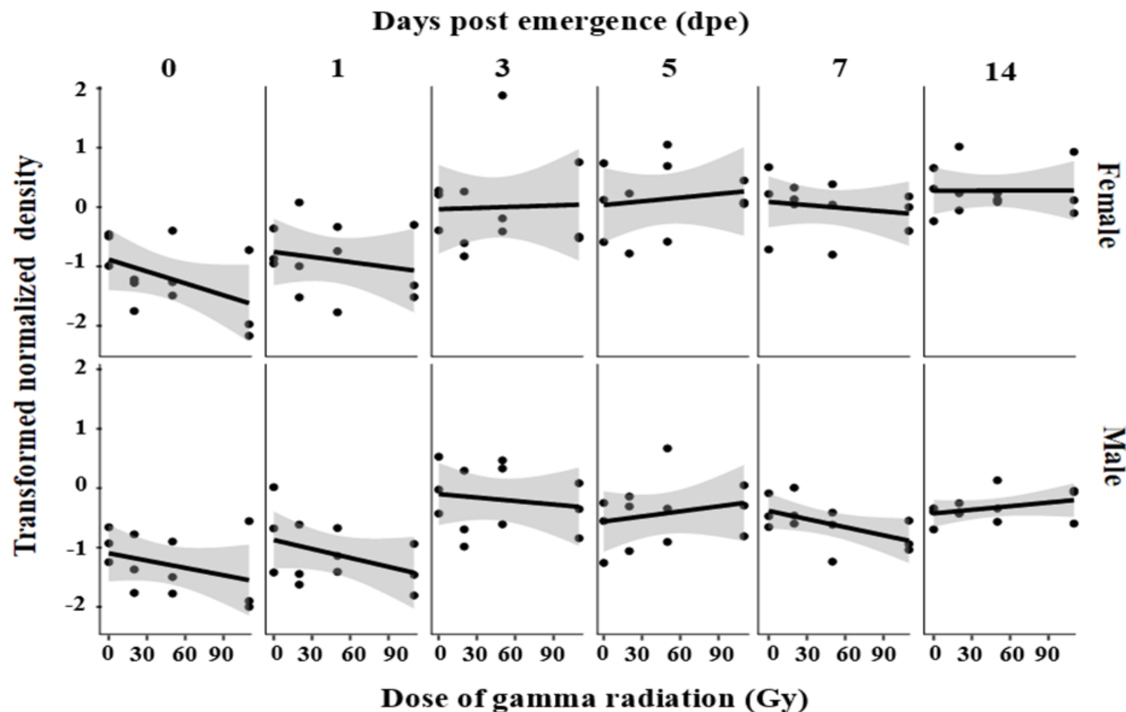

Figure 5. Impact of different ionizing radiation doses on *Sodalis* density in adult flies emerged from irradiated 22-day old puparia at different time post emergence.

In male flies, *Sodalis* density was in general not affected by dose, but was significantly affected by day post emergence when analyzed all data was together (**Figure 5, Supplementary Table 2).** However, when analyzed on each day post emergence, increasing doses induced a decrease in *Sodalis* density on day 0, 1, 3 and 7 post emergence. Contrary, *Sodalis* density increased with increasing radiation doses on day 5 and 14 post emergence (**Figure 5, Supplementary Table 3**). The rate of increase of *Sodalis* density in non-irradiated control was not significant with time but was significant for the 20, 50 and 110 Gy-treatment groups (**Supplementary Table 4, Supplementary Figure 4B**).

In general, the density of *Sodalis* was higher in female than in male flies and was independent of radiation dose, but increased significantly with time (**Figure 5, Supplementary Table 2**). However, the rate of increase of *Sodalis* density with time in the samples irradiated with 20 and 110 Gy was much higher than the rate of increase in the samples irradiated with 50 Gy and non-irradiated control (**Supplementary Table 4, Supplementary Figure 4A**).

Impact of tsetse developmental stage during irradiation on Sodalis density in *G. m. morsitans* males

Comparing the *Sodalis* density in the non-irradiated control with that in males irradiated with 110 Gy on day 22 and 29 post larviposition and adults, indicated that on day 7 post emergence, the *Sodalis* density in 110 irradiated males was significantly lower than in non-irradiated males (P = 0.002) irrespective of the developmental stage at the time of irradiation. On day 14 post emergence while the density of *Sodalis* in males irradiated with 110 Gy as adults males was significantly lower than non-irradiated males (P < 0.001). *Sodalis* density was not significantly different in males emerged from puparia irradiated on day 29 and day 22 post larviposition as compared with non-irradiated control flies. It is worth noting that the highest and lowest density of *Sodalis* was observed in males in the irradiated adult treatment in controls and 110 Gy respectively (**Figure 6**).

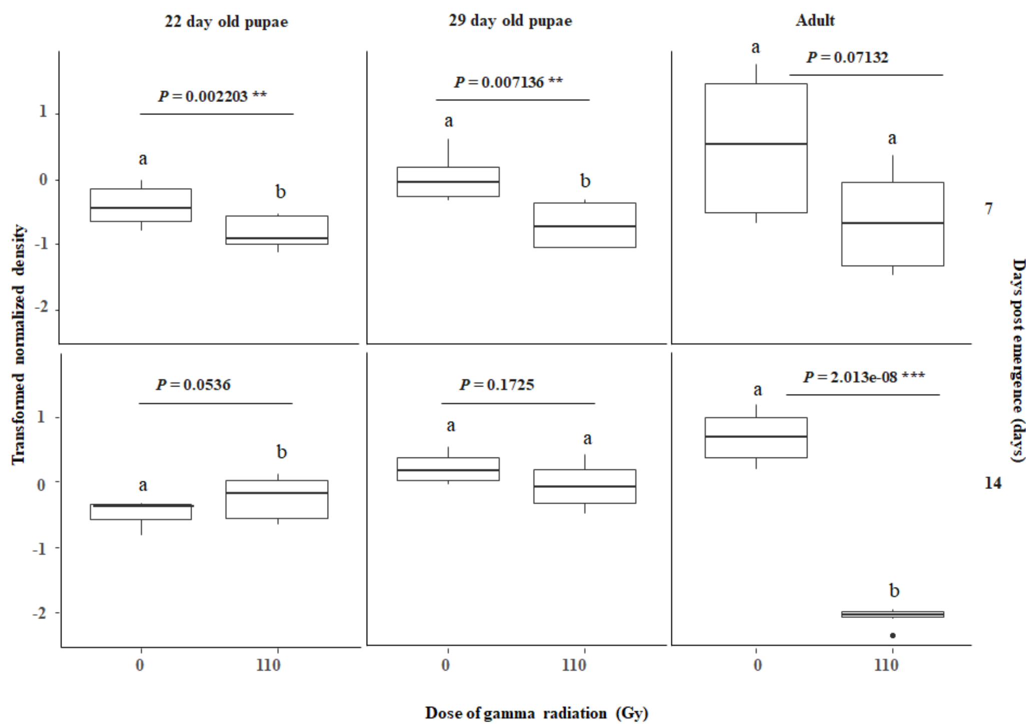

Figure 6. Impact of tsetse developmental stage during irradiation with 110 Gy on *Sodalis* density in *G. m. morsitans* males.

Impact of irradiation of 22-day old puparia on *Wigglesworthia* and *Wolbachia* density in *G. m. morsitans* flies

Wigglesworthia and *Wolbachia* densities were significantly different in female flies as compared with male flies, (**Supplementary Table 2**). In addition there was a significant interaction between sex and treatment in *Wolbachia*, therefore the data of both males and females were analyzed separately **(Supplementary Table 2**).

In males, increasing irradiation dose and time did not cause significant changes in *Wigglesworthia* density **(Figure 7, Supplementary Table 2).** In general, the density of *Wigglesworthia* was reduced with increasing dose regardless of the time post emergence, whoever this negative regression was only significant on days 0, 3 and 14 post emergence **(Supplementary Table 3)**. It is important to note that unlike *Sodalis*, the *Wigglesworthia* density did not significantly change with time in non-irradiated males or males irradiated with 50 Gy. In males irradiated with 20 and 110 Gy the *Wigglesworthia* density decreased significantly with time **(Figure 6, Supplementary Table 4, Supplementary Figure 5B).** The density of *Wolbachia* in male flies was not significantly affected by the radiation dose **(Supplementary Table 2)**. *Wolbachia* density increased with increasing doses on the day of emergence. This positive correlation turned into a significant negative correlation on day 3 post emergence **(Supplementary Table 3, Figure 7)**. The density of *Wolbachia* did not change significantly over time for non-irradiated males and for irradiated males irradiated **(Figure 6, Supplementary Table 2, Supplementary Figure 6B).**

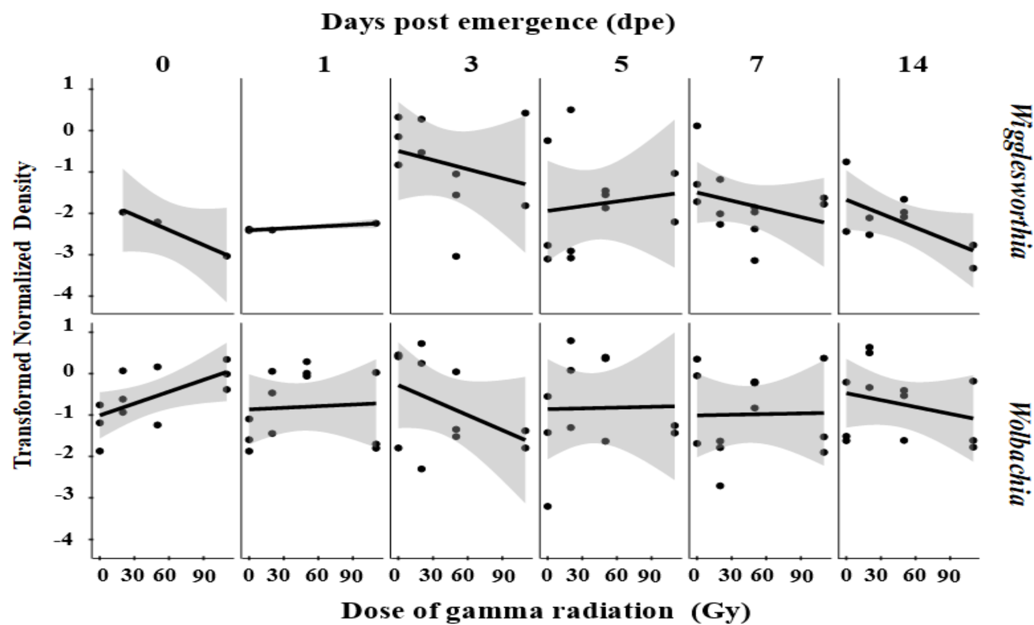

Figure 7. Impact of different ionizing radiation doses on *Wigglesworthia and Wolbachia* density in males emerged from irradiated 22-day old puparia at different time post emergence.

In females flies increasing radiation dose or time post emergence did not causes significant changes in the density of *Wigglesworthia* (**Supplementary Figure 1 and Supplementary Table 2**). In general the density of *Wigglesworthia* was reduced with increasing dose up to day 7 post emergence but seemed to increase with increasing dose on day 14 post emergence (**Supplementary Table 3**). There was no significant correlation between density of *Wigglesworthia* and time in non-irradiated control flies, whereas significant positive correlation was observed in the 110 Gy treatment group (**Supplementary Table 4, Supplementary Figure 5A**).

Unlike *Wigglesworthia,* the density of *Wolbachia* in female flies was significantly affected by radiation dose and time post emergence **(Supplementary Figure 1, Supplementary Table 2**). In general, similar to *Wigglesworthia*, the density of *Wolbachia* decreased with increasing doses, with the exception on the day of emergence when the density of *Wolbachia* increased. The decrease in *Wolbachia* density was significant on days 1, 3, 5, 7 and 14 post emergence **(Supplementary Table 3).** Over time, the *Wolbachia* density increased significantly only in 20 Gy treated females **(Supplementary Table 4, Supplementary Figure 6A and B).**

Impact of irradiation on the tsetse fly's susceptibility towards trypanosomes

Following *per os* challenge with trypanosomes in their 1st blood meal, 14.7 % and 6 % of non-irradiated and irradiated *G. m. morsitans* adults, respectively developed a mature trypanosome infection in their salivary glands **(Table 1)**. At the midgut level a similar infection ratio was observed, i.e. 18 % and 6 % in non-irradiated and irradiated flies, respectively. However, the observed differences in infection rates between irradiated and non-irradiated flies in both the salivary gland and the midgut were not statistically significant **(Table 1)**. Results from this experiment show that the establishment of a trypanosome infection in the tsetse's midgut and the subsequent maturation of this infection were not significantly affected by irradiation.

Table 1. Microscopic evaluation of the proportion of male irradiated and non-irradiated control *G. m. morsitans* flies infected with *T. b. brucei.*

Treatment	**Infected/ total # flies**		**Maturation**	***P* values**	
	Midgut glands	Salivary gland rate		Midgut glands	Salivary gland rate
Non-irradiated	11/61	9/61	0.82	-	-
Irradiated	3/50	3/50	1	0.0839	0.219

p values were obtained by comparing the infection prevalence of the irradiated group to the infection prevalence of non-treated control flies using a two-sided Fisher's exact test.
* Maturation rate: salivary gland/midgut infected flies

Discussion

The implementation of the SIT in the context of an area-wide integrated pest management strategy was successful in eradicating a population of *Glossina austeni* from Unguja Island of Zanzibar [60]. However, the release of large numbers of sterile male flies bears a potential risk of temporarily increasing disease transmission during the initial release phase of the programme [61]. To date, the release of sterile male tsetse flies has only been implemented in areas without HAT. Before their release, the sterile males were offered blood meals mixed with an anti-trypanosomal drug (isometamidium chloride) , and although this protocol reduced the risk of increased trypanosome transmission, there are reports that claim that it does not completely prevent it [17, 18]. Therefore, the implementation of a programme with an SIT component in an a HAT endemic area will require additional measures to eleminate the risk of increased trypanosome transmission.

One possibility would be to use paratransgenesis to develop tsetse flies refractory to trypanosome infection by exploiting the presense of symbiotic bacteria associated with the flies. It has been suggested to modify the symbiotic bacteria *Sodalis* to produce anti-trypanosome factors [44, 47, 54, 62], and important recent progress can be reported with the development of paratransgenic tsetse flies [54, 63, 64] for use in SIT programmes [61]. However, as the males destined for release need to be irradiated to make them sterile, the impact of the irradiation treatment on the *Sodalis* community needed to be assessed. Therefore, we investigated the effect of different radiation doses administered during different life stages on the density of *Sodalis* in *G. m. morsitans* flies.

It is known that the SIT becomes more effective when only males are released, but separating tsetse male from female puparia is currently not possible at an operational scale. In operational SIT programmes implemented so far, tsetse fly males have been seperated from females using one the following methods: (i) manual separation of the adults based on the morphological differences, and (ii) exploiting the difference in pupal period (female emergence two days earlier than males) [65, 66]. A third method is based on the use of near infrared light [67] to separate the puparia 8-10 days before adults emergence, but this is still being investigated. The above mentioned methods offer opportunities to irradiate male flies as adults (methods 1) or pupae (method 2 and 3) and to sterilize them for the release in an SIT program. Hence, the importance of analysing the impact of irradiation on tsetse symbionts at these different developmental phases. The selected of a male separation method depends on the conditions of each SIT program: (1) in the programme that successfully eradicated a population of *G. austeni* from Unguja Island of Zanzibar [68], adult males were separated manually from adult females, and the males irradiated and released as adults. A similar strategy was used for the programme against *Glossina palpalis gambiensis* and *Glossina tachinoides* in Sidéradougou, Burkina Faso [68] and against *G. fuscipes fuscipes and G. pallidipes* in Ethiopia [69] . A different apprach was adopted in the pilot SIT programme against *Glossina morsitans* in Tanzania, where the flies were irradiated and released as pupae in release stations [70](Williamson et al. 1983) . Another approach was adopted in the program in Senegal against *G. p. gambiensis* where the male puparia were collected on day 29 post laviposition after the emergence of females, irradiated and shipped under chilled conditions from different countries to Dakar, Senegal. [71]. Upon arrival, the pupae were left to emerge and the male flies were released as adults in the target area [71]. In the latter case, it is important to point out that separating male and female puparia during the mid-pupal phase

(between days 15-25 post larviposition) would be much appreciated in SIT programs as it would allow the irradiation and shipment of male puparia under ideal environmental condions (e.g. 23 °C) which would result in better quality males. With insects like the Mediterranean fruit fly *Ceratitis capitata* the problem was solved through the development of genetic sexing strains (GSS), which enabled eleminating the females at the embryonic or pupal stage. This approach greatly increased the efficacy of SIT programmes against this pest and significantly reduced its cost [72, 73]. Unfortunately such an approach is still not available for tsetse flies.

The use of ionizing radiation to sterilize male insects is a simple process that is easy and safe to apply [74]. Radiation causes single- and double-strand breaks in the chromosomes of both somatic and germ line cells [75], resulting in the formation of dominant lethal mutations in eggs and sperm [74]. However, as a result of the irradiation free radicals originating from water radiolysis, mainly OH free radicals, H atoms and solvated electrons e_{aqu} are formed in the treated insect that interact with intra- or extracellular molecules. The free radicals affect the microbial communities associated with irradiated flies as an indirect effect of irradiation. The negative impact of irradiation on reducing the gut microbiota was previously demonstrated in humans [76], but the impact on the microbiota associated with insects has so far was not been reported.

