WORKING MATERIAL

ENHANCING VECTOR REFRACTORINESS TO TRYPANOSOME INFECTION

SECOND RESEARCH COORDINATION MEETING

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1. INTRODUCTION AND CURRENT STATUS

Tsetse and African trypanosomosis.
Tsetse flies (Diptera: Glossinidae) are the only cyclical vectors of African trypanosomes, protozoan parasites that cause sleeping sickness in humans (HAT) and Animal African Trypanosomosis (AAT) in domesticated animals. HAT is endemic to 36 countries in sub-Saharan Africa with about 70 million of the inhabitants at risk. In 2009, the number of new cases of HAT reported to WHO dropped below the symbolic number of 10,000. However, given that the disease affects hard to reach rural populations, and that active surveillance in war-torn areas is non-existent, the disease prevalence numbers are undoubtedly a gross underestimation. The related disease AAT causes estimated losses to African agriculture of US$ 4,5 billion per year and has a profound effect on development of the continent.

Most economically important African trypanosomes are transmitted during the bite of the tsetse fly. Humans are only infected by *Trypanosoma brucei rhodesiense* and *T.b.gambiense*. The 'nagana' causing related trypanosomatids *T. vivax, T. congolense* and *T. brucei brucei* are major pathogens of livestock.

Following the success of the SIT programme in Zanzibar and the PATTEC initiative of the AU, there interest in the use of SIT for tsetse and trypanosomosis control is increasing. To date, the IAEA supported SIT projects have been in areas without human sleeping sickness, but future projects could include areas of actual or potential human disease transmission. In such projects it would be ethically unacceptable to release flies capable of transmitting the infection to humans and so it is essential to understand the mechanisms that limit the development of infections in tsetse and how these may be enhanced. This CRP proposal addresses this issue, in particular through the interaction of the fly, its immune system, the trypanosomes and the other microorganisms associated with tsetse.

Tsetse-trypanosome interactions.
The transmission cycle starts when a tsetse fly feeds on an infected host. Here, the various trypanosome species have a different developmental cycle in various tissues/ organs of the tsetse fly vector. *T. vivax* has the simplest life cycle with a development exclusively in the mouthparts. So far, only limited information is available on factors that affect *T. vivax* development in the tsetse fly. For *T. congolense* and *T. brucei* initial establishment of infection occurs in the fly midgut with a subsequent maturation in the tsetse fly proboscis and salivary gland respectively. During this journey, these parasites undergo several rounds of differentiation and proliferation, finally ending in the metacyclic form that is the end-stage infective to the mammalian host. The success rate of trypanosome colonization in the tsetse fly is low and often fails during initial establishment in the vector midgut. Tsetse flies mount innate immune responses and down regulation of the Imd pathway increases the efficiency of midgut colonization. Similarly, RNAi silencing of a tsetse fly immune-responsive glutamine/proline-rich (EP) protein promotes trypanosome establishment. Reactive oxygen species are important determinants of resistance. From the midgut, trypanosome differentiation and migration to the salivary glands represents a significant bottleneck that remains to be fully understood. The proventriculus, which is an immune-active tissue, transcribes nitric oxide synthase and contains increased levels of nitric oxide, reactive oxygen intermediates and hydrogen peroxide (H_2O_2). From the proventriculus, only a few trypanosomes will achieve an elusive journey to the salivary glands. The tsetse fly salivary gland plays a key role as a biotope where the parasites adhere, multiply and undergo the final re-programming into the infectious metacyclic forms. Information on the saliva composition is scanty and mainly based on *in silico* interpretation of the salivary gland EST library and a limited 'proteomics' analysis. Although many

different ESTs are predicted to encode for immune defense or pattern-recognition associated proteins, no information is available on the salivary glands as immune-responsive tissue.

No information is available so far about the underlying mechanisms of how trypanosomes adapt to the different tsetse fly microenvironments and to the insect immune responses they circumvent during their developmental journey.

Insect Symbiosis.

Symbiosis is ubiquitous in nature and has had significant consequences in promoting evolution and biodiversity. This is particularly true for insects that are masters in establishing symbiotic associations both inside and outside their bodies. These symbiotic associations are just starting to be understood. Symbiotic microorganisms seem to affect different aspects of the insect life cycle and physiology, including development, nutrition, reproduction, speciation, defense against natural enemies, and host preference.