The results show that the density of *Sodalis* in untreated male and female *G. m. morsitans* significantly increased with time. Non irradiated female *G. m. morsitans* had a higher *Sodalis* density than male flies during a period of 30 days after emergence. This contrasts with earlier work that showed that *Sodalis* densities in male *G. p. gambiensis* were always higher than in female flies over a period of 80 days [77], and this difference might be due to a species-specific impact on *Sodalis* density. In general, the density of *Sodalis* infection in somatic tissues increased with the age of the fly but varied with species and sex [26]. In addition, our results indicate that the *Sodalis* population was significantly reduced after irradiation of 7-day old adult males, with no significant recovery on day 14 post irradiation. In contrast, the recovery of *Sodalis* density was significant in adult flies treated as 22 or 29 day-old puparia. The recovery in *Sodalis* density was most prominent in female flies when treated as 29-day old puparia, and in male flies when treated as 22-day old puparia. The observed recovery in *Sodalis* density in adult flies treated as pupae might be due to the relative longer period available for multiplication of *Sodalis* individuals after irradiation in comparison with the shorter period available in irradiated adult males. It is important to note that *Sodalis* has a

relatively slow growth rate (~15 hours for cell population doubling times *in vitro*) and therefore a relatively longer period is needed to increase its density in the irradiated host [62].

The recovery of *Sodalis* density in males treated as 22 day-old puparia to similar or even higher levels as observed in non-irradiated males opens the opportunity to use paratransgenesis to develop tsetse strains that are refractory to trypanosome infection. Although this study was conducted on non-modified *Sodalis*, it can at this stage be assumed that the response of modified *Sodalis* to irradiation would be similar to wild *Sodalis*, but this will need to be confirmed by further research. In our study both puparia and adult flies were irradiated to estimate the optimal dose and effects on *Sodalis* density, and the results clearly indicate that irradiating adult flies prohibits the use of paratransgenesis to develop tsetse strains that are refractory to trypanosome infection. Therefore, the most effective use of paratransgenesis in SIT programs will be achieved when separating the male from the female puparia on day 22 post larviposition using near infrared light, at least for *G. m. morsitans* [67]. This method however, is still under development and it is important to note that the successful development and use of paratransgenesis in SIT programs might be species dependent and is most certainly closely linked to an optimization of male and female pupal separation protocols.

The results also indicate a general reduction in the density of *Wigglesworthia* and *Wolbachia* in irradiated flies, especially when the dose was a high as 110 Gy. Whereas *Wigglesworthia* has a clear role in tsetse females as it provides vitamins necessary for female fertility [78], the role of *Wigglesworthia* in males is not clear and therefore we cannot speculate on the impact of a reduction in *Wigglesworthia* density in irradiated males. On the other hand, the reduction in *Wolbachia* density might affect the release of *Wolbachia* infected males to combine the cytoplasmic incompatibility with SIT programmes for tsetse as recenly implemented in mosquitoes [36, 40, 79-81]. The reduction in *Wolbachia* denisty after gamma radiation treatment was previously reported in *Brugia malayi* adult worms [82]. On the other hand, an enhancement effect on *Sodalis*, *Wigglesworthia* and *Wolbachia* densities was observed with of lower radiation doses.

In the vector competence experiment, our results from adults treated as puparia on day 22 post larviposition show that the establishment of a trypanosome infection in the tsetse's midgut and the subsequent maturation of this infection was not affected by the irradiation. This might be due to the low infection rate as the majority of insects are capable of eliminating pathogens in

their midgut shortly after getting a blood meal. An important factor for vector competence is the proficiency of the immune responses of the host insect [28]. On the other hand, the gut microbiome has a strong influence on host immunity and some of the bacteria species such as *Serratia* and *Enterobacter* present in the midgut of *Anopheles* mosquitos directly interfere with the mosquito vector competence [83]. Therefore, the pending research question will be to determine the vectorial capacity of adult tsetse flies infected with modified *Sodalis* after irradiation as 22 day-old puparia.

To date, no previous study has been conducted to assess the effect of ionizing radiation on the tsetse flies' symbiont density. This study determined the impact of irradiating puparia and adult *G. m. morsitans* flies on the density of *Sodalis, Wigglesworthia* and *Wolbachia*. Our data indicate that irradiation does not affect the vectorial capacity of the released sterile males, and hence, measures are needed to address this problem. The data of this study are encouraging to use paratransgensis to develop strains that are refractory to trypanosome infection, which will reduce or eliminate any potential risk that might be associated with the release of sterile males in HAT endemic areas.

Conclusion

This study provides the first demonstration of the functional impact of irradiation on *Sodalis glossinidus* and the vectorial capacity of treated flies. When puparia are irradiated between day 22 and 29 post larviposition, a significant recovery in *Sodalis* density occurs in the adult flies, but the vectorial capacity of adult males is not affected. Moreover, irradiaton induces a significant reduction in the density of *Wigglesworthia* and *Wolbachia*. The current study also reinforces the idea for the potential use of *Sodalis* to be developed into a paratransgenic platform that can be combined with SIT to block transmission of trypanosomes.

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Author Contributions

AMMA, AGP, RLM: designed, supervised the research and writing of the manuscript. GDU, LDV, AGP, AMMA: conducted the experiments, collected and analyzed data and prepared the figs. GDU, LDV, AGP, MJBV, JVDA, RLM, AMMA: participated in the writing of the manuscript. All authors have read and agreed to its content and that the manuscript conforms to the journal's policies.

Competing interests

The authors declare that they have no competing interests

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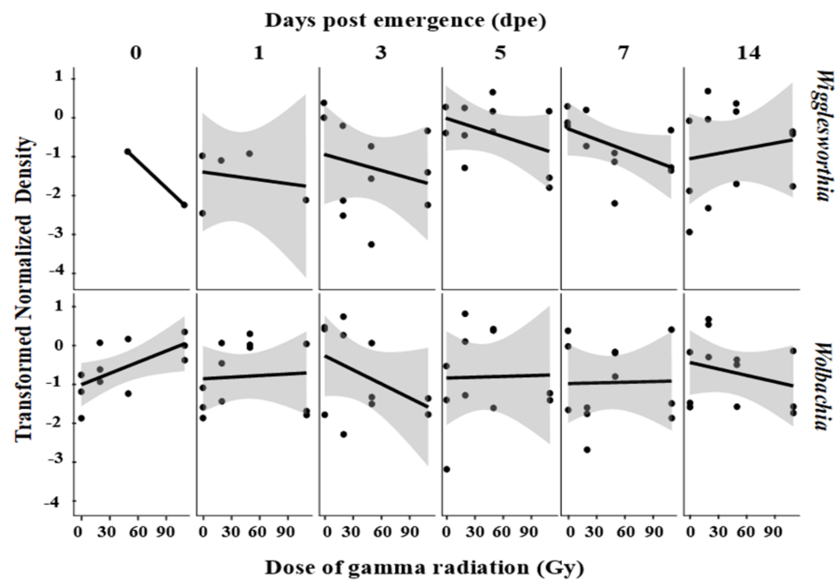
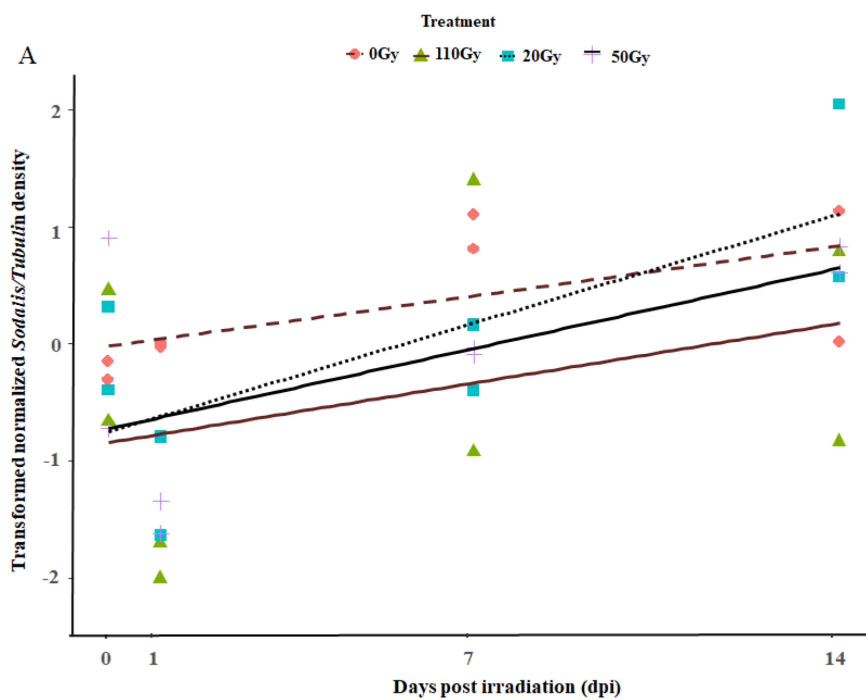
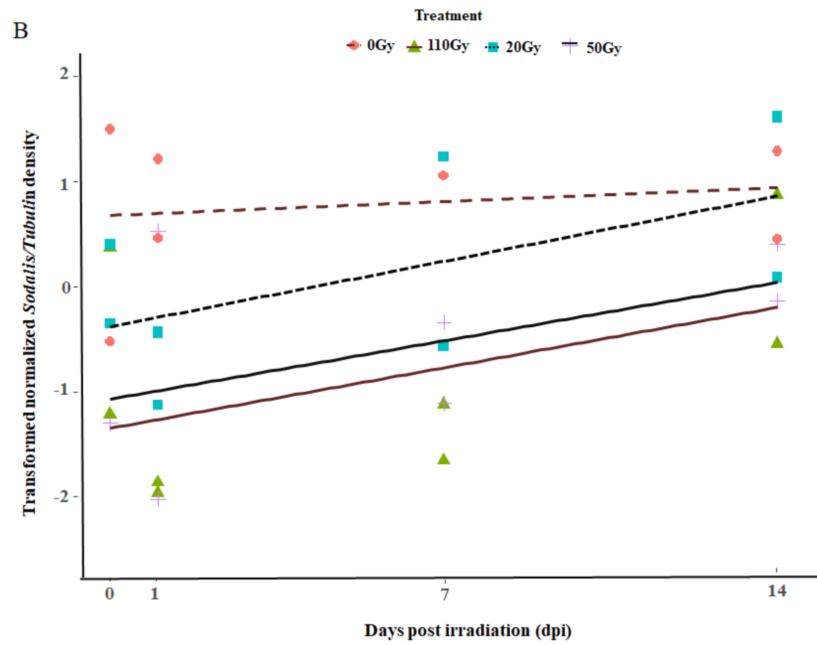
Supplementary Figure Legends



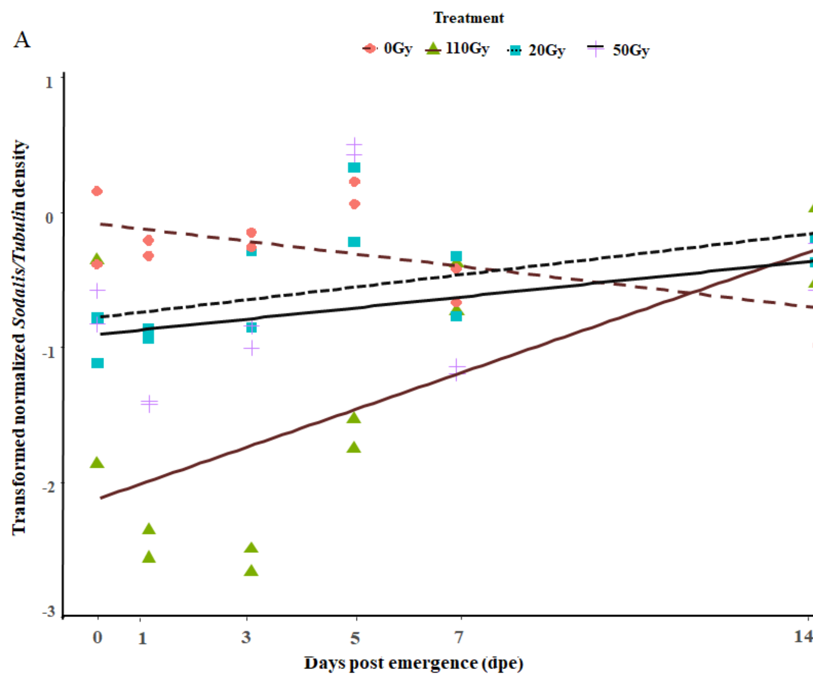
Supplementary Figure 1. Impact of different ionizing radiation doses on *Wigglesworthia and Wolbachia* density in females emerged from irradiated 22-day old puparia at different time post emergence.



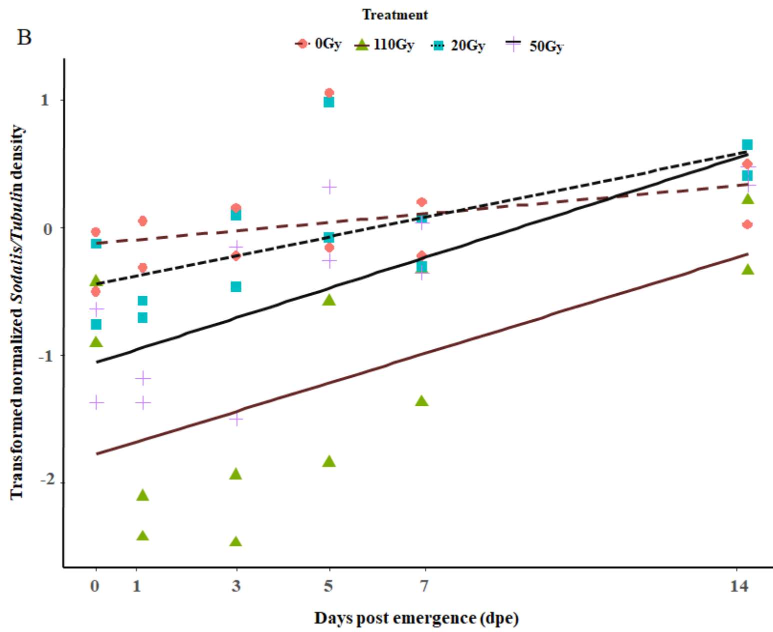
Supplementary Figure 2A. Impact of time post irradiation on *Sodalis* density in adult flies irradiated at 7-day post emergence treated with different doses for Females.



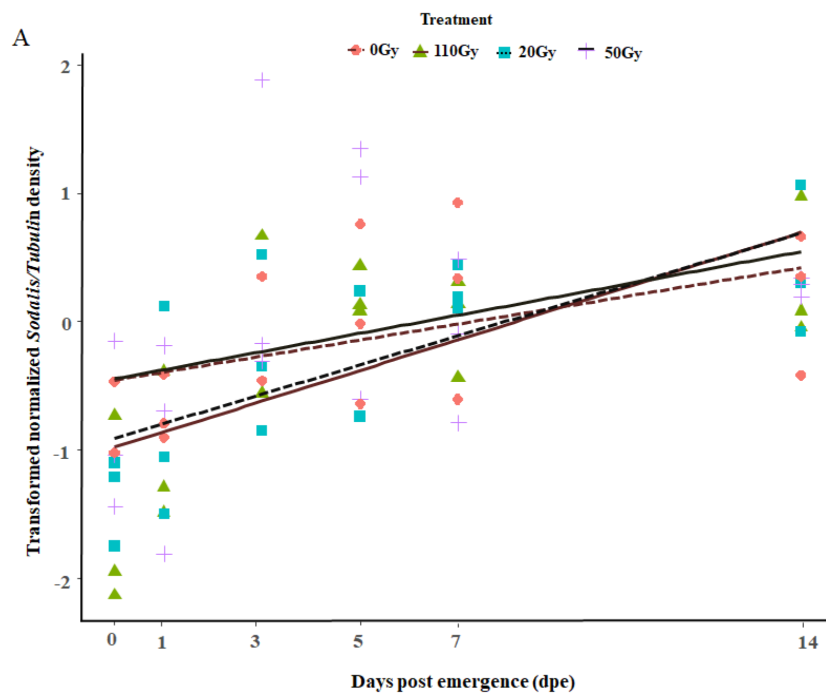
Supplementary Figure 2B. Impact of time post irradiation on *Sodalis* density in adult flies irradiated at 7-day post emergence treated with different doses for males.



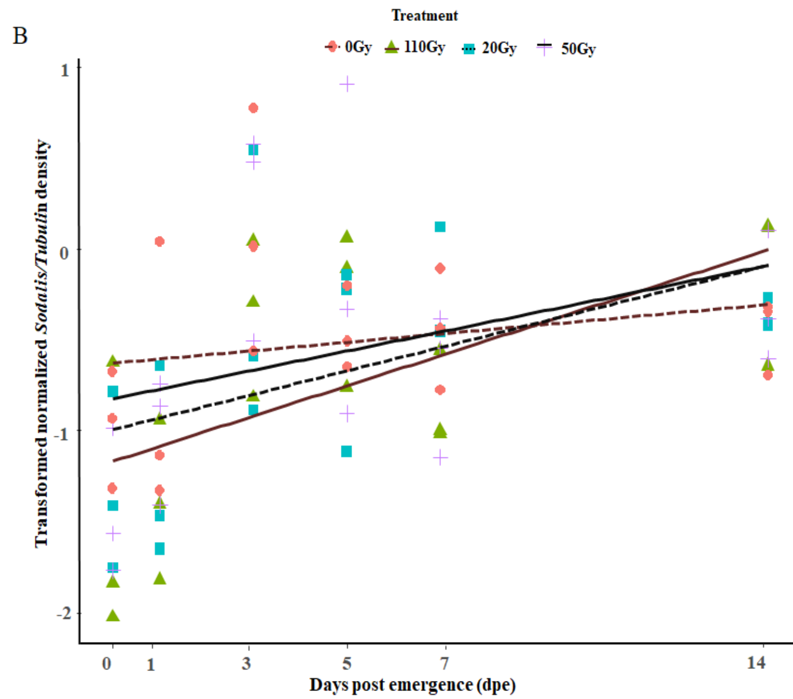
Supplementary Figure 3A. Impact of time post emergence on *Sodalis* density in adult flies emerged from irradiated 29-day old pupae treated with different doses for females.