Currently, insect symbiotic associations are divided artificially into three categories. The first category includes symbionts that provide nutrients such as amino acids and vitamins to their hosts through mutualistic associations. The second category includes symbionts that provide their hosts with the ability to survive heat stress, to develop resistance to parasitic wasps and/or microbial pathogens, and to exhibit altered host preference. The third category includes symbionts that manipulate the reproductive properties of their hosts, inducing phenomena such as parthenogenesis, feminization, male-killing and cytoplasmic incompatibility (CI).

Tsetse Symbiosis.

All tsetse flies, examined to date, harbor an obligate symbiont of the genus *Wigglesworthia*. This relationship is ancient (50-80 million years), and likely serves to complement tsetse's vertebrate blood-specific diet. In fact, *Wigglesworthia*'s highly reduced genome encodes several vitamin biosynthesis pathways, the products of which are absent from vertebrate blood.

Two populations of *Wigglesworthia* exist in tsetse. The first is found within bacteriocytes, which collectively comprise an organ called the 'bacteriome' that is located immediately adjacent to tsetse's midgut. This population of *Wigglesworthia* presumably supplements metabolites absent from its host's diet. Tsetse's second population of *Wigglesworthia* is found extracellularly in the female milk gland. This milk-associated population of cells colonizes developing intrauterine larvae.

Recent experiments demonstrate that *Wigglesworthia* also serves an immuno-modulatory role in tsetse. Adult tsetse that lack this symbiont (Gmm^{Wgm-}) exhibit a immuno-compromised phenotype compared to their wild type counterparts (Gmm^{WT}). When challenged with trypanosomes, gut infections are established in a large percentage of Gmm^{Wgm-} flies. Conversely, Gmm^{WT} flies are highly resistant and can efficiently clear parasite infections. Interestingly, *Wigglesworthia* does not directly enhance immunity in wild type individuals, as elimination of this bacterium from adult flies via antibiotic tetracycline treatment does not result in an immune compromised phenotype. Instead, this obligate must be present during the larval immature stages in order for tsetse's immune system to develop and function properly during adulthood.

Tsetse's second symbiont, *Sodalis*, is a commensal bacterium found in all lab-colonized tsetse lines and some natural populations. This bacterium is polytropic, and is found intra- and extracellularly in tsetse's gut, hemolymph and salivary glands. 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. Furthermore, *Sodalis*' cohabits tsetse's gut along with pathogenic

trypanosomes, and is amenable to genetic manipulation. In addition, recent data suggest that the tsetse fly midgut microbiota (*Sodalis* and *Wigglesworthia*) can also modulate trypanosome development. These characteristics make *Sodalis* an ideal candidate for use in tsetse paratransgenesis.

Tsetse's third symbiont is the alpha-proteobacterium *Wolbachia*. This bacterium is the most successful symbiont on earth, where it infects up to 40% of arthropods, including insects, terrestrial crustaceans, spiders, scorpions and springtails, as well as filarial nematodes. *Wolbachia*´s lifestyle is strictly intracellular and they are mainly transmitted maternally to progeny with the egg cytoplasm, although more and more reports emerge in literature showing that horizontal transfer events are more common than earlier anticipated. In the course of their intimate associations with their hosts, *Wolbachia* has evolved different levels of symbiotic interactions ranging from reproductive parasitism to commensalism to obligatory mutualism. In order to enhance their own spreading throughout populations, these maternally transmitted bacteria cause a number of reproductive phenotypes in many arthropods, such as cytoplasmic incompatibility (CI), male killing, feminization, and parthenogenesis. Furthermore, as part of their co-evolutionary physiological interrelations at cellular and metabolic levels, *Wolbachia* can manipulate host fitness and fecundity, immunity and longevity, development and even sexual behaviour. As recently shown in hybrids between closely related *Drosophila* hosts, mutualistic *Wolbachia* can lose host-directed replication control, overreplicate and thereby transform into pathogens. Such artificial hybrid-host systems can serve as sensitive biomonitors for studying symbiont dynamics in *vivo*.

As demonstrated recently, *Wolbachia* are involved in more host-pathways than was earlier anticipated. For example, *Wolbachia* protect *D. melanogaster* from infections with RNA viruses, whereas similar studies on the *Wolbachia* protection from entomopathogenic fungi in *D. melanogaster* are still inconclusive. The analyses of *Wolbachia*-mediated protective effects against microbes have recently been extended from the *Drosophila* host system to the mosquito *Aedes aegypti* and *Anopheles stephensi*. In this case, the *Wolbachia* infection interferes with the establishment of viral infections, as well as *Plasmodium* and filarial nematodes.

In addition, *Wolbachia*-infected *Ae. aegypti* have reduced lifespan, as expected from the phenotype in its original host. *Wolbachia*-infected *Ae. aegypti* are less likely to transmit viral diseases such as dengue, that require specific incubation periods in the mosquito vectors before transmission to humans can occur. The application of this virulent *Wolbachia* is therefore a novel strategy for fighting vector-borne diseases, such as dengue, malaria and filariasis, by interfering with the vector biology. As recently demonstrated, such artificially infected mosquitoes spread rapidly in the wild and provide dengue protection to their novel host. Because of these seminal discoveries, *Wolbachia* continue to attract 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.

Since many tsetse fly species and populations harbour closely related but distinctive *Wolbachia* strains at different titres and, infection prevalence.. In addition, there is evidence of chromosomal symbiont-insertions in a number of tsetse species. In the laboratory line of *Glossina morsitans morsitans,* crossing studies between *Wolbachia* infected and uninfected individuals have demonstrated the expression of strong CI. It is also of pivotal interest to survey the potential capacity of natural *Wolbachia* infections to functionally interfere with trypanosome transmission.

Symbiont-based control strategies
Because of the high embryonic lethality caused by CI-inducing *Wolbachia* strains, this symbiont can be applied for population suppression of insect pests and disease vectors, in a way analogous to SIT, by releasing *Wolbachia*-infected males expressing strong CI (IIT). As recently shown, strong and promiscuous CI-inducing *Wolbachia* strains can be used as a novel environment-friendly tool for the control of insect populations such as the Mediterranean fruit fly (*Ceratitis capitata*), the

olive fly (*Bactrocera oleae*) and mosquitoes (*Culex pipiens, Aedes aegypti*). *Wolbachia* induced CI can also be used to augment the sterility caused by irradiation and enhance the efficacy of current SIT methods.

Furthermore, *Wolbachia*-induced CI can be also applied as a natural driver system for spreading transgenic insects and maternally transmitted modified symbionts (in paratransgenesis approaches, see below) throughout populations. Because transgenic organisms are likely to be less fit than their wild-type counterparts, transgenic traits must be actively driven into the population in spite of fitness costs by means of population replacement. By a variety of mechanisms, *Wolbachia*-infected females have a reproductive advantage relative to uninfected females, allowing infection to spread rapidly through host populations to high frequency in spite of fitness costs. Hence, *Wolbachia* can be exploited to drive costly transgenes or modified symbionts into vector populations for disease control.

The symbiotic population of tsetse flies also offers the potential to use paratransgenesis as an approach to produce refractoriness for infection with trypanosome parasites. At least one genetically amenable tsetse symbiont has been identified (*Sodalis*) and transformed. Expression of parasite resistance genes in *Sodalis* and its introduction into laboratory reared sterile populations prior to release in the field can now be harnessed for disease control. *Sodalis* paratransgenesis, coupled with SIT, would further decrease the risk of disease transmission to the human population in areas under control.