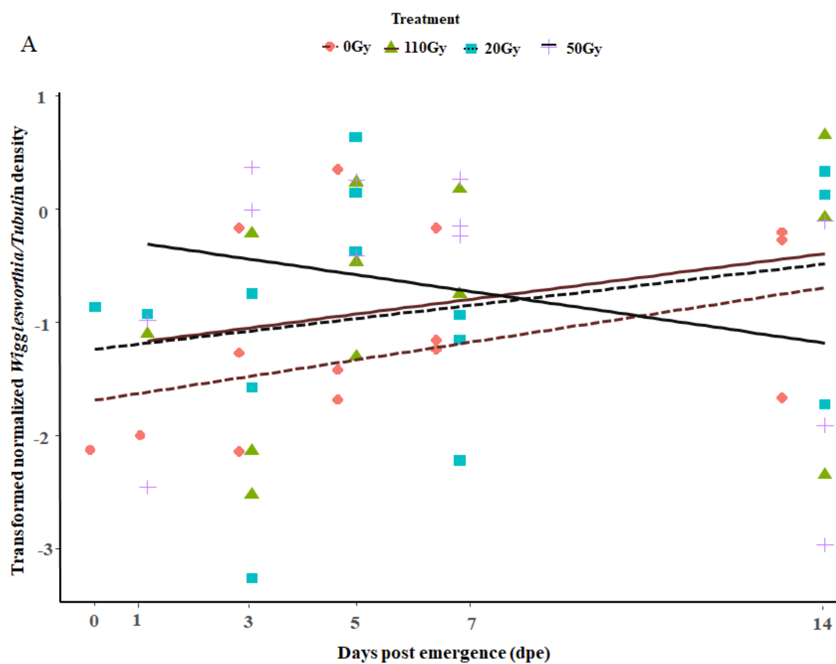
Supplementary Figure 3B. Impact of time post emergence on *Sodalis* density in adult flies emerged from irradiated 29-day old pupae treated with different doses for males.



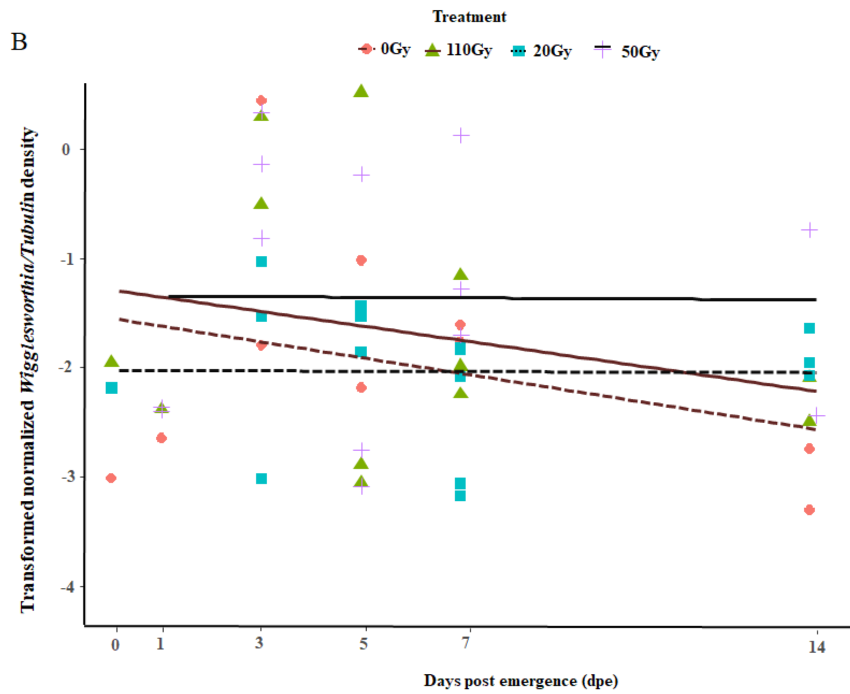
Supplementary Figure 4A. Impact of time post emergence on *Sodalis* density in adult flies emerged from irradiated 22-day old puparia treated with different doses for females.



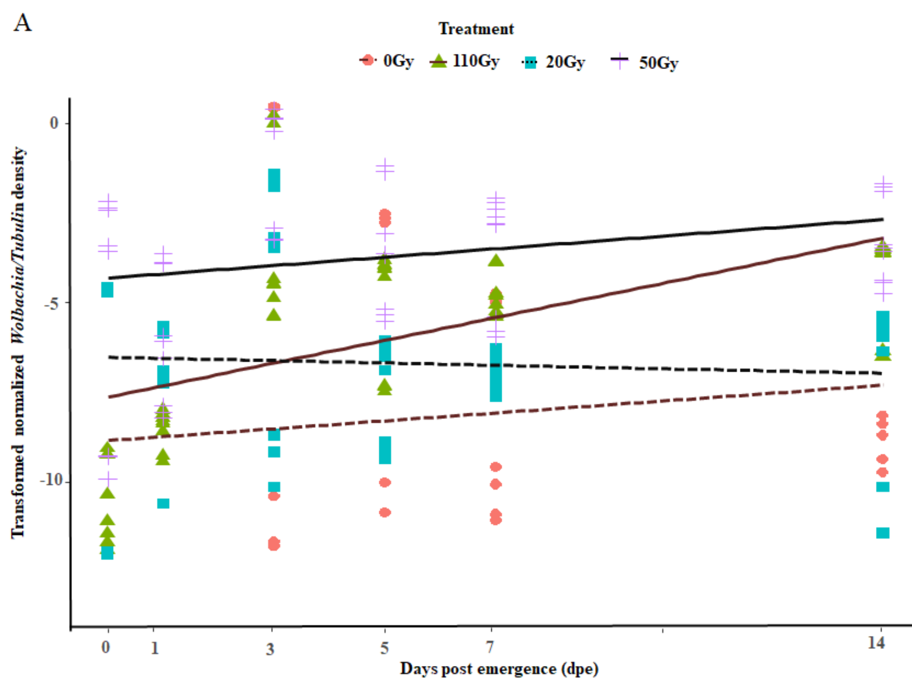
Supplementary Figure 4B. Impact of time post emergence on *Sodalis* density in adult flies emerged from irradiated 22-day old puparia treated with different doses for males.



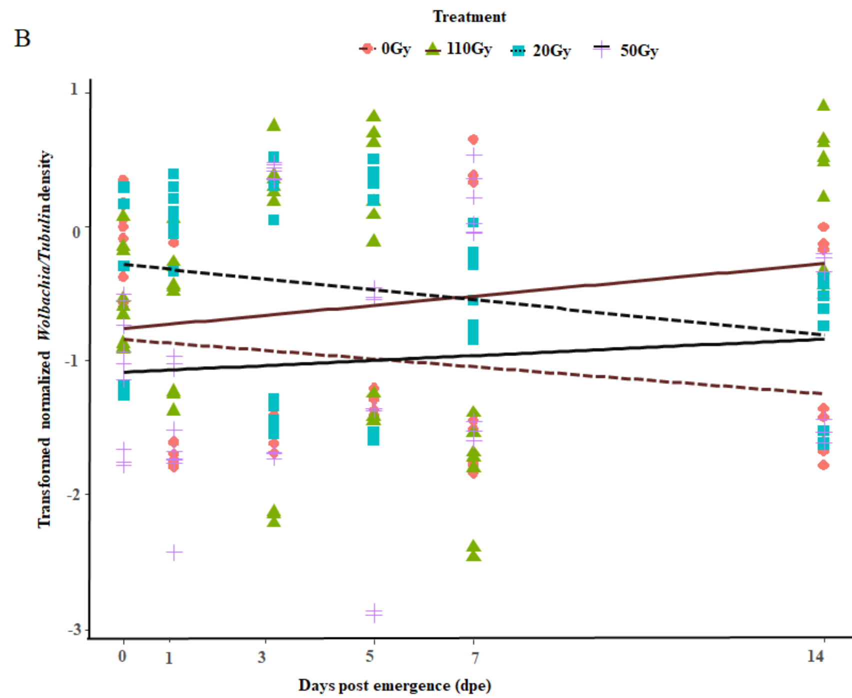
Supplementary Figure 5A. Impact of time post emergence on *Wigglesworthia* density in adult flies emerged from irradiated 22-day old puparia treated with different doses for females.



Supplementary Figure 5B. Impact of time post emergence on *Wigglesworthia* density in adult flies emerged from irradiated 22-day old puparia treated with different doses for males.



Supplementary Figure 6A. Impact of time post emergence on *Wolbachia* density in adult flies emerged from irradiated 22-day old puparia treated with different doses for females



Supplementary Figure 6B. Impact of time post emergence on *Wolbachia* density in adult flies emerged from irradiated 22-day old puparia treated with different doses for males

Table S1. List of Primers used for quantitative PCR (qPCR) analyses of microbiome in *Glossina* species

Target Gene	Primer Name	Primer Sequence (Listed 5- to -3)	Annealing Temperature (°C)	Amplicon Size (bp)	References
fliC (flagellin) (*Sodalis*)	sodqPCR-FliCF	GAA GCC ACC GAT CCT GTA AC	55	508	[1]
	sodqPCR-FliCR	CAT CTT TGC CCG TAG AAA TCA C			
Codhoc (*Wigglesworthia*)	WiggqPCRcodhocF2	GACTTGTACGTGATATTTCCAAGC	60	645	[2]
	WiggqPCRcodhocR2	GACATCAAATCGCGTTACTGG			
Wolbachia 16S rRNA(*Wolbachia*)	Wsp fwd	YATACCTATTCGAAGGGATAG	60	438	[3, 4]
	Woltse- cyt R	GGATTAGCTTAGCCTCGC			
β-tubulin (Tsetse Fly)	Tsetse-tubulinF	GATGGTCAAGTGCGATCCT	55	355	[5]
	Tsetse-tubulinR	TGAGAACTCGCCTTCTTC C			

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Supplementary Table 2. ANOVA Statistics for Interaction

Treatment	Interactions	Both Female and Male			Female			Male		
		F	df	P value	F	df	P value	F	df	P value
Impact of irradiation on *Sodalis* in tsetse irradiated as Adults	Sex	0.40	1, 178	0.527867	-	-	-	-	-	-
	Time	59.73	1, 178	7.654e-13	42.20	1, 92	4.146e-09	21.37	1, 86	1.320e-05
	Treatment	53.11	1, 178	9.856e-12	9.39	1, 92	0.002	49.19	1, 86	4.968e-10
	Sex – Time	0.93	1, 178	0.33	-	-	-	-	-	-
	Sex - Treatment	9.12	1, 178	0.002888	-	-	-	-	-	-
	Time – Treatment	0.60	1, 178	0.43	0.05	1, 92	0.81	1.39	1, 86	0.24
	Sex- Time- Treatments	0.74	1, 178	0.38	-	-	-	-	-	-
Impact of irradiation on *Sodalis* in tsetse irradiated as 29-day old pupae	Sex	31.12	1, 279	5.721e-08	-	-	-	-	-	-
	Time	86.74	1, 279	< 2.2e-16	6.33	1, 44	0.01	25.81	1, 44	7.370e-06
	Treatment	232.04	1, 279	< 2.2e-16	28.49	1, 44	3.137e-06	40.77	1, 44	9.133e-08
	Sex – Time	5.009	1, 279	0.02	-	-	-	-	-	-
	Sex - Treatment	0.07	1, 279	0.79	-	-	-	-	-	-
	Time – Treatment	37.46	1, 279	3.148e-09	11.60	1, 44	0.001	2.73	1, 44	0.1054
	Sex- Time- Treatments	3.30	1, 279	0.07	-	-	-	-	-	-
Impact of irradiation on *Sodalis* in tsetse irradiated as 22-day old pupae	Sex	44.33	1, 417	8.743e-11	-	-	-	-	-	-
	Time	134.20	1, 417	< 2.2e-16	27.27	1, 63	2.116e-06	14.28	1, 68	0.0003
	Treatment	4.55	1, 417	0.03	0.74	1, 63	0.52	1.11	1, 68	0.29
	Sex – Time	9.21	1, 417	0.002546	-	-	-	-	-	-
	Sex - Treatment	0.01	1, 417	0.90	-	-	-	-	-	-
	Time – Treatment	5.57	1, 417	0.01	0.68	1, 63	0.56	1.51	1, 68	0.222
	Sex- Time- Treatments	0.001	1, 417	0.97192	-	-	-	-	-	-
Impact of	Sex	537.58	1,389	< 2.2e-16	-	-	-	-	-	-
	Time	1.69	1,389	0.19	8.47	1, 187	0.004	0.02	1, 202	0.86

Treatment	Interactions	Both Female and Male			Female			Male		
		F	df	P value	F	df	P value	F	df	P value
irradiation on *Wolbachia* in tsetse irradiated as 22-day old pupae	Treatment	12.69	1,389	0.0004114	52.13	1, 187	1.268e-11	0.36	1, 202	0.546
	Sex – Time	1.53	1,389	0.21	-	-	-	-	-	-
	Sex - Treatment	20.49	1,389	7.97e-06	-	-	-	-	-	-
	Time – Treatment	3.81	1,389	0.05	0.880	1, 187	0.34	3.21	1, 202	0.07
	Sex- Time-Treatments	0.002	1,389	0.9595567	-	-	-	-	-	-
Impact of irradiation on *Wigglesworthia* in tsetse irradiated as 22-day old pupae	Sex	18.40	1, 94	4.339e-05	-	-	-	-	-	-
	Time	0.02	1, 94	0.87	0.96	1, 48	0.32	1.15	1, 46	0.28
	Treatment	3.15	1, 94	0.07	2.12	1, 48	0.15	2.23	1, 46	0.14
	Sex – Time	2.09	1, 94	0.15	-	-	-	-	-	-
	Sex - Treatment	0.01	1, 94	0.91	-	-	-	-	-	-
	Time – Treatment	0.43	1, 94	0.511	1.64	1, 48	0.20	0.24	1, 46	0.62
	Sex- Time-Treatments	1.45	1, 94	0.23	-	-	-	-	-	-
Sodalis density of normal colony flies in tsetse irradiated as Adults	Sex	36.46	1, 62	9.581e-08						
	Time	79.42	1, 62	1.053e-12	30.26	1, 31	5.102e-06	43.256	1, 31	2.404e-07
	Treatment	-	-	-	-	-	-	-	-	-
	Sex – Time	0.35	1, 62	0.5543						
	Sex - Treatment	-	-	-	-	-	-		-	-
	Time – Treatment	-	-	-	-	-	-	-	-	-
	Sex- Time-Treatments	-	-	-	-	-	-	-	-	-

Supplementary Table 3. Regression Statistics

Treatment	Days Post Emergence	Female							Male						
		Coefficient	**t value**	**df**	***P* value**	**Intercept t value**	**Intercept *P* value**	**R-squared (R^2)**	**Coefficient**	**t value**	**df**	***P* value**	**Intercept t value**	**Intercept *P* value**	**R-squared (R^2)**
Impact of irradiation on *Sodalis* in tsetse irradiated as Adults	0	0.000	0.327	22	0.74	-1.327	0.198	-0.0404	-0.0085	-1.780	19	0.091	0.046	0.963	0.0977
	1	- 0.013	-7.060	22	4.4e-07	-4.294	0.0002	0.6799	-0.0215	-5.487	22	1.63e-05	0.952	0.351	0.5586
	7	-0.0028	-0.812	22	0.42	1.856	0.07	-0.0150	-0.0227	-6.038	19	8.29e-06	3.135	0.0054	0.6393
	14	-0.0071	-2.126	22	0.04	4.235	0.0003	0.1327	-0.0067	-2.326	22	0.02	4.187	0.0003	0.1973
Impact of irradiation on *Sodalis* in tsetse irradiated as 29-day old pupae	0	-0.0056	-2.822	22	0.009	-3.365	0.002	0.2324	-0.0034	-2.218	22	0.03	-3.399	0.002	0.1455
	1	-0.0124	-10.424	22	5.64e-10	-5.627	1.17e-05	0.824	-0.0108	-12.072	22	3.54e-11	-5.387	2.07e-05	0.8629
	3	-0.0123	-15.987	22	1.35e-13	-6.923	5.97e-07	0.9171	-0.0142	-5.949	22	5.49e-06	4.561	0.0001	0.5993
	5	-0.0139	-5.911	22	5.99e-06	3.398	0.002	0.5961	-0.01302	-5.303	21	2.94e-05	3.877	0.0008	0.5521
	7	-0.0018	-1.668	22	0.109	-8.102	4.78e-08	0.0719	-0.0067	-4.860	22	7.39e-05	1.142	0.266	0.4959
	14	0.0007	0.724	22	0.477	-6.783	8.15e-07	-0.0211	-0.0030	-2.291	22	0.03	4.286	0.0003	0.1559
Impact of irradiation on *Sodalis* in tsetse irradiated as 22-day old pupae	0	-0.0062	-3.161	34	0.003	-7.184	2.62e-08	0.2044	-0.0037	0.003	34	0.04	-9.669	2.74e-11	0.0880
	1	-0.0028	-1.283	33	0.208	-5.241	9.06e-06	0.01864	-0.0038	0.208	33	0.05	-8.005	3.1e-09	0.0774
	3	0.0007	0.241	33	0.811	-0.122	0.904	-0.0284	-0.0020	-0.957	33	0.34	-0.843	0.405	-0.0024
	5	0.0020	0.896	33	0.377	0.256	0.800	-0.0058	0.0030	1.339	34	0.18	-4.191	0.0001	0.0221
	7	-0.0018	-1.038	34	0.306	0.867	0.392	0.0022	-0.0044	-3.725	34	0.0007	-5.163	1.06e-05	0.2689
	14	-0.0003	-0.165	26	0.870	2.406	0.023	-0.0373	0.0019	2.202	33	0.03	-7.887	4.3e-09	0.1017