Viral Pathogens

An essential facet of SIT is the ability to establish large colonies of tsetse lines. Several species of tsetse flies are infected by the *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV). Infection can cause salivary gland hypertrophy (SGH) and significantly reduce the fecundity of the infected flies. Although the SGH syndrome prevalence is likely very low in the trapped wild tsetse population (0.5-5%), in the mass rearing facilities prevalence can reach up to 80% and cause colony collapse. For example, high prevalence of SGH led to the collapse of the *G. pallidipes* colony in Seibersdorf, Austria in 1978 and 2002. The first report of the SGH was described in wild populations of *G. pallidipes* but later observed in other tsetse fly species from different African countries. The causative agent of this syndrome was described initially as a rod-shaped, enveloped DNA virus averaging 70 by 640 nm in size. This virus was associated also with testicular degeneration and ovarian abnormalities, and its presence affected the development, survival, fertility, and fecundity of naturally or experimentally infected flies. Mother-to-offspring transmission, either transovum or through infected milk glands, is thought to be the mode of virus transmission in natural tsetse fly populations. In tsetse colonies horizontal virus transmission, facilitated by the membrane feeding technique used for large-scale feeding of tsetse flies, constitutes the main route of virus transmission. The SGH syndrome has been observed in two other dipterans, the narcissus bulb fly *Merodon equestris* and the housefly *Musca domestica*

Although the PCR detection of GpSGHV in old *G. pallidipes* shows widespread asymptomatic virus infection (100%), only 5-10% of the infected flies develop SGH symptoms. It is unclear why some infected flies show symptomatic infection whereas others remain asymptomatically infected. Although a positive correlation was found between the SGH symptom and the increase in virus copy number (which indicate an accumulation effect of the virus related to the SGH symptoms) another unknown factor related to the fly's genetics and interaction with its microbiota cannot be excluded. Preliminary data indicate a possible negative correlation between virus infection and *Wolbachia*. Therefore it will be interesting to analyze the interaction between this virus infection and tsetse's other associated microorganisms (*Wolbachia, Sodalis, Wigglesworthia* and trypanosome) in different tsetse species.

Dissection of tsetse-microbial associations towards vector and disease control.
Microbial organisms are important during all tsetse life stages. They also impact vectorial capacity and efficiency of SIT programs. We seek to resolve four key questions related to tsetse's microbial community:

- Can the elucidation of tsetse-trypanosome molecular interactions help develop methods and approaches to reduce the transmission of trypanosomes by tsetse?

- Can tsetse symbionts be used to develop novel vector and disease control tools, complementary to the SIT?

- Can the characterization of tsetse symbiome and viral pathogens improve the efficacy of SIT?

- Are tsetse symbionts affected by radiation?

These questions are expanded in the following paragraphs.

1. Tsetse-trypanosome molecular interactions
The transmission of the major medically and veterinary important trypanosome species (*T. brucei* ssp., *T. congolense* and *T. vivax*) relies on the specific biological relationship between the parasites and the blood feeding the tsetse fly. Indeed, depending on the trypanosome species, the parasite has to go through an obligatory developmental cycle that varies from a short cycle in the mouthparts of the fly (*T. vivax*) to a longer, more complex life cycle in the tsetse fly midgut and mouthparts (*T. congolense*) or the midgut, mouthparts and salivary glands for the *T. brucei* species. For both *T. congolense* and *T. brucei*, the molecular interplay at different developmental stages in the fly will determine if the parasite develops to it's final infective stage. The elucidation of these interactions is essential to understand the determinants of tsetse vector competence for a given trypanosome population and how they can be affected. High resistance to trypanosome infection has been demonstrated both in lab lines and natural populations. Understanding the genetic basis of tsetse's resistance (vector competence) will help to develop tools to enhance refractoriness of tsetse to trypanosome infection.

2. Tsetse microbiota
Laboratory colonies of tsetse flies may harbour three distinct, maternally-transmitted bacterial endosymbionts and a virus. Additionally, field-captured flies are colonized with a taxonomically complex microbiota acquired from their environment. Gut microbiota has been shown in other systems to prevent establishment and/or transmission of pathogens. Thus, acquiring a better understanding of the symbionts and pathogens present in the tsetse vector, and the physiology that underlies their interactions, may allow us to manipulate tsetse flies so that they exhibit increased refractoriness to trypanosome infection.

3. Symbiotic organisms and novel control tools
The Incompatible Insect Technique (IIT), which is based on the mechanism of *Wolbachia*-induced cytoplasmic incompatibility, has been successfully tested for the population control of the agricultural pests, the Mediterranean fruit fly (*Ceratitis capitata*) and the olive fly (*Bactrocera oleae*), under laboratory conditions. Also, *Wolbachia*-induced CI has been successfully used as a driving mechanism of desirable traits in mosquito *Aedes aegypti* populations under laboratory and field conditions in Australia. Such approaches should be considered, alone and/or in conjunction with the SIT, for the control of tsetse flies and trypanosomosis.