Supplementary Table 4. **Slope values for Regression Statistics**

Treatment	DOSE	Female							Male						
		Coefficient	t value	df	*P* value	Intercept t value	Intercept *P* value	R-squared (R^2)	Coefficient	t value	df	*P* value	Intercept t value	Intercept *P* value	R-squared (R^2)
Impact of irradiation on *Sodalis* in tsetse irradiated as Adults	0 Gy	0.056	3.185	22	0.004	-0.973	0.34131	0.284	0.020	0.849	19	0.406	3.194	0.004	-0.014
	20 Gy	0.120	5.678	22	1.04e-05	-4.242	0.000334	0.575	0.094	3.37	22	0.002	-2.59	0.016	0.310
	50 Gy	0.107	3.944	22	0.000691	-3.286	0.003372	0.387	0.091	2.867	19	0.009	-4.339	0.0003	0.265
	110 Gy	0.068	1.807	22	0.08	-2.888	0.00854	0.089	0.089	2.748	22	0.011	-6.022	4.63e-06	0.221
Impact of irradiation on *Sodalis* in tsetse irradiated as 29-day old pupae	0 Gy	-0.035	-2.686	34	0.011	-0.207	0.8372	0.150	0.024	1.754	34	0.088	-0.540	0.592	0.05598
	20 Gy	0.047	4.427	34	9.37e-05	-11.014	9.30e-13	0.347	0.064	4.760	33	3.74e-05	-4.256	0.0001	0.389
	50 Gy	0.033	1.841	34	0.07	-6.101	6.36e-07	0.063	0.086	6.405	34	2.58e-07	-8.465	6.93e-10	0.533
	110 Gy	0.077	5.129	34	1.17e-05	-14.506	3.99e-16	0.419	0.072	4.782	34	3.29e-05	-11.580	2.40e-13	0.384
Impact of irradiation on *Sodalis* in tsetse irradiated as 22-day old pupae	0 Gy	0.062	4.411	51	5.33e-05	-4.710	1.95e-05	0.262	0.022	1.392	52	0.17	-6.051	1.6e-07	0.017
	20 Gy	0.115	7.334	51	1.62e-09	-8.849	7.03e-12	0.503	0.064	4.631	52	2.47e-05	-10.925	4.48e-15	0.278
	50 Gy	0.071	2.912	51	0.005	-3.275	0.00190	0.125	0.055	2.927	50	0.00514	-7.173	3.22e-09	0.1292
	110 Gy	0.121	5.965	51	2.33e-07	-7.300	1.83e-09	0.399	0.075	4.637	51	2.49e-05	-10.271	5.15e-14	0.282
Impact of irradiation on *Wigglesworthia* in tsetse irradiated as 22-day old pupae	0 Gy	-0.060	-1.863	32	0.07	-0.677	0.5035	0.069	-0.017	-0.249	34	0.804	-3.115	0.003	-0.02753
	20 Gy	0.063	1.675	31	0.10	-3.868	0.000526	0.053	-0.163	-2.264	28	0.031	-2.088	0.046	0.1246
	50 Gy	0.046	1.591	36	0.12	-4.615	4.84e-05	0.039	-0.019	-0.515	32	0.610	-7.792	6.9e-09	-0.022
	110 Gy	0.070	2.725	39	0.009	-8.026	8.75e-10	0.138	-0.129	-1.934	24	0.064	-3.578	0.001	0.098
Impact of irradiation on *Wolbachia* in tsetse irradiated as 22-day old pupae	0 Gy	0.116	1.605	52	0.115	-8.679	1.09e-11	0.028	0.017	0.63	51	0.531	-5.81	4.07e-07	-0.011
	20 Gy	0.317	4.262	51	8.76e-05	-14.837	$< 2e-16$	0.248	0.034	1.219	52	0.228	-3.941	0.0002	0.009
	50 Gy	-0.033	-0.41	45	0.68	-11.43	6.74e-15	-0.018	-0.037	-1.79	49	0.079	-1.92	0.060	0.042
	110 Gy	0.109	0.781	35	0.44	-9.871	1.19e-11	-0.010	-0.029	-1.255	46	0.216	-5.063	7.1e-06	0.012

Chapter 7

Effect of antibiotic treatment and gamma-irradiation on cuticular hydrocarbon profiles and mate choice in tsetse flies (*Glossina m. morsitans*)

Effect of antibiotic treatment and gamma-irradiation on cuticular hydrocarbon profiles and mate choice in tsetse flies (*Glossina m. morsitans*)

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Keywords: *Glossina morsitans*, Tsetse, endosymbiont, *Wigglesworthia*, cuticular hydrocarbons, mate choice

Abstract

Symbiotic microbes represent a driving force of evolutionary innovation by conferring novel ecological traits to their hosts. Many insects are associated with microbial symbionts that contribute to their host's nutrition, digestion, detoxification, reproduction, immune homeostasis, and defense. In addition, recent studies suggest a microbial involvement in chemical communication and mating behavior, which can ultimately impact reproductive isolation and, hence, speciation. Here we investigated whether a disruption of the microbiota through antibiotic treatment or irradiation affects cuticular hydrocarbon profiles, and possibly mate choice behavior in the tsetse fly, *Glossina morsitans morsitans*. Four independent experiments that differentially knock down the multiple bacterial symbionts of tsetse flies were conducted by subjecting tsetse flies to ampicillin, tetracycline, or gamma-irradiation and analyzing their cuticular hydrocarbon profiles in comparison to untreated controls by gas chromatography – mass spectrometry. In two of the antibiotic experiments, flies were mass-reared, while individual rearing was done for the third experiment to avoid possible chemical cross-contamination between individual flies. All three antibiotic experiments yielded significant effects of antibiotic treatment (particularly tetracycline) on cuticular hydrocarbon profiles in both female and male *G. m. morsitans*, while irradiation itself had no effect on the CHC profiles. Importantly, tetracycline reduced relative amounts of 15,19,23-trimethyl-heptatriacontane, a known compound of the female contact sex pheromone, in two of the three experiments, suggesting a possible implication of microbiota disturbance on mate choice decisions. Concordantly, both female and male flies preferred non-treated over tetracycline-treated flies in direct choice assays. While we cannot exclude the possibility that antibiotic treatment had a directly detrimental effect on fly vigor as we are unable recolonize antibiotic treated flies with individual symbiont taxa, our results are consistent with an effect of the microbiota, particularly the obligate nutritional endosymbiont *Wigglesworthia*, on CHC profiles and mate choice behavior. These findings highlight the importance of considering host-microbiota interactions when studying chemical communication and mate choice in insects.

Background

Cuticular hydrocarbons (CHCs) are ubiquitous and both structurally and functionally diverse in insects [1]. Although the primary function of CHCs is the protection of the insect from water loss, they have secondarily adopted a multitude of functions in intra- and interspecific communication in a solitary as well as social context [1-5]. In particular, CHCs play an important role in mate attraction, species and sex recognition, courtship, and mate choice in many insect species [1, 6].

Most insects are associated with obligate and/or facultative microbial symbionts that can affect physiology, ecology, and evolution of their hosts in a multitude of ways [7-9], including direct or indirect effects on chemical communication and mate choice [10]. Notably, experiments in locusts revealed a direct contribution of microbial gut symbionts to the production of the host's cohesion pheromone [11, 12], and studies in fruit flies suggested that members of the microbiota can alter the CHC profile of the host and thereby affect mate choice decisions under certain circumstances [13-15]. Such pheromonal changes may constitute the first steps towards premating isolation and hence initiate speciation processes [10, 16].

Tsetse flies (*Glossina* spp., Diptera, Glossinidae) are associated with a taxonomically diverse microbial community. These microbes include environmentally acquired gut-associated microbes [17-19] as well as two bacterial symbionts (obligate mutualistic *Wigglesworthia glossinidia* and commensal *Sodalis glossinidius*) that are transmitted from pregnant females to their intrauterine larval offspring via maternal milk gland secretions [20, 21]. Some tsetse flies also house the reproductive symbiont *Wolbachia* [maternally transmitted through the germ line; 22] as well as viral and protozoan pathogens [22]. *Wigglesworthia* is an intracellular mutualist that serves important functions in tsetse, including supplementation of B-complex vitamins absent from vertebrate blood [23], and actuation of the development of tsetse's immune system [24, 25]. While *Sodalis* is consistently present in flies, its function is not yet well established. *Wolbachia* is less prevalent, but is known to affect host reproduction across a wide range of insect hosts, including tsetse flies, where it causes cytoplasmic incompatibility [26-28].

While the effects of the microbial symbionts on tsetse fly metabolism and reproduction have been studied in detail, their possible impact on chemical communication and mate choice remains unknown. The CHCs of tsetse flies are characterized by a sex-specific blend of

mono-, di-, and tri-methyl alkanes [29, 30]. Some of the long-chain methyl-branched CHCs have been implicated in eliciting sexual behavior of males upon contact with the females [31-35]. In *G. m. morsitans*, male contact with female-produced 15,19,23-trimethyl-heptatriacontane is necessary and sufficient to trigger male sexual behavior, provided that the compound is presented on a fly-like visual stimulus [31]. However, it remains elusive whether male CHCs also play a role for female mate choice decisions in tsetse.

Here, we set out to investigate the impact of bacterial symbionts on CHC profiles of *Glossina morsitans morsitans* and their possible influence on sexual selection and mating success. We used gas chromatography coupled to mass spectrometry (GC-MS) to analyze CHC profiles [36] of tsetse flies after antibiotic- as well as irradiation-mediated perturbations of the host-symbiont equilibrium [26, 37]. In addition, we assessed the effect of antibiotics on mating success of male and female *G. m. morsitans*.

Materials and methods

Sampling and treatments

Glossina morsitans morsitans for antibiotic treatments were reared on bovine blood (Hemostat laboratories, Dixon, CA) in the laboratory at Yale University at 24°C and on a 14hr/10hr light/dark photoregime. Two fly treatment groups were established by feeding pregnant females a diet supplemented with either ampicillin (Amp; 50µg/ml blood; Pais et al., 2008) or tetracycline (Tet; 25µg/ml blood; Alam et al., 2011). Tet-treated females were also supplemented with yeast extract [1% w/v; 24] to partially restore reproductive sterility that occurs in the absence of obligate *Wigglesworthia* [37]. Amp treatment of pregnant tsetse flies eliminates only *Wigglesworthia* from milk secretions such that larval offspring undergo their entire developmental program in the absence of this obligate symbiont but in the presence of *Sodalis* and *Wolbachia* [37]. Tet treatment eliminates all bacteria from pregnant females so that larvae undergo their entire developmental program in the absence of all bacteria [26]. Offspring from Amp and Tet treated mothers, which were used to test the impact of symbiont titer knockdown on tsetse's CHC profile, are hereafter designated Gmm^{Wgm-} and Gmm^{Apo} (Apo = aposymbiotic), respectively. Gmm^{Wgm-} and Gmm^{Apo} flies were reared either collectively (experiment 1 and 2) or individually (experiment 3) on antibiotic-free bovine blood and sampled for chemical analyses. For experiment 3, only control and Gmm^{Apo} individuals were generated because rearing tsetse individually is untenable on a large scale.

For each treatment group, 10 unmated male and 10 virgin female flies were sampled at day 10 (experiments 1+3) or day 5 (experiment 2) after adult emergence.

Glossina morsitans morsitans flies used for irradiation treatments were originally from Zimbabwe and maintained since 1997 at the Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria. Tsetse flies were maintained a temperature of 23 ± 1^0C, a relative humidity of 75-80% under on a 12hr/12hr light/dark photoregime. Experimental flies were fed on defibrinated bovine blood using an artificial (*in vitro*) membrane feeding system for 15-20 minutes three times per week [38]. Male flies were either irradiated as 22-day old pupae (early), at the very late pupal stage at which females had already emerged (29-days old (late)), or as 5-day old adults (adult). The irradiation treatment was performed using a Gammacell 220 60Co irradiator (Nordion Ltd., Ottawa, Canada) by exposing the samples for different time periods to receive an irradiation dose of 110, 50 or 20 Gy. Non irradiated flies were used as a control (0 Gy). Twenty two-day and 29-day old pupae were irradiated in a 9 cm diameter petri-dish while the 5-day old males were irradiated in individual small cages (4 cm diameter x 6 cm high) (one male/cage). Irradiated pupae were separated and reared individually in a pill sorter until emergence. After emergence each male was individually placed in a small cage and maintained until day 10 after emergence. Depending on the time and dosage, irradiation treatment has variable effects on *Sodalis* and *Wolbachia*, but not *Wigglesworthia* titers [39]. Specifically, in adult flies emerging from early irradiated (22-day old) pupae, *Sodalis* density was decreased at 24 hours post emergence and recovered over time until day 14 post eclosure, while the *Wigglesworthia* titer did not differ between treatment and control groups, and *Wolbachia* density was increased at emergence but decreased again over time. In the males emerging from late irradiated (29-day old) pupae, both *Sodalis* and *Wolbachia* density was reduced during the first week after emergence and then recovered over time. In males irradiated as adults, *Sodalis* density decreased after irradiation while the *Wolbachia* density increased at 24 post irradiation and then decreased again over time.

Extraction of samples and GC-MS analysis

Individual flies were extracted in hexane. 2 µg of heneicosane was added as internal standard to allow for later quantification of hydrocarbons. Extracts were evaporated to about 20-30µl of hexane under a constant stream of argon and transferred to a 150µl GC-µ-vial (CZT, Kriftel, Germany) for GC-MS analysis. An aliquot of 1µl of each sample was injected into a

Varian 450GC gas chromatograph coupled to a Varian 240MS mass spectrometer (Agilent Technologies, Böblingen, Germany) using a split/splitless injector at 250°C with the purge valve opened after 60s. The GC was equipped with a DB5-MS capillary column (30m x 0.25mm diameter, film thickness: 0.25µm, Agilent Technologies) and programmed from 150 to 300°C at 15°C/min with a 27 min. final isothermal hold. Helium was used as carrier gas, with a constant flow rate of 1ml/min. Mass spectra were recorded using electron ionization (EI-MS). Data acquisition and quantifications were achieved with MS Workstation Version 6.9.3 Software (Agilent Technologies). The peaks were identified by their mass spectra in comparison to previously published analyses of *G. m. morsitans* cuticular hydrocarbon profiles [29]. Peak areas were automatically integrated using the MS Workstation Software. Finally, the success of this integration was controlled manually for every peak. Some substances had to be combined for the analysis, as the peaks were not always clearly separated in the chromatograms.

Mate choice assays

Individual control males or females were given a simultaneous choice between one control and one *Gmm*^{Apo} mate, respectively. All flies were 5 days old adults. To later distinguish the individuals, the last tarsal segment was cut from either the right or left mid leg. The control and *Gmm*^{Apo} mate were set up in the clean round colony cage with 20 cm of diameter and height of 5 cm, one day post feeding and six hours before the actual experiment. An individual control male/female was inserted into the middle of the cage while the potential mates were held on the opposite side of cage by shading them with a black blanket. After the removal of the blanket, the control fly was given the ability to come into contact with both potential mates before choosing a mating partner. Matings were scored visually by observing the cage for 3 hours or until the end of a successful mating, which lasts in *G. m. morsitans* 2 hours [40]. For male choice, 30 replicates were performed, while 17 replicates were done for female choice assays due to the availability of flies.

Statistical analysis

Since CHC profiles of tsetse flies are sex-specific, the profiles of males and females were analyzed separately. To compare absolute amounts of hydrocarbons across treatment groups, the total amount of all compounds (combined) was calculated from the combined peak areas by comparison to the peak area of the internal standard (=2µg). For the known contact sex

pheromone of female *G. m. morsitans*, 15,19,23-trimethyl-heptatriacontane [31], absolute and relative amounts were calculated for each individual, based on the internal standard and the total peak area of all hydrocarbons, respectively. The resulting values were compared among antibiotic treatment groups by ANOVA with Tukey post-hoc comparisons. Irradiation treatment groups were analyzed in a two-factorial ANOVA to test for effects of the dosage and age/developmental stage at which the flies were subjected to irradiation.

For all other analyses, relative amounts were calculated from the peak areas and then log-ratio-transformed according to Aitchison [41]. In order to test for differences in chemical profiles across groups, principal component analyses (PCAs) were performed to reduce the number of variables, and the resulting PCs (with Eigenvalues > 0.9) were used for discriminant analyses (DAs) to test for among-group differences. Chi-squared tests were performed for the mate choice assays. All statistical analyses were done with SPSS 17.0.

Results

CHC composition in G. *m. morsitans*

As described earlier [29], CHC profiles of *G. m. morsitans* were dominated by mono-, di-, and tri-methyl alkanes, and there were distinct sex-specific differences, with females generally showing more compounds with longer carbon backbones (**Tables 1-3**). The main components of female CHC profiles were 2-methyl-triacontane, 15,19- and 17,21-dimethyl-heptatriacontane, and 15,19,23-trimethyl-heptatriacontane, which together accounted for about 70% of the complete CHCs in control flies (**Table 1**). In males, 2-methyl-triacontane and 11,15-dimethyl-tritriacontane dominated, amounting to about 40% of the total CHC profile in control flies from Vienna and 70% in control flies from Yale (**Table 2-3**). In addition to these differences in the dominant compounds, males reared in Vienna showed slightly more of the longer carbon backbone compounds then males reared at Yale (**Table 2-3**).

Table 1: CHC profiles of 5- and 10-day old antibiotic-treated (*Gmm*$^{Wgm-}$=ampicillin; *Gmm*^{Apo} =tetracycline) and control female *G. m. morsitans* after mass- or individual rearing. Given are relative amounts of CHCs (in percent) +/- standard deviation, as well as the total absolute amount of CHCs as determined by comparison with an internal standard. Me=methyl.

	5-day old, mass-rearing			**10-day old, mass-rearing**			**10-day old, individual rearing**	
Compound	**Control**	*Gmm*$^{Wgm-}$	*Gmm*^{Apo}	**Control**	*Gmm*$^{Wgm-}$	*Gmm*^{Apo}	**Control**	*Gmm*^{Apo}
2Me-C28	0.41±0.13	0.58±0.19	0.83±0.62	0.28±0.11	0.80±0.70	0.75±0.40	0.46±0.14	1.16±0.32
2Me-C29	0.77±0.23	1.81±0.60	1.32±0.76	1.26±0.42	1.78±0.70	1.89±0.45	1.88±0.70	3.56±0.61
2Me-C30	13.16±2.71	21.57±6.70	13.89±5.51	20.72±4.04	20.75±4.21	26.15±9.91	31.02±6.64	37.94±3.87
2Me-C31	0.47±0.20	0.80±0.32	0.57±0.29	0.63±0.16	0.80±0.78	0.97±0.48	1.20±0.38	2.19±1.00
2Me-C32	0.89±0.25	1.55±0.50	1.13±0.65	0.89±0.26	0.90±0.30	1.12±0.36	1.47±0.32	2.09±0.70
2Me-C34	0.08±0.05	0.14±0.12	0.10±0.07	0.06±0.04	0.12±0.07	0.11±0.07	0.05±0.02	0.12±0.08
11,15-diMe-C33	0.46±0.14	1.04±0.32	0.96±0.53	0.43±0.13	0.46±0.33	0.99±1.23	0.18±0.04	0.82±0.70
diMe-C34	0.36±0.09	0.44±0.09	0.45±0.14	0.20±0.04	0.22±0.10	0.24±0.21	0.32±0.07	0.34±0.16
15,19-diMe-C35	9.13±1.31	8.51±1.15	8.06±1.33	5.54±0.56	5.42±0.08	5.62±2.51	7.76±0.98	5.84±1.71
15,19+16,20-diMe-C36	6.85±0.54	6.35±0.43	7.03±0.77	4.35±0.54	3.74±0.45	4.43±1.22	5.66±0.66	4.32±0.71
15,19+17,21-diMe-C37	21.80±3.82	17.61±2.71	17.60±2.35	13.72±1.89	13.30±3.84	15.11±3.36	13.67±2.63	10.19±2.21
diMe-C38	1.57±0.19	1.47±0.24	1.83±0.58	0.90±0.21	0.66±0.30	1.06±0.23	0.74±0.22	0.64±0.12
13,17,21-triMe-C35	0.64±0.15	0.51±0.08	0.69±0.10	0.57±0.13	1.19±1.27	0.42±0.14	0.85±0.19	0.96±0.13
triMe-C36	1.86±0.44	1.65±0.29	2.17±0.33	1.81±0.36	1.85±0.63	1.39±0.35	2.16±0.53	1.94±0.28

	5-day old, mass-rearing			10-day old, mass-rearing			10-day old, individual rearing	
Compound	**Control**	*Gmm*$^{Wgm-}$	*Gmm*^{Apo}	**Control**	*Gmm*$^{Wgm-}$	*Gmm*^{Apo}	**Control**	*Gmm*^{Apo}
15,19,23-triMe-C37	27.35±3.18	23.05±2.69	27.85±3.55	36.58±2.52	37.67±6.04	28.88±7.64	25.10±3.29	21.91±2.63
15,19,23-triMe-C38	8.40±1.42	7.90±1.51	9.93±2.06	7.88±1.83	6.76±1.86	7.01±0.55	6.52±1.82	5.14±0.98
15,19,23-triMe-C39	5.41±1.16	4.46±1.32	5.24±1.58	3.78±1.00	2.89±0.92	3.43±0.67	0.24±0.15	0.19±0.06
unknown	0.37±0.15	0.53±0.22	0.36±0.15	0.41±0.19	0.70±0.68	0.45±0.27	0.70±0.30	0.63±0.20
Total CHC amount (μg)	35.04±9.36	29.70±26.66	43.32±18.73	33.43±29.28	24.17±20.06	18.48±14.72	26.98±10.48	28.22±6.44

Table 2: CHC profiles of 5- and 10-day old antibiotic-treated (*Gmm*$^{Wgm-}$=ampicillin; *Gmm*^{Apo} =tetracycline) and control male *G. m. morsitans* after mass- or individual rearing. Given are relative amounts of CHCs (in percent), as well as the total absolute amount of CHCs as determined by comparison with an internal standard. Me=methyl.

	5-day old, mass-rearing			**10-day old, mass-rearing**			**10-day old, individual rearing**	
Compound	**Control**	*Gmm*$^{Wgm-}$	*Gmm*^{Apo}	**Control**	*Gmm*$^{Wgm-}$	*Gmm*^{Apo}	**Control**	*Gmm*^{Apo}
2Me-C28	0.42±0.18	1.36±0.91	1.20±0.37	0.45±0.18	0.84±0.42	2.15±0.89	0.58±0.10	1.29±0.49
2Me-C29	4.36±1.03	6.21±2.17	5.74±1.00	8.83±1.77	8.10±3.03	7.59±0.52	8.80±1.11	9.24±1.69
2Me-C30	19.52±1.67	21.89±3.40	21.81±1.70	20.42±2.82	22.96±2.01	29.22±4.91	22.12±2.07	29.23±4.85
2Me-C31+9,13-diMe-C31	4.39±075.	5.59±1.70	4.62±0.52	3.79±0.24	5.11±0.87	5.03±0.87	6.54±0.40	7.46±1.70
2Me-C32+diMe-C32	4.47±0.37	5.79±0.82	5.48±0.43	3.32±0.35	3.78±0.48	4.40±0.25	5.57±0.67	5.73±1.32
11,15-diMe-C33	49.51±2.60	37.92±7.16	39.70±2.55	51.62±3.77	46.12±4.19	36.18±3.99	47.04±2.04	36.00±9.06
12,16-diMe-C34	5.59±0.47	5.85±1.02	5.20±0.31	3.30±0.47	2.96±0.51	3.77±0.51	4.24±0.65	3.53±0.60
15,19-diMe-C35+8-Me-C35:1	9.28±2.61	11.70±2.03	12.64±2.40	5.36±0.94	5.09±0.93	8.17±1.53	1.96±0.45	2.07±0.84
7,11,15-triMe-C33	1.06±0.18	1.45±0.37	1.48±0.21	0.91±0.17	2.00±0.91	1.27±0.22	1.10±0.19	1.78±0.86
unknown1	0.11±0.08	0.18±0.09	0.13±0.08	0.75±0.38	0.10±0.09	0.23±0.08	0.29±0.12	0.32±0.12
unknown2	0.39±0.10	0.61±0.15	0.62±0.07	0.37±0.07	1.17±0.68	0.69±0.09	0.62±0.15	1.56±1.11
unknown3	0.28±0.05	0.38±0.13	0.38±0.04	0.20±0.04	0.52±0.22	0.30±0.09	0.37±0.10	0.65±0.40
unknown4	0.62±0.20	1.07±0.32	0.99±0.19	0.68±0.05	1.26±0.46	0.99±0.11	0.77±0.12	1.14±0.49
Total CHC amount (µg)	17.17±7.91	20.95±7.10	15.89±4.53	25.04±12.89	19.62±10.43	4.30±1.82	38.29±8.94	36.25±19.02

Table 3: CHC profiles of male *Glossina m. morsitans* treated with different gamma-irradiation doses at three time points (early and late pupae, and young adults, respectively). Compounds are sorted by class (mono-, di-, and trimethyl-alkanes). Values indicate average relative amounts (in percent) +/- standard deviation of individual substances within groups, as well as the average absolute total amount of hydrocarbons per fly.

Compound	Irradiated as early pupae				Irradiated as late pupae				Irradiated as young adults			
	0Gy	**20Gy**	**50Gy**	**110Gy**	**0Gy**	**20Gy**	**50Gy**	**110Gy**	**0Gy**	**20Gy**	**50Gy**	**110Gy**
2Me-C28	0.54 ±0.44	0.37 ±0.19	0.62 ±0.46	0.79 ±0.88	0.77 ±0.98	0.96 ±1.11	0.78 ±0.6	0.68 ±1.1	1.09 ±1.59	0.62 ±0.6	0.91 ±0.97	0.81 ±0.82
2Me-C29	8.74 ±2.18	9.28 ±1.24	9.74 ±2.07	9.1 ±1.79	8.4 ±3.57	8.07 ±2.02	8.59 ±2.92	7.18 ±2.19	8.48 ±2.28	9.46 ±1.63	9.1 ±1.09	9.84 ±2.51
2Me-C30	20.64 ±4.4	20.74±2.49	23.11±3.66	21.95±3.29	24.62±5.23	22.38±5.71	21.76±5.94	19.6 ±7.91	23.88±8.78	22.61 ±3.14	21.79±4.14	23.15 ±4.83
2Me-C31+9,13-diMe-C31	6.08 ±0.91	6.14 ±0.97	6.1 ±0.81	5.8 ±0.94	5.84 ±0.85	5.55 ±0.65	5.96 ±0.89	5.62 ±1.4	6.19 ±1.24	6.26 ±0.67	6.34 ±0.62	6.08 ±0.44
2Me-C32+diMe-C32	4.52 ±0.56	4.67 ±0.65	3.96 ±0.52	4.46 ±0.68	4.41 ±0.78	4.49 ±0.65	4.82 ±1.3	4.7 ±0.82	4.53 ±0.89	4.43 ±0.58	4.59 ±0.71	4.09 ±0.51
11,15-diMe-C33	47.6 ±4.02	47.49±2.75	46.07±4.83	46.56±3.72	45.02±4.17	47.63±4.79	44.95±5.86	47.46 ±12.05	44.48±7.63	45.47 ±3.97	45.52±2.74	43.91 ±6.32
12,16-diMe-C34	3.21 ±0.47	3.15 ±0.38	2.88 ±0.27	3.09 ±0.73	3.01 ±0.48	3.15 ±0.5	3.46 ±0.82	3.62 ±1.01	3.06 ±0.65	3.08 ±0.3	3.21 ±0.61	3.06 ±0.62
15,19-diMe-C35 +8-Me-C35:1	4.73 ±0.98	4.22 ±0.72	4.42 ±0.75	4.88 ±0.69	4.32 ±1.03	4.08 ±0.85	5.29 ±0.86	5.41 ±1.3	4.56 ±1.47	3.98 ±0.45	4.24 ±1.13	4.6 ±0.77
15,19+16,20-diMe-C36	0.17 ±0.1	0.13 ±0.07	0.14 ±0.03	0.17 ±0.05	0.17 ±0.07	0.16 ±0.04	0.2 ±0.04	0.39 ±0.7	0.15 ±0.05	0.11 ±0.03	0.13 ±0.03	0.16 ±0.08
15,19+17,21-diMe-C37	0.12 ±0.09	0.06 ±0.03	0.12 ±0.06	0.12 ±0.05	0.13 ±0.05	0.12 ±0.05	0.17 ±0.08	0.96 ±2.69	0.12 ±0.09	0.09 ±0.04	0.09 ±0.03	0.11 ±0.06

Compound	Irradiated as early pupae				Irradiated as late pupae				Irradiated as young adults			
	0Gy	20Gy	50Gy	110Gy	0Gy	20Gy	50Gy	110Gy	0Gy	20Gy	50Gy	110Gy
7,11,15-triMe-C33	0.92 ±0.18	0.93 ±0.12	0.77 ±0.16	0.88 ±0.2	0.91 ±0.35	0.93 ±0.15	1.12 ±0.32	0.95 ±0.3	0.86 ±0.21	0.95 ±0.11	1.06 ±0.21	1.06 ±0.22
15,19,23-triMe-C37	0.06 ±0.06	0.03 ±0.02	0.04 ±0.02	0.04 ±0.03	0.05 ±0.03	0.05 ±0.03	0.06 ±0.03	0.84 ±2.51	0.05 ±0.06	0.03 ±0.01	0.03 ±0.02	0.07 ±0.06
unknown1	1.21 ±0.53	1.34 ±0.42	0.77 ±0.49	0.76 ±0.41	0.84 ±0.55	0.91 ±0.55	0.97 ±0.6	0.92 ±0.33	1.06 ±0.92	1.2 ±0.46	1.1 ±0.51	1.27 ±0.7
unknown2	0.47 ±0.18	0.47 ±0.12	0.42 ±0.12	0.46 ±0.15	0.54 ±0.29	0.53 ±0.15	0.61 ±0.19	0.46 ±0.12	0.46 ±0.09	0.52 ±0.11	0.65 ±0.19	0.56 ±0.14
unknown3	0.27 ±0.07	0.26 ±0.08	0.22 ±0.05	0.24 ±0.06	0.28 ±0.12	0.29 ±0.04	0.33 ±0.13	0.29 ±0.09	0.27 ±0.07	0.29 ±0.05	0.3 ±0.09	0.27 ±0.06
unknown4	0.73 ±0.15	0.72 ±0.12	0.62 ±0.12	0.7 ±0.14	0.68 ±0.19	0.7 ±0.09	0.94 ±0.29	0.94 ±0.27	0.77 ±0.21	0.9 ±0.13	0.94 ±0.19	0.95 ±0.21
Total CHC amount (µg)	33.48 ±11.08	34.83 ±11.81	32.02 ±9.02	35.22 ±11.92	35.26 ±12.03	26.06 ±10.17	37.03 ±21.52	31.60 ±12.73	24.17 ±10.09	31.01 ±8.98	27.63 ±8.21	25.20 ±12.97

Influence of antibiotic treatment on CHC profiles in mass-reared female flies

Antibiotic treatment had no effect on the total amount of CHCs in 10-day-old females (**Figure 1a**; ANOVA, $F_{2,27}$=1.154, p=0.330). *Gmm*^{Apo} females showed a non-significant tendency towards lower absolute amounts of 15,19,23-trimethyl-heptatriacontane (**Figure 1c**; ANOVA, $F_{2,27}$=1.267, p=0.298). A comparison of the relative amounts of 15,19,23-trimethyl-heptatriacontane revealed significantly lower proportions of sex pheromone in *Gmm*^{Apo} females as compared to control and *Gmm*$^{Wgm-}$ flies (**Figure 1d**; ANOVA, $F_{2,27}$=6.291, p=0.006).

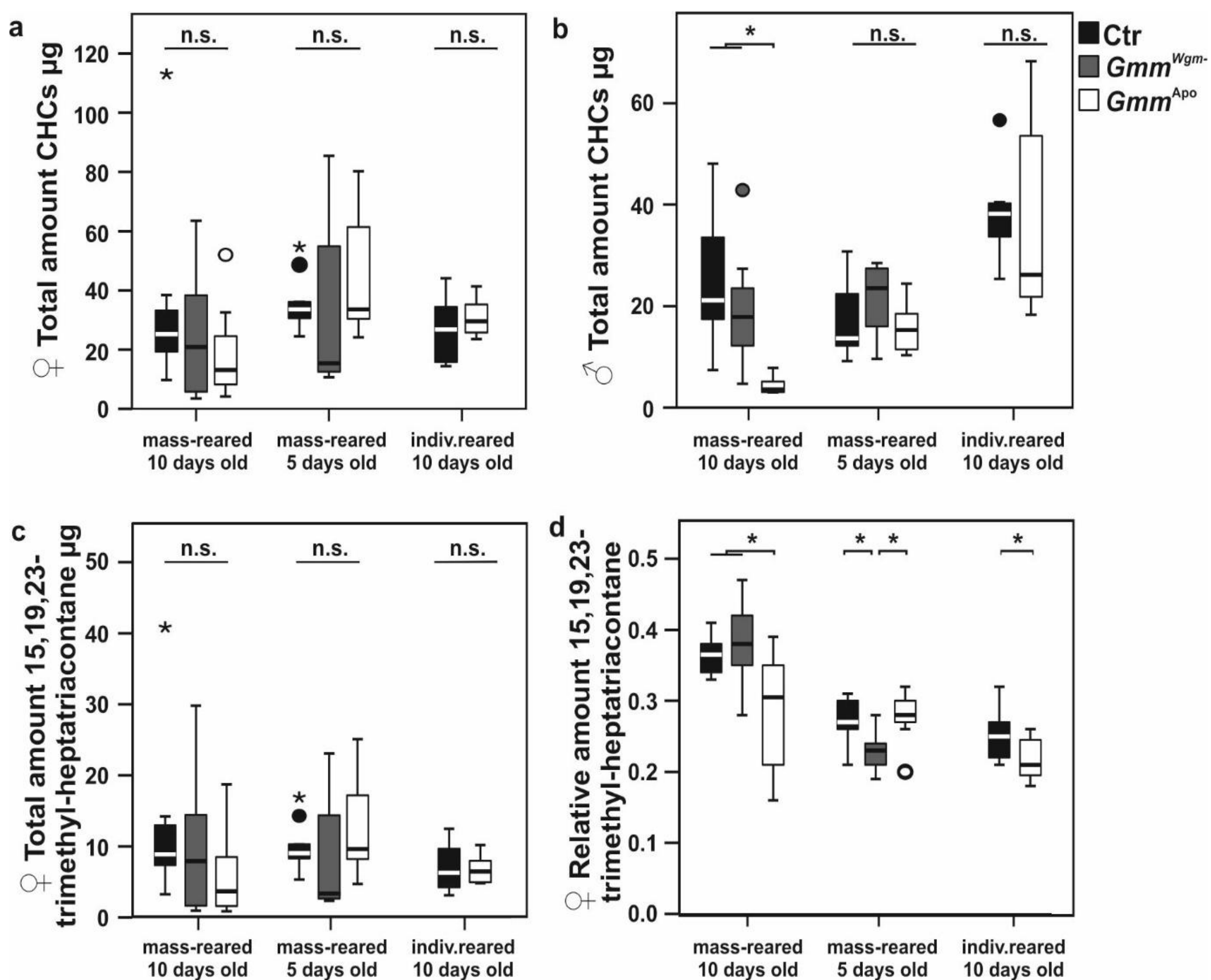

Figure 1: Effect of antibiotic treatment on the total amount of hydrocarbons in female (**a**) and male (**b**) *Glossina m. morsitans*, as well as on the absolute (**c**) and relative (**d**) amount of the females' contact sex pheromone 15,19,23-trimethyl-heptatriacontane. Ctr=Control (without antibiotics), *Gmm*$^{Wgm-}$ =ampicillin-treated, *Gmm*^{Apo}=tetracycline-treated. Lines represent medians, boxes comprise interquartile ranges, and whiskers denote minimum and maximum values, except for outliers that lie further away from a quartile than 1.5 times (circles) or 3 times (asterisks) the interquartile range. An asterisk above lines connecting single treatments indicates a significant difference at p<0.05.