At present, SIT has been proven effective in eradicating isolated tsetse populations. This method relies on the massive release of sterile male tsetse flies resulting in a temporary increase in the number of potential trypanosome vectors in the release zones. As such, the use of tsetse flies with a trypanosome-resistant phenotype would render this SIT approach much less controversial vector control strategy.

The tsetse fly commensal bacterium, *Sodalis glossinidius*, is ideally suited for its use in paratransgenesis because: 1) it resides in different tsetse tissues (midgut, haemolymph, salivary glands) that are in close proximity to the pathogenic trypanosomes; 2) it can be cultured and genetically modified *in vitro*; 3) transformed *Sodalis* can be re-introduced into the tsetse; 4) *Sodalis* bacteria are maternally transmitted to the offspring; and 5) due to large-scale gene erosion, *Sodalis* is metabolically dependent on its tsetse host niche, suggesting that this bacterial symbiont is a safe candidate for the use in paratransgenic strategies. A key component is the identification of anti-trypanosome targets as effector transgenes. The tsetse-trypanosome interaction research proposed in this CRP will provide several potential candidates as anti-trypanosome effector molecules to be delivered by the *Sodalis* bacterium.

4. Effects of radiation on symbionts and pathogens

Before their release into the field for SIT, tsetse fly males are irradiated to render them sterile. It can be assumed that this radiation treatment may alter the bacterial community of the fly creating an imbalance between the different bacterial communities in the tsetse fly gut and other organs/tissues. Hence, this could have an impact on the tsetse fly physiology and vector competence. Understanding the effects of radiation may enable us to design responses that address them in a manner that optimizes the SIT efficiency. In addition, radiation may also result in the development and isolation of mutant strains of endogenous symbiotic bacteria leading to novel insect symbiotic associations that may affect tsetse fly vector competence for trypanosome infection. Moreover, in the context of the possible application of a paratransgenic approach to enhance refractoriness to trypanosome infection in the released tsetse flies, it is essential to evaluate the impact of radiation on the transformed bacterial community.

In conclusion, the elucidation of the molecular interactions between the host, it's symbionts, and associated pathogens can have profound effects on the development and application of efficient control strategies for tsetse flies and trypanosomosis. This CRP aims at the characterization and harnessing of the tripartite association of tsetse flies – trypanosomes – symbionts in order to: (a) unravel the molecular interplay among tsetse flies, symbionts, and trypanosomes, (b) characterize tsetse microbiota under field and laboratory conditions as well as in the presence / absence of tsetse pathogens and trypanosomes, (c) develop novel, symbiont-based and SIT-compatible control tools for tsetse flies and trypanosomosis and (d) determine the effects of radiation on the tsetse symbionts and pathogens. We believe that this initiative will lead to better and more cost-effective SIT programmes against tsetse flies and trypanosomosis. A better understanding of the vector-trypanosomes-symbionts tripartite association is essential to develop methodologies that will lead to the enhancement of refractoriness of the tsetse flies to trypanosome infection.

5. Genomics and transcriptomic research

The Glossina Genome Cluster project has completed the whole genome sequencing of *G. pallidipes, G. austeni, G. brevipalpis, G. fuscipes fuscipes* and *G. palpalis gambiensis*. There has also been tissue-specific transcriptomes to assist with the genome assembly and annotation. There will be a network meeting planned to assist with data dissemination as well as comparative genomics analysis of the different genomes in March 2015. The genomes of the Ugandan and Ethiopian strains of GpSGHV and the MDSGHV have been completed and validated with transcriptomic analysis.