Based on the 19 quantified peaks, four principal components were extracted, capturing 83.9% of the total variance. A discriminant analysis (DA) based on the four PCs including all three treatment groups yielded a significant difference in CHC profiles across groups (**Figure 2a**; Wilks' Lambda=0.354, X^2=26.5, df=8, p=0.001). Based on the two discriminant functions, 60% of the cases were correctly classified (30% would be expected by chance). Subsequent DAs of pairwise combinations of the three groups revealed no significant difference between control and *Gmm*$^{Wgm-}$flies (Wilks' Lambda=0.595, X^2=8.30, df=4, p=0.081), but significant differences between control and *Gmm*$^{\mathrm{Apo}}$ flies (Wilks' Lambda=0.498, X^2=11.1, df=4, p=0.025) and between *Gmm*$^{Wgm-}$ and *Gmm*$^{\mathrm{Apo}}$ flies (Wilks' Lambda=0.402, X^2=14.6, df=4, p=0.006), respectively.

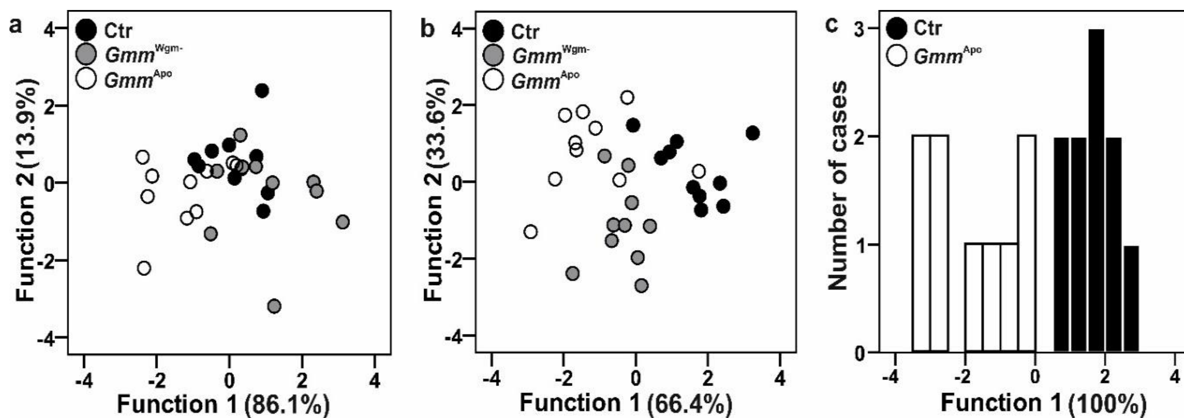

Figure 2: Effect of antibiotic treatment on CHC profiles of female tsetse flies (*G. m. morsitans*). Discriminant analyses based on log-ratio transformed relative amounts of (**a**) CHCs of mass-reared, 10 day old females, (**b**) mass-reared 5 day old females, and (**c**) individually reared, 10 day old females. Ctr=Control (without antibiotics), *Gmm*$^{\mathrm{Wgm-}}$=ampicillin-treated, *Gmm*$^{\mathrm{Apo}}$ =tetracycline-treated.

In 5-day-old females, there was also no difference in total amount of CHCs across groups (**Figure 1a**; ANOVA, $F_{2,27}$=1.234, p=0.307). *Gmm*$^{Wgm-}$ females showed a non-significant tendency towards lower absolute amounts of 15,19,23-trimethyl-heptatriacontane (**Figure 1c**; ANOVA, $F_{2,27}$=1.785, p=0.187). A comparison of the relative amounts of 15,19,23-trimethyl-heptatriacontane revealed significantly lower proportions of sex pheromone in *Gmm*$^{Wgm-}$ females as compared to control and *Gmm*$^{\mathrm{Apo}}$ flies (**Figure 1d**; ANOVA, $F_{2,27}$=6.981, p=0.004; Tukey HSD p=0.014 for control-*Gmm*$^{Wgm-}$ and p=0.001 for *Gmm*$^{Wgm-}$-*Gmm*$^{\mathrm{Apo}}$).

Based on the 18 quantified peaks, four principal components were extracted, capturing 85.0% of the total variance. A discriminant analysis (DA) based on the four PCs including all three treatment groups yielded a significant difference in CHC profiles across groups (**Figure 2b**; Wilks' Lambda=0.224, X^2=38.1, df=8, p<0.001). Based on the two discriminant functions, 83.3% of the cases were correctly classified (30% would be expected by chance). Subsequent DAs of pairwise combinations of the three groups revealed a significant difference between control and Gmm^{Wgm-} flies (Wilks' Lambda=0.232, X^2=23.37, df=4, p<0.001), between control and Gmm^{Apo} flies (Wilks' Lambda=0.367, X^2=16.0, df=4, p=0.003) and also between Gmm^{Wgm-}- and Gmm^{Apo} flies (Wilks' Lambda=0.405, X^2=14.5, df=4, p=0.006), respectively.

Influence of antibiotic treatment on CHC profiles in mass-reared male flies

In 10-day-old male flies, control and Gmm^{Wgm-} individuals showed on average 5-6 times higher total amounts of CHCs than did Gmm^{Apo} flies (**Figure 1b**; ANOVA, $F_{2,25}$=10.03, p=0.001). Post-hoc comparisons (Tukey HSD) revealed these differences to be significant (p=0.001 and p=0.009 for control-Gmm^{Apo} and Gmm^{Wgm-}-Gmm^{Apo}, respectively), while there was no difference between control and Gmm^{Wgm-} flies (p=0.457). Based on the 13 quantified peaks, four principal components were extracted, capturing 84.3% of the total variance. A discriminant analysis (DA) based on the four PCs including all three treatment groups yielded a significant difference in CHC profiles across groups (**Figure 3a**; Wilks' Lambda=0.046, X^2=72.5, df=8, p<0.001). Based on the two discriminant functions, 96.4% of the cases were correctly classified (30% would be expected by chance). Subsequent DAs of all pairwise combinations of the three groups revealed significant differences between all groups: control vs. Gmm^{Wgm-}: Wilks' Lambda=0.233, X^2=23.3, df=4, p<0.001; control vs. Gmm^{Apo}: Wilks' Lambda=0.076, X^2=36.0, df=4, p<0.001; Gmm^{Wgm-}- vs. Gmm^{Apo}: Wilks' Lambda=0.177, X^2=24.2, df=4, p<0.001.

Antibiotic treatment had no effect on the total amount of CHCs in 5-day-old males (**Figure 1b**; ANOVA, $F_{2,27}$=1.565, p=0.227). Based on the 13 quantified peaks, five principal components were extracted, capturing 88.8% of the total variance. A discriminant analysis (DA) based on the four PCs including all three treatment groups yielded a significant difference in CHC profiles across groups (**Figure 3b**; Wilks' Lambda=0.207, X^2=39.3, df=10, p<0.001). Based on the two discriminant functions, 70.0% of the cases were correctly classified (30% would be expected by chance). Subsequent DAs of all pairwise combinations of the three groups revealed significant differences between control vs. Gmm^{Wgm-} males

(Wilks' Lambda=0.253, X^2=21.3, df=5, p<0.001); control vs. *Gmm*^{Apo} males (Wilks' Lambda=0.146, X^2=29.8, df=5, p<0.001), but not between *Gmm*$^{Wgm-}$ and *Gmm*^{Apo} males (Wilks' Lambda=0.727, X^2=4.9, df=5, p<0.424).

Influence of antibiotic treatment in individually reared flies

In individually reared 10-day-old females, there was no difference in the total amount of CHCs between control and *Gmm*^{Apo} flies (**Figure 1a**; t-test, T=-0.888, df=16, p=0.388), nor in the absolute amount of female sex pheromone (**Figure 1c**; t-test, T=0.170, df=16, p=0.868). A comparison of the relative amounts of 15,19,23-trimethyl-heptatriacontane revealed significantly lower proportions of sex pheromone in *Gmm*^{Apo} females as compared to control flies (**Figure 1d**; t-test, T=2.080, df=17, p=0.044). Based on the 18 quantified peaks, four principal components were extracted, capturing 87.2% of the total variance. A discriminant analysis (DA) based on the four PCs including yielded a significant difference in CHC profiles between control and *Gmm*^{Apo} females (**Figure 2c**; Wilks' Lambda=0.233, X^2=21.8, df=4, p<0.001). Based on the first discriminant functions, 94.7% of the cases were correctly classified (50% would be expected by chance).

As in females, individually reared 10-day-old males showed no difference in the total amount of CHCs between control and *Gmm*^{Apo} flies (**Figure 1b**; t-test for non-equal variances, T=0.287, df=11.653, p=0.779). Based on the 13 quantified peaks, four principal components were extracted, capturing 87.2% of the total variance. A discriminant analysis (DA) based on the four PCs yielded a significant difference in CHC profiles across groups (**Figure 3c**; Wilks' Lambda=0.246, X^2=18.2, df=4, p=0.0011). Based on one discriminant function, 94.1% of the cases were correctly classified (50% would be expected by chance).

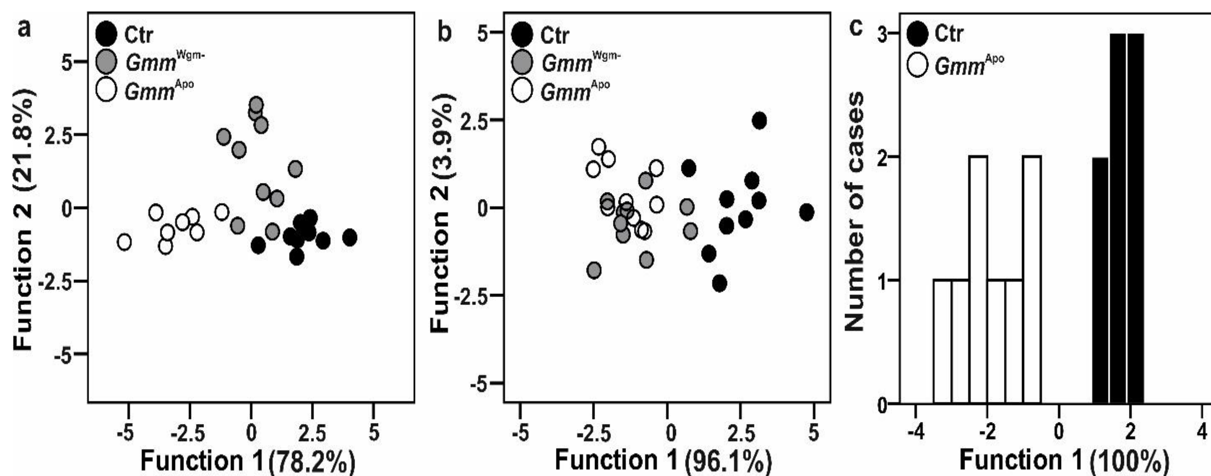

Figure 3: Effect of antibiotic treatment on CHC profiles of male tsetse flies (*G. m. morsitans*). Discriminant analyses based on log-ratio transformed relative amounts of (**a**) CHCs of mass-reared, 10 day old males, (**b**) mass-reared 5 day old males, and (**c**) individually reared, 10 day old males. Ctr=Control (without antibiotics), *Gmm*$^{Wgm-}$=ampicillin-treated, *Gmm*^{Apo} =tetracycline-treated.

Comparison of CHC profiles between antibiotic experiments

A comparison of chemical profiles of *Gmm*^{Apo} and control flies between the three experiments revealed significant differences between treatments and experiments for both females (**Supplementary file 1a**; Wilks' Lambda=0.027, X^2=192.2, df=20, p<0.001) and males (**Supplementary file 1b**; Wilks' Lambda=0.005, X^2=250.6, df=20, p<0.001). In particular, fly profiles were very well separated into the three experiments in the discriminant analysis.

Influence of gamma-irradiaton on male flies

In a full factorial model, no overall differences in total CHC amounts could be detected between treatment groups (ANOVA $F_{11,108}$=1.292, p=0.239; time points: $F_{2,108}$=3.577, p=0.031; irradiation dose: $F_{3,108}$=0.114, p=0.952; interaction: $F_{6,108}$=1.119, p=0.356; **Figure 4**).

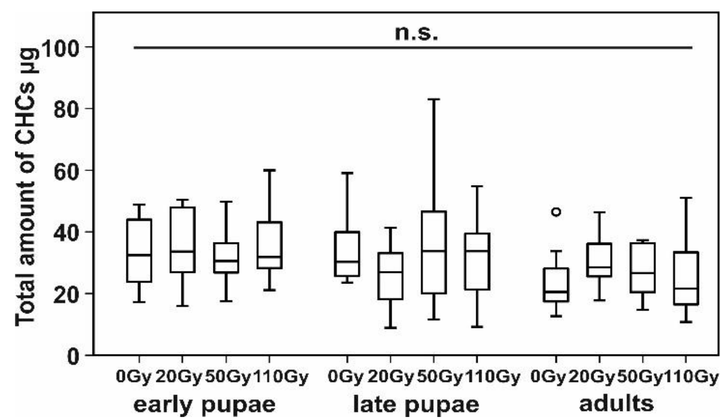

Figure 4: Effect of gamma-irradiation on the total amount of CHCs in male *Glossina m. morsitans* flies. 0Gy = control (without irradiation). Male flies were irradiated at one of three different time points (early pupae, age 22 days; late pupae, 29 days; or young adults, 5 days). Lines represent medians, boxes comprise interquartile ranges, and whiskers denote minimum and maximum values, except for outliers that lie further away from a quartile than 1.5 times (circles) or 3 times (asterisks) the interquartile range.

Based on the 16 quantified peaks and the 12 treatment groups, three principal components were extracted, capturing 80.0% of the total variance. A discriminant analysis (DA) based on the three PCs including all twelve treatment groups yielded a significant difference in CHC profiles across groups (Wilks' Lambda = 0.592, X^2=58.5, df=33, p=0.004; **Supplementary file 2**), but only 28.3% of the cases were classified correctly based on both discriminant functions. When treatment time point was used as a grouping variable, the groups also differed significantly (Wilks' Lambda = 0.781, X^2=28.7, df=6, p<0.001; **Supplementary file 3**), with 60% of the cases being classified correctly. Subsequent DAs of irradiation treatments at single time points revealed no significant difference between irradiation treatments for any of the three time points (including the 0Gy control; early: Wilks' Lambda = 0.677, X^2=13.9, df=9, p=0.127; late: Wilks' Lambda = 0.693, X^2=13.0, df=6, p=0.126; adult: Wilks' Lambda = 0.799, X^2=8.0, df=6, p=0.539; **Figure 5a-c**).

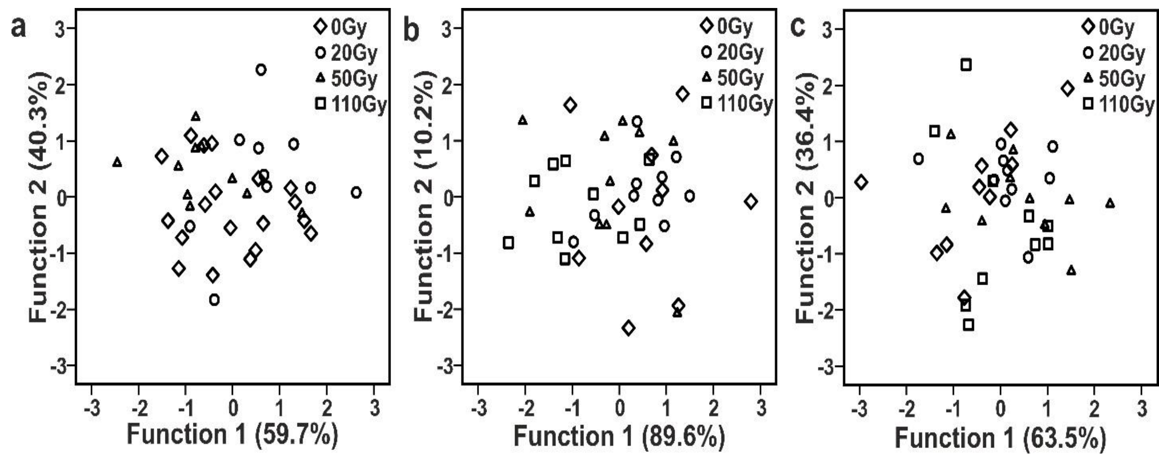

Figure 5: Effect of gamma-irradiation dose on CHC profiles of male tsetse flies (*G. m. morsitans*). Discriminant analyses based on log-ratio transformed relative amounts of individually reared 10 day old males that were treated with different irradiation doses (0Gy, 20Gy, 50Gy, 110Gy) **a**) during early pupal development (at day 22), **b**) during late pupal development (after females emerged) and **c**) to adult males (day 5 post-eclosion).