2. REVISED LOGICAL FRAME WORK

Project Design Elements	Verifiable Indicators	Means of Verification	Important Assumptions
Overall Objective: The objective of the project is to elucidate the molecular interactions between host-symbionts-pathogens to improve SIT and SIT-compatible interventions for the control of trypanosomosis by enhancing vector refractoriness to allow SIT to be expanded into areas of potential human disease transmission.	N/A	N/A	African countries continue to suffer from tsetse fly-vectored sleeping sickness in humans (HAT) and Animal African Trypanosomosis (AAT) in domesticated animals. Prevention and treatment measures remain ineffective. Drug resistance remains a problem. The increasing demand for area-wide integrated vector management approaches to control vector-borne diseases, including where appropriate the SIT as non-polluting suppression/eradication component, and SIT will be expanded into areas of potential human disease transmission. Financial and human resources are available. Novel approaches will be accepted on ethical ground.
Specific Objectives: 1. To decipher the molecular interplay between vector-symbiont-trypanosomes	1. Mechanisms and molecules that cause parasite resistance identified in one model species.	1. Reports and publications.	1. Tools and experimental models for functional research are available.
2. To characterize	2. Diversity and	2. Scientific	2. Biological (field and

Project Design Elements	Verifiable Indicators	Means of Verification	Important Assumptions
tsetse symbiome including gut microflora.	composition of tsetse symbiome identified	reports and peer reviewed publications.	laboratory) material and next generation sequencing and bioinformatics tools are available.
3. To determine the effect of radiation on vector symbiome	3. Effects of radiation on vector symbionts assessed.	3. Scientific reports and peer reviewed publications.	3. Radiation services are available.
4. To develop innovative symbiont-based strategies, in conjunction with SIT, to control African trypanosomosis.	4. Symbiont-based approaches, in conjunction with SIT, developed and validated.	4. Scientific reports and peer reviewed publications.	4. Symbionts can be manipulated as tools for the control of African Trypanosomosis.
Outcomes:			
1. Molecular cross-talking between vector-symbiont-trypanosomes characterized	1. Factors and mechanisms involved in molecular interplay determined.	1. Scientific reports and peer reviewed publications.	1. Tools and experimental models are adequate and valid.
2. Vector symbionts characterized and impact on host physiology identified.	2. Vector's symbiotic partners determined and impact identified.	2. Scientific reports and peer reviewed publications.	2. Available genomics and bioinformatics tools are optimal.
3. The impact of radiation on vectors and symbionts.	3. Qualitative and quantitative changes on the experimental systems determined.	3. Scientific reports and peer reviewed publications.	3. Experimental tools are available.

Project Design Elements	Verifiable Indicators	Means of Verification	Important Assumptions
4. SIT used in areas with potential human sleeping sickness transmission through the release of refractory tsetse flies	4. SIT in areas of potential human sleeping sickness adopted.	4. Projects implemented.	4. Technology adopted
Outputs: 1.a. Molecular interplay of *G. m. morsitans*-pathogens-symbionts characterized.	1.a. Impact of pathogen and/or bacterial symbiont infection in relation to immunity, sex, behaviour and fitness characterized.	1.a. Reports and peer reviewed publications.	1.a. Molecular tools and experimental models available.
1.b. Comparative genomics and transcriptomics of *Glossina* species and outgroups performed to elucidate vector competence.	1.b. Immunity relevant genes identified	1.b. Reports and peer reviewed publications.	1.b. Funding, molecular tools and experimental models available.
1.c. Factors that affect *T. vivax* infections in tsetse flies determined.	1.c. Detecting field populations of *T. vivax* in SIT-target species	1.c. Reports and peer reviewed publications.	1.c. Molecular tools available.
2a. Symbionts of multiple tryp infected and uninfected tsetse species and hybrid colonies determined.	2a. Symbionts in laboratory and field populations identified and characterized.	2.a. Reports and peer reviewed publications.	2.a. Biological material, genomics and bioinformatics tools are available.