Mate choice assays with mass-reared flies

Out of the 30 male mate choice assays, the males chose females with their native microbiota in 20 cases and *Gmm*^{Apo} females in 8 cases (**Figure 6a**). Two males remained unmated. Excluding the unmated males, this distribution differs significantly from random mating (df=1, Chi²=5.14, p=0.02). Of the 17 females, 10 mated with males with their native microbiota, two with *Gmm*^{Apo} males, and five remained unmated (**Figure 6b**). Excluding the unmated females, this distribution differed significantly from random mating (df=1, Chi²=5.33, p=0.02).

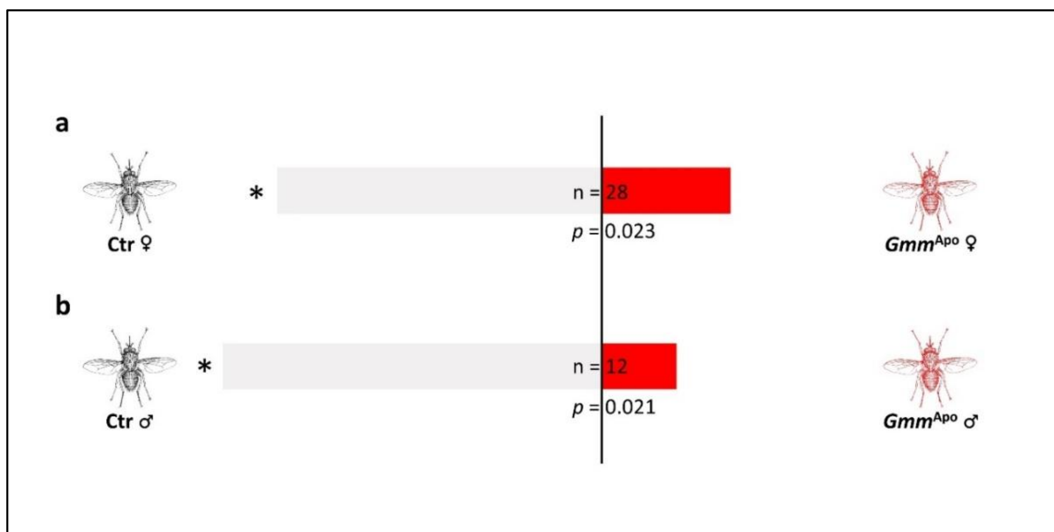

Figure 6: Effect of tetracycline treatment on mating success of (**a**) male and (**b**) female tsetse flies (*G. m. morsitans*). An untreated individual of the opposite sex was given a simultaneous choice between a *Gmm*^{Apo} and an untreated individual. Ctr=Control (without antibiotics), *Gmm*^{Apo} =tetracycline-treated.

Discussion

We assessed the impact of antibiotic treatment on the CHC profiles and mating success of male and female tsetse flies. Neither the absolute amount of all CHCs in females, nor the absolute amount of the female sex pheromone 15,19,23-trimethyl-heptatriacontane was effected by Amp or Tet treatment under any rearing condition. However, the relative amount of the sex pheromone was significantly reduced after Tet treatment. In males, the total amount of CHCs was significantly reduced in mass-reared Gmm^{Apo} males, but not in Gmm^{Wgm-} and individually reared Gmm^{Apo} males. The CHC profiles of both females and males differed significantly between treatments under all rearing conditions except for mass reared Gmm^{Wgm-} vs. Gmm^{Apo} 5-day old males. Further, gamma-irradiation of male pupae or young adults did not affect the CHC profile of 10-day old males, even though a previous study has shown that the treatment with 110gy causes significant, yet variable effects on the three symbiont titers, based on irradiation time [39]. Finally, both male and female flies with their native microbiota discriminated against Gmm^{Apo} flies in mate choice assays.

The bacterial symbionts harbored by tsetse flies exhibit differential sensitivity to antibiotics and irradiation. Only *Wigglesworthia* is sensitive to both Amp and Tet [26, 37], whereas all three symbionts are affected by Tet [26]. Finally, irradiation significantly affects *Sodalis* and *Wolbachia*, but not *Wigglesworthia* titers [39]. Thus, our treatments include tsetse flies with their full microbiota (untreated controls of both antibiotic and irradiation experiments), flies with normal *Sodalis* and *Wolbachia* titers but without *Wigglesworthia* (Gmm^{Wgm-} resulting from Amp treatment), flies with normal *Wigglesworthia,* but reduced *Sodalis* and *Wolbachia* titers ($Gmm^{\text{Sod-Wlb-}}$, resulting from some of the irradiation treatments) and fully aposymbiotic flies (Gmm^{Apo}, resulting from Tet treatment). Tet treatment, which clears all symbionts, had the strongest and most consistent effect on CHC profiles of males and females, as well as on the relative amount of the female sex pheromone. Furthermore, males mate preferentially with untreated females, possibly because their CHC profile contains a higher relative amount of 15,19,23-trimethyl-heptatriacontane, the female sex pheromone. Amp treatment also affected CHC profiles in males and 5-day old females, but not in 10-day old females, while irradiation which disturbs *Sodalis* and *Wolbachia* did not affect the CHC profiles of male flies. However, we cannot exclude the possibility that other time points of irradiation would yield different results, given the complex interaction effects of irradiation dosage and time on symbiont titers [39]. Nevertheless, taken together, these results suggest that *Wigglesworthia* has the strongest effect on CHC profiles of *G. m. morsitans.*

There is increasing evidence that symbiotic bacteria can under certain circumstances influence pheromone communication and mate choice of their insect host [13-15, 42-48], which can ultimately result in reproductive isolation and, hence, speciation [10, 16, 43]. While reproductive manipulators like *Wolbachia* are prime suspects for the modification of their host's chemical communication and mate choice [10, 16, 43], several gut associated microbes are also known to be involved in the production of host pheromone components. By contrast, nutritional endosymbionts like *Wigglesworthia* were so far not implicated in changes of host mating signals or mate choice. However, as *Wigglesworthia* provides essential vitamins [23] and is involved in the maturation of the immune system [24, 25, 37] direct or indirect effects on other metabolic processes such as the synthesis and distribution of hydrocarbons or their precursors seem plausible and could explain the modification of CHC profiles upon antibiotic treatment observed in our study.

Although our results are consistent with the hypothesis that an effect of *Wigglesworthia* on CHC profiles modulates mate choice, we could not test this effect on mate choice directly, nor is it currently possible to exclude direct effects of the antibiotic treatment itself on the fly's physiology, CHC profile, overall vigor, and behavior. Antibiotics influence several life history parameters of insects. For example, treatment of the black bean aphid [*Aphis fabae*; 47, 48], the mustard aphid [*Lipaphis erysimi*; 49], the walnut husk fly [*Rhagoletis complete*; 50] and the melonfly [*Dacus cucurbitae*; 51] with Tet derivatives in particular causes diverse side effects including reduced larval development rate, adult size, weight, reproduction and longevity. However, as all these aphids harbor the obligate intracellular mutualist *Buchnera aphidicola* [52], and the gut microbiota of diverse true fruit flies also has a significant influence on host fitness [53, 54], a direct influence of the tested antibiotics on host physiology cannot be differentiated from an indirect influence via symbiont depletion in these studies. A few studies have succeeded in implicating the insect microbiota in CHC profile modulation, without the involvement of antibiotics. Guo et al. [55] demonstrated that the gut microbiota of termites provides precursors for the synthesis of methyl-branched CHCs through the incorporation of ^{14}C-labelled succinate. Furthermore, Dosmann et al. [56] investigated a possible microbial modulation of nest mate recognition in harvester ants by altering the external microbiome through antibiotic treatment as well as application of cultured bacteria to the ant cuticle. While the application of cultured microbes influenced nest mate recognition, treatment with rifampicin did not [56]. Thus, while direct contributions of

obligate symbionts to nest mate recognition cues in harvester ants are possible, the results remain inconclusive.

Another factor that warrants careful interpretation of the presented results is that differences in CHC profiles across experiments were more pronounced than between treatments within each experiment (**Supplementary file 1**). Hence, the age of the flies, the rearing conditions (individual vs. mass-rearing), and possibly fluctuations in rearing conditions (e.g. diet, temperature, humidity) as well as variation in the genetic composition of the starting populations may influence CHC composition. Furthermore, the *Sodalis* and *Wolbachia* depleted flies resulting from gamma-irradiation were treated themselves, as opposed to analyzing the offspring of treated flies, as in the antibiotic experiments. Flies thus have experienced a different ontogeny, with *Sodalis* and *Wolbachia* present during part of their development. Despite the fact that cuticular hydrocarbons usually display a fast turnover, enabling insects to adapt within hours to days, it cannot be excluded that the late time point of symbiont depletion in the irradiation treatment was responsible for the lack of an effect on CHC profiles. Age and ontogeny-dependent changes in CHCs have been described across different insect species [57-61] and may serve as reliable age indicators for mate choice [62]. Diet and host genetics influence CHCs via fatty acid metabolism [63, 64], whereas fluctuations in temperature and humidity can stimulate insects to adjust their CHC profiles to improve desiccation resistance under the current conditions [65-67]. Thus, under natural settings, microbial symbionts may be one of several different factors affecting insect CHC profiles and thereby mate choice and sexual selection.

Conclusion

Our results provide first insights into changes in CHC profiles upon symbiont depletion by antibiotic and gamma-irradiation treatment in *G. m. morsitans*. Individual rearing corroborated the results obtained from mass-rearing, excluding potential pseudoreplication artifacts by flies exchanging CHCs through direct contact under mass-rearing conditions. Mate choice assays indicate that antibiotic treatment not only affects CHC composition, but also impairs mating success of both males and females. However, the link between mating success, CHC profiles, and *Wigglesworthia* as the causative agent for the observed changes remains speculative at this point. Further studies are needed to pinpoint single symbiont contributions to CHC synthesis and mate choice. Nevertheless, our results indicate that the chemical ecology of tsetse flies should be taken into account when investigating the effects of

symbionts on host fitness or manipulating the symbiosis to enhance refractoriness to trypanosome infection. Furthermore, we could show that gamma-irradiation, which is routinely employed to create sterile males for the sterile-insect-technique to control *G. m. morsitans* populations, does not alter the CHC profiles of males. Hence, irradiated males might not suffer a competitive disadvantage after their release into the field, if females use chemical cues for mate choice. Finally, if symbiont or parasite infection predictably affects CHC profiles, chemical analyses may also provide a simple and cost-efficient alternative to molecular screenings for the assessment of symbiont/parasite infection status.

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Availability of data

Data presented in this article is available on figshare under doi:10.6084/m9.figshare.5472682 and doi: 10.6084/m9.figshare.5473153.

Competing interests

The authors declare no competing interests.

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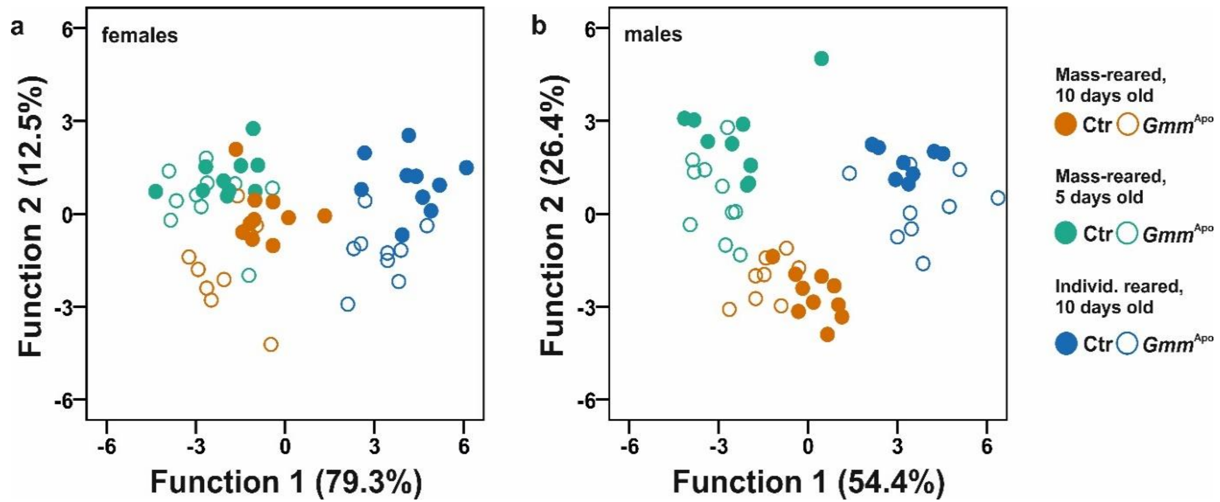
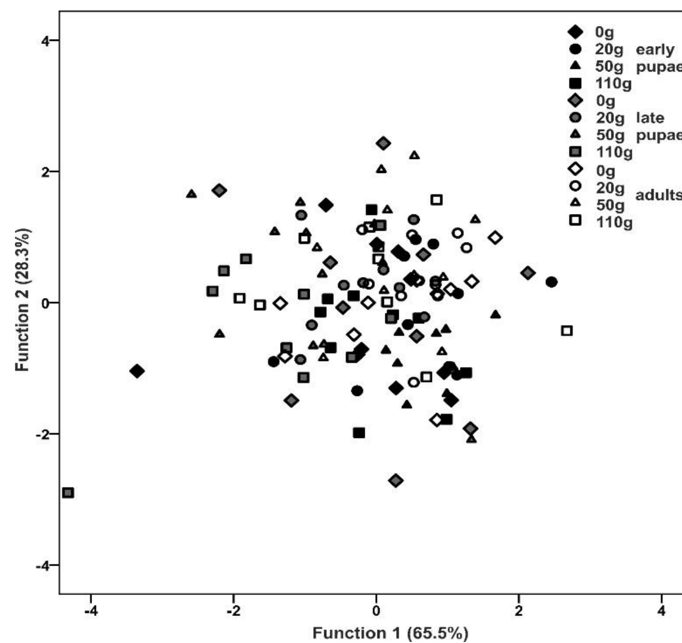
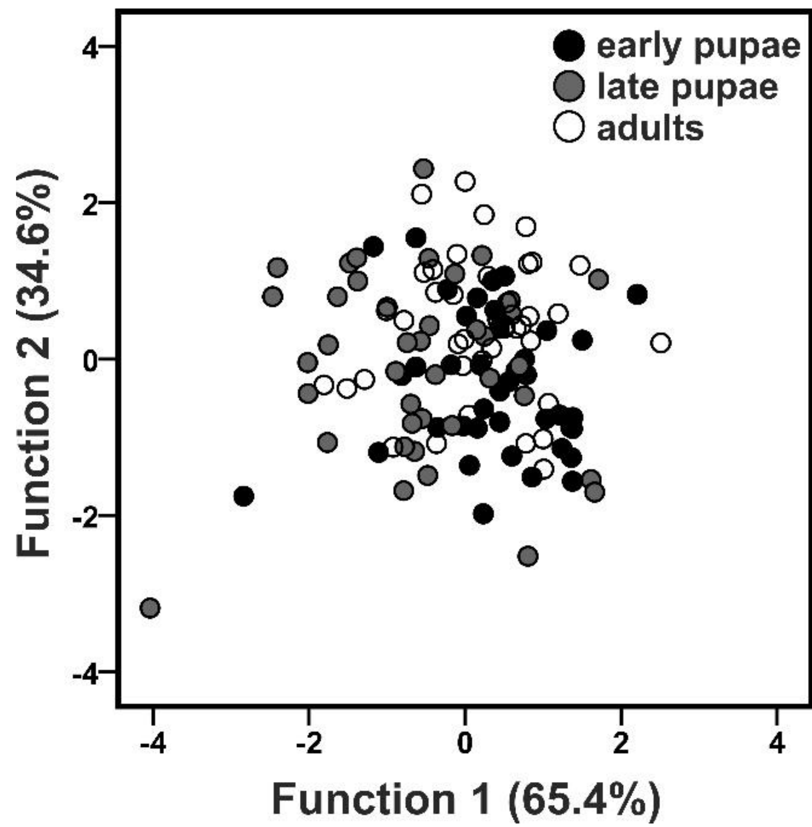
Supplementary files



Supplementary file 1: Comparison of CHC profiles of untreated (Ctr) and tetracycline-treated (Tet) (**a**) female and (**b**) male tsetse flies (*G. m. morsitans*) across the three different experiments.



Supplementary file 2: Effect of gamma-irradiation dose and time point on CHC profiles of 10 day old individually reared adult *G. m. morsitans* males. Discriminant analysis based on log-ratio transformed relative amounts across all treatment groups (time points and irradiation doses).



Supplementary file 3: Effect of the time point of gamma-irradiation on CHC profiles of individually reared 10 day old adult *G. m. morsitans* males. Discriminant analysis based on log-ratio transformed relative amounts across irradiation time points (early and late pupal development and as young adults).

Chapter 8

General Discussion and Conclusion

General Discussion and Conclusion

In the frame of our attempts to improve the efficiency of SIT programs for tsetse fly control, this Ph.D. dissertation has addressed some questions of important relevance. Some of the important milestones for effective implementation of SIT for tsetse are (i) the economic mass-production of high-quality sterile males and (ii) reducing the risk of increasing the disease incidence that might be associated with the initial release of sterile males. The mass-production of high-quality sterile males in a complicated process that can be affected by many factors such as the feeding nature, the productive capacity, the mass rearing condition, irradiation treatment, packaging and handling during transport and the release process. However, above all, and because SIT is a species-specific tool, it is imperative to start the mass rearing with the correct tsetse species that will be targeted by the SIT program to ensure mating compatibility. In addition, tsetse mass rearing can be affected by pathogens such as the salivary gland hypertrophy virus (SGHV) and therefore protection against these pathogens in tsetse mass-rearing is needed. Moreover, as the radiation step is fundamental to sterile males before release, reducing the impact of the quality of the male and investigating the effect of irradiation treatment on the male mating capability and it vectorial capacity is important. Finally, attempts to reduce the risk of increasing the disease incidence through the possible combination of paratransgenesis to produce males refractory to trypanosome infection and SIT were investigated. Under the above-mentioned objectives, this thesis was conducted on the following topics: (i) identification of tsetse species using molecular biological tools, (ii) evaluation of the prevalence and co-infection of trypanosome, *Wolbachia* and SGHV in wild tsetse population, (iii) assessment of the impact of SGHV infection of different tsetse species, (iv) investigation of the impact of irradiation and antibiotic treatment impact on hydrocarbon profiles and mate choice in tsetse flies and (v) irradiation impact on symbiont from different developmental stages of tsetse flies in order to use further the paratransgenesis approach combination with SIT to make flies refractory against trypanosomiosis transmission.

Early studies have reported that *Glossina* species were identified using their morphological characters based on their genitalia, habitat and host choice [13] but this is a challenge for the development of a successful implementation of SIT due to morphological similarities. During the last decades, generic and molecular markers have been used for identification studies. In Chapter 2, we described more reliable, fast and cheap identification approaches with the integral use of Nuclear Markers (ITS1), selected microsatellite markers and *Wolbachia* status

(chromosomal introgression and cytoplasmic infection) to differentiate various Glossina species. The results showed that ITS1 can distinguish five different tsetse species based on amplicon size among eight different tsetse species tested. To distinguish the un-identified remaining species, several microsatellite markers and additional symbiotic markers were also used. When there was a lack of identification using the ITS1 marker, the A10 microsatellite marker helped to separate *G. p. gambiensis* and *G. tachinoides* species from each other. Additionally, with the help of the Gmm14 microsatellite marker, *G. brevipalpis* species can easily be separated from other tsetse species. According to differences in *Wolbachia* infection status in *G. pallidipes* from two different countries (Uganda and Ethiopia), it was understood that the geographical origin of species might have an impact on species differences. Similarly, the absence and presence of *Wolbachia* in the same species from different geographic areas are also reported [110, 117]. Our results also demonstrated that *Wolbachia* is present in laboratory *G. m. morsitans* species based on a 16S RNA gene-based PCR assay. No other field and laboratory collections of any other taxons studies agree with this specific chromosomal insertion. This study shows that the reliable molecular marker ITS1 tool can distinguish species easily from each other. Similar to our study, species have also been differentiated for ITS1 sequences species in the past [139-141].

The trypanosome, virus and *Wolbachia* presence, co or/and triplicate infection, synergetic and antagonistic effects investigated in natural tsetse populations from West Africa **(Chapter 3)** after confirmed the tsetse species identity as described in chapter 2. The results indicate a high prevalence of trypanosomes in tsetse flies, however, the prevalence of SGHV and *Wolbachia* were low, but trypanosome was high, and their prevalences differ between different geographical locations and species. Yet, no significant differences were determined for the sex of flies. In some cases, mixed trypanosome and SGHV infection or/and double or triplicate trypanosome infections were determined. No triplicate infection of SGHV, *Wolbachia* and trypanosome were detectable in the natural population.

The results of this study indicate the relatively high prevalence of trypanosomes in tsetse flies in West Africa, most probably AAT [142], and therefore this region should be a priority for tsetse a SIT control program where possible. It also indicates the possibility of using molecular tools to screen tsetse flies for trypanosome infection as indirect evaluation of the disease risk rather that screening both humans and animals which require more expensive preparation. The low prevalence of both SGHV indicates the low risk of virus infection if tsetse flies are collected from this region to start tsetse mass rearing. The low prevalence of

Wolbachia might allow for the opportunity to use cytoplasmic incompatibility combined with SIT in these regions by releasing sterile males infected with *Wolbachia* [143]

Although a virus management strategy to control the SGHV infection was developed and proved effective in eliminating the virus risk from *G. pallidipes* colonies [76]. The impact of the virus infection on other tsetse species was unknown. Therefore the work presented in **Chapter 4 and 5** focused on the assessment of the virus host range on different tsetse species as well as the impact of the virus infection on the quality control parameters on *G. f. fuscipes* infected flies. The results indicate that the virus can infect other tsetse species and affect the flies' fitness and performance although the trans-generation transmission was only active in *G. pallidipes*. These results highlight the importance of screening wild flies from any tsetse species for virus infection before using it to start mass rearing facilities. In addition, it highlights the importance of implementing virus management strategies for all tsetse species, mainly reducing the number of feeding cycles per membrane and periodic checks for virus infection. Implementing these guidelines in mass rearing colonies will avoid sudden increases of virus infection. It might also be important to conduct periodic screening for tsetse mass rearing colonies for other pathogenic agents.

The treatment is a must to sterilize tsetse males for SIT, but to maintain the high quality of sterile males released during the SIT program, the effect of this treatment should be minimum and not affect the males' performance and its appeal to wild females. One of the factors that might affect the mating choice of sterile males is the cuticular hydrocarbon profiles (CHC). To ensure that the irradiation treatment did not have a direct or indirect impact by affecting tsetse microbiota on tsetse CHC profiles, we analyzed the tsetse males CHC profiles after both irradiation and antibiotic treatment. The results presented in **Chapter 6** indicate that Tetracycline and ampicillin antibiotics have a significant impact on CHC profiles in both female and male *G. m.* morsitans and mating choice, while irradiation had no significant impact. These results are of importance as the irradiation did not directly affect the appeal of sterile male to wild females by affecting the CHC profiles. However, care should be taken to reduce the impact of irradiation on tsetse symbionts.

The second milestone for tsetse SIT is reducing the potential risk of increasing the disease incidence that might be associated with the release of a large number of sterile males. To this end the possibility of using the tsetse strain refractory to trypanosome infection produced by paratransgenesis by modifying symbiont [144, 145] in the SIT program was investigated. To

assess the feasibility of this approach, we assessed the impact of irradiation on *Sodalis* density **(Chapter 7)**. The results indicate that conducting radiation during the pupae stage rather than the adult stage reduces the negative impact on *Sodalis* density and allows for significant recovery of *Sodalis* which is most probably enough to produce anti-trypanosome factors to maintain males refractory to trypanosome infection. These results open the door to combining paratransgenesis approach with SIT and to reduce and eliminate the risk of increasing the disease incidence after releasing sterile males for SIT programs. The results also indicate that the male's vectorial capacity are not affected by irradiation treatment.

Conclusion

The work presented in this thesis provides information of important relevance for SIT and represents a significant step to improve the implementation of the SIT program. Improving tsetse species identification, improving the mass-rearing for sterile males and reducing or eliminating the risk of disease incidence after releasing a large number of sterile males for SIT programs will undoubtedly enhance the implementation of SIT. The successful implementation of SIT for tsetse and Trypanosomoses will have a very positive impact on the socio-economic impact of countries in the sub-Saharan area. This might also have a significant positive impact on the environment and human health by reducing the use of chemical pesticides for the control of tsetse flies.

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Curriculum Vitae

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EDUCATION

Vienna University of Technology 2014-2018
Ph.D. in Natural Science in Technical Chemistry at the
Institute of Chemical, Environmental and Bioscience Engineering Vienna, Austria

Yeditepe University 2009-2012
Master of Science in Biotechnology Istanbul, Turkey

Ankara University 2001-2006
Bachelor of Science in Biology (Molecular Biology and Genetics) Ankara, Turkey

RESEARCH WORK EXPERIENCE

Consultant 2012-Present
Joint FAO/IAEA Agriculture and Biotechnology Laboratories Seibersdorf, Austria
United Nations

Research Assistant 2009-2012
Yeditepe University Istanbul, Turkey

Intern 2008-2009
Bogazıcı University Istanbul, Turkey

Intern 2005
Gazi University Ankara, Turkey

RESEARCH SKILLS AND EXPERTISE

- Molecular Techniques including DNA isolation, PCR, qPCR, gel electrophoresis, SNP analysis, molecular cloning
- Tsetse fly rearing and colony management
- Virus and symbiont screening in insect vectors
- Cell Culture Techniques, Cytotoxicity Assays,
- Protein, Antioxidant Assays. ELISA
- Microbial Identification Techniques (Biol og® System, MIDI, API, BD® Crystal)

- Basic Microbiological Techniques including aerobic and anaerobic bacterial growth and biochemical characterization of microorganisms using anaerobic cabinet
- Phototherapy for applications in biotechnology
- Various plant extraction methods
- Drug delivery design and nanotechnological applications
- Encapsulation application

TECHNICAL TRAININGS AND CONFERENCE PARTICIPATION

- Radiation and Dosimetry Course, Joint IAEA/FAO Agriculture and Biotechnology Laboratories, Seibersdorf, Austria, 2016.
- IAEA-Bio-Linux VMs and GWAS training course in IAEA, 5-6 December 2016 ,Vienna, Austria
- Bi-Linux 8 and Sequence assembly training in IAEA, 14-16 December 2015,Vienna, Austria
- Mutation, DNA and Protein Application Training, Tubitak MAM, , 4-28 July 2009, Istanbul, Turkey.
- EMBO Young Scientists 'Forum, Yeditepe University, 14-16 June 2012, Istanbul, Turkey
- Certificate of Animal Use in Experimental Research, Yeditepe University, 14-24 May 2012 Istanbul, Turkey
- Phyrosequencing Training QIAGEN 26 April 2102 İstanbul, Turkey
- Rotor Gene &Software Training. QIAGEN, 26 April 2102, İstanbul, Turkey
- GC /GC MS Training –Thermo Scientific İstanbul, 24 March 2012 İstanbul, Turkey
- HPLC Training- Thermo Scientific İstanbul,10 March 2012 İstanbul, Turkey
- III. International Entomopathogens and Microbial Control Symposium-18-22, September 2011 Istanbul, Turkey
- B-D Brand Cyristal Panel Viewer Bacteria Reading Light Operator Training-18 April 2011, Istanbul, Turkey
- VI Molecular Biology and Genetics Weekend. 9-10 April 2011, Istanbul Turkey.
- Personalized Medicine Seminar 05/06/2010, Istanbul, Turkey
- Array Technologies for BSL3 and BSL4 Pathogens 05-06 May 2010, Istanbul, Turkey
- Attendance Certificate, 1st International Multidisciplinary Cancer Research Congress, May 2009, Antalya, Turkey,

HONORS & AWARDS

- Yeditepe University Scholarship award for Masters studies. Istanbul, Turkey, 2009-2012
- Government of Turkey scholarship for BSc studies. University of Ankara, Turkey, 2001-2005
- Private Honor Donation scholarship for BSc studies. University of Ankara, Turkey, 2001-2005

CONFERENCE PROCEEDINGS AND PUBLICATIONS

PUBLICATIONS

1. **Güler Demirbas Uzel, Henry M. Kariithi, Andrew G. Parker, Marc J. B. Vreysen, Robert L. Mach and Adly M. M. Abd-Alla. "**Comparative Susceptibilities of Tsetse Species to *Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV)", **(Frontier Microbiology. 2017, under revision)**

2. **Güler Demirbas Uzel, Henry M. Kariithi, Andrew G. Parker, Marc J. B. Vreysen, Jeremy Bouyer, Robert L. Mach and Adly M. M. Abd-Alla. "**Impact of Glossina pallidipes salivary gland hypertrophy virus (GpSGHV) on a heterologous host, tsetse fly *G. f. fuscipes***"(Accepted to BMC Microbiology, 2018***)*

3. **Güler Demirbas Uzel, Avgoustnos, A Andrew G. Parker, Marc J. B. Vreysen, Robert L. Mach, Bourtzis K and Adly M. M. Abd-Alla. "**Interactions between *Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV) and endosymbiont of different tsetse species**", (Under preparation to Frontier Microbiology, 2018)**

4. **Güler Demirbas Uzel, Andrew Parker, Marc Vreysen, Robert L Mach, Adly Abd-Alla, "**Combining paratransgenesis with SIT; Impact of ionizing radiation on the prevalence of *Sodalis glossinidius",* **(Submitted to BMC Microbiology, 2018***).*

5. **Güler Demirbas Uzel, Andrew Parker, Marc Vreysen, Robert L Mach, Adly Abd-Alla, "**Sterile Insects to enhance SIT: Quality of sterile male *G. m. morsitans* after transport as irradiated pupae*",* **(Under preparation for Parasite and Vector, 2017).**

6. **Tobias Engl, Veronika Michalkova, Brian L. Weiss, Güler Demirbas Uzel ,Peter Takac, Wolfgang J. Miller, Adly M. M. Abd-Alla**, **Serap Aksoy**, **Martin Kaltenpoth,** 'Effect of antibiotic treatment and gamma-irradiation on cuticular hydrocarbon profiles and mate choice in tsetse flies (*Glossina m. morsitans*) **(Accepted to BMC Microbiology, 2017***)*

7. **Ouedraogo G.M.S, Güler Demirbas Uzel, Traore A, Rayaisse J.B, Avgoustnos, A , Parker A .,Sidibe, I, Ouedraogo, A.G, Vreysen MJB, Bourtzis K. and Abd-Alla M.M.A .**"Prevalence of salivary gland hypertrophy virus *Wolbachia* and trypanosome in wild population of tsetse flies from West Africa*"*(**Submitted to BMC Microbiology, 2017***)*

8. **Augustinos AA†, Meki I†, Güler Demirbas Uzel, Saridaki A, Tsiamis G, van Oers, MM, Vreysen M, Parker A, Abd-Alla A, Bourtzis K**, "Nuclear, mitochondrial and Wolbachia-based approaches for the quick and accurate identification of tsetse species, (**Submitted to BMC Microbiology, 2018)**

9. **Kariithi, HM; Boucias, DG; Murungi, EK ; Meki, IK; Uzel, Demirbas Guler ; van Oers, MM ; Vreysen, MJB; Abd-Alla, AMM and Vlak, JM "**Coevolution of Hytrosaviruses and Host Immune Responses***"***,** (**Accepted to BMC Microbiology, 2017**)

10. **Guy J. Hallman, Lincong Wan, Teresa Vera, Güler Demirbas Uzel, Carlos, Scott Myers, Marc Vreysen** "Comparison of Populations of Ceratitis capitata (Diptera: Tephritidae) from Three Continents for Susceptibility to Cold Phytosanitary Treatment and Implications for Generic Treatments*",***(Under Preparation)**

11. **Vanessa Simoes Dias, Carlos B. Caceres, Andrew G. Parker, Rui C. Pereira, Nicholas M. Teers, Güler Demirbas Uzel, Abd-Alla M.M.A, Al Handler and Daniel A. Hahn**. **"**Combining enzymatic overexpression and low-oxygen conditioning hormesis to improve sexual performance of gamma- irradiated Anastrepha suspensa males", **(Under preparation for Plos One).**

12. **Kariithi, HM,†; Meki, IK†; Schneider, DI†; De Vooght, L†; Khamis, FM†; Geiger, A†; Demirbas Uzel, Guler†; Vlak, JM; iNCE, ikbal Agah; Kelm, S, and Abd-Alla, AMM.** "Enhancing Vector Refractoriness to Trypanosome Infection: Achievements, Challenges and Perspectives" (**Accepted to BMC Microbiology, 2018**)

POSTER AND ORAL PRESENTATIONS

1. **Güler Demirbas Uzel and Adly M. M. Abd-Alla. "**Current research activities in at IPCL; Irradiation impact on tsetse symbiont*"* (**Oral Presentation**), " Enhancing vector refractoriness to trypanosome infection", Final Reserach Coordinating Meeting of the Joint FAO/IAEA Division of nuclear tecniques in food and agriculture. Tanga, Tanzania, December, 2017.

2. **Güler Demirbas Uzel, Henry M. Kariithi, Andrew G. Parker, Marc J. B. Vreysen, Jeremy Bouyer, Robert L. Mach and Adly M. M. Abd-Alla.** "Impact of

Glossina pallidipes salivary gland hypertrophy virus (GpSGHV) on a heterologous host, tsetse fly *G. f. fuscipes",* (**Oral Presentation**), 50th Annual Meeting of Society for Invertebrate Pathology (SIP) Congress, 13- 17 August 2017, in San Diego, California, USA.

3. **Güler Demirbas Uzel, Henry M. Kariithi, Andrew G. Parker, Marc J. B. Vreysen, Jeremy Bouyer, Robert L. Mach and Adly M. M. Abd-Alla.** "Impact of Glossina pallidipes salivary gland hypertrophy virus(GpSGHV) on a heterologous host, tsetse fly *G. f. fuscipes",* (**Poster Presentation**), FEMS Microbiology Congress, 9-13 July 2017, Valencia, Spain.

4. **Guler Demirbas Uzel, Andrew Parker, Marc Vreysen, Robert L Mach, Kostas Bourtzis, and Adly Abd-Alla,** "Analysis of the impact of irradiation treatment on the establishment of *Sodalis* in ***Glossina morsistans morsitans*** Species"; (**Poster Presentation**), Third FAO–IAEA International Conference on Area-wide Management of Insect Pests: Integrating the Sterile Insect and Related Nuclear and Other Techniques, 22-26 May 2017, Vienna, Austria.

5. **Augustinos AA , Meki I , 2, Saridaki A , Guler Demirbas Uzel, Tsiamis G, van Oers, MM, Vreysen M, Parker A, Abd-Alla A, Bourtzis K**, "Contributing to the resolution of taxonomic puzzles: multiple molecular tools and development of protocols for the accurate identification of tsetse species", (**Poster Presentation**), Third FAO–IAEA International Conference on Area-wide Management of Insect Pests: Integrating the Sterile Insect and Related Nuclear and Other Techniques, 22-26 May 2017, Vienna, Austria.

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