Project Design Elements	Verifiable Indicators	Means of Verification	Important Assumptions
2b. Interaction of trypanosome and symbiome in model tsetse species and hybrid colonies determined.	2b. Bidirectional effects of the symbiome and trypanosomes characterized.	2.b. Reports and peer reviewed publications.	2.b. Biological material, genomics and bioinformatics tools are available.
2c. Impact of viral pathology on the tsetse symbionts .	2c. Symbionts of SGHV symptomatic and asymptomatic tsetse hosts characterized.	2c. Reports and peer reviewed publications.	2c. Biological material, genomics and bioinformatics tools are available.
3a. The effect of radiation on tsetse vectors, their symbionts and pathogens determined.	3a. Impact of irradiation in mass-reared species determined.	3a. Reports and peer reviewed publications.	3a. Irradiation damages hosts, symbionts and pathogens in a dose and species-specific manner.
3b. The mutagenic effect of radiation on paratransgenesis.	3b. Effect in at least 1 target species assessed.	3b. Reports and publications	3b. Paratransgenesis developed.
4a. *Wolbachia*-based population suppression and/or replacement strategies assessed.	4a. CI in target species characterized and *Wolbachia* impact on mating behaviour assessed.	4a. Reports and publications	4a. *Wolbachia* is amenable to characterization by classical genetic and molecular approaches.
4b. Develop parasite resistant paratransgenic tsetse.	4b. Anti-trypanosome molecules identified and expressed in paratransgenic tsetse.	4b. Reports and peer reviewed publications.	4b. Symbiont-based trypanosome control strategies can be developed against target vector species.

Project Design Elements	Verifiable Indicators	Means of Verification	Important Assumptions
ACTIVITIES: 1. Submit CRP proposal.	1. Consultants meeting held October 2011.	1. Report of Consultants Meeting and CRP proposal prepared.	1. CRP proposal approved by IAEA committee.
2. Announce project amongst established vector entomologists, trypanosome biologists, virologists and symbiologists	2. CRP announced, and research contracts and agreements submitted, evaluated and forwarded to IAEA committee.	2. Issued contracts and agreements.	2. Suitable proposals submitted and approved by IAEA committee.
3. Organize first RCM to plan, coordinate and review research activities	3. 1st RCM held.	3. Working material printed and distributed for 1st RCM.	3. Research activities started. Reports published and distributed following each RCM.
4. Carry out R&D.	4. Research carried out by contract and agreement holders.	4. Reports and publications.	4. Renewal requests and continued funding of RCM's and CRP.

Project Design Elements	Verifiable Indicators	Means of Verification	Important Assumptions
5. Second RCM to analyse data and draft technical protocols as required	5. 2nd RCM held.	5. Working material printed and distributed for 2nd RCM; Research published in scientific literature and disseminated to member states and scientific community.	5. Research activities continue, progress satisfactory.
6. Continue R&D.	6. Research carried out by contract and agreement holders.	6. Reports and publications.	6. Renewal requests and continued funding of RCM's and CRP.
7. Review the CRP after its third year.	7. Mid-CRP review carried out.	7. Report of mid-CRP review.	7. Mid-CRP review by Agency committee is positive.
8. Convene third RCM to evaluate and standardize protocols.	8. 3rd RCM held beginning of June 6-10, 2016 in Lyon, France.	8. Working material printed and distributed for 3rd RCM; Research published in scientific literature and disseminated to member states and scientific community.	8. Research activities continue, progress satisfactory.

Project Design Elements	Verifiable Indicators	Means of Verification	Important Assumptions
9. Hold workshops on "Microbial bioinformatic methods and *in silico* methods and fluorescence microscopy."	9. Workshop held May 30th to June 5, 2016 in Lyon, France. Harmonized procedures and trainees capable of implementing novel techniques	9. Workshop report.	9. There is need for training; techniques, equipment and instructors are available.
10. Continue R&D.	10. Research carried out by contract and agreement holders.	10. Reports and publications.	10. Renewal requests and continued funding of RCM's and CRP.
11. Hold final RCM to review data and reach consensus.	11. Final RCM held.	11. Final CRP report.	11. Research and dissemination activities concluded.
12. Evaluate the CRP and submit evaluation report.	12. CRP evaluation carried out.	12. CRP evaluation report.	12. CRP evaluation by Agency committee is positive.
13. Summarize and publish advances of CRP in a series of joint publications.	13. CRP members submit papers summarizing activities.	13. Publication in scientific literature.	13. Manuscripts accepted and published.

3. INDIVIDUAL WORK PLANS FOR THE NEXT 18 MONTHS

Activities / Names	Drion Boucias	Imna Malele	Khosi Motloang	Jean Maniania	Gisele Ouedraogo	Just Vlak	Florence Wamwiri	Jan Abeele	Serap Aksoy	Flobert Njokou	Abdelaziz Heddi	Anna Malacrida	Peter Takac	Martin Kaltenpoth	Sorge Kelm	Fleur Ponton	George Tsiamis	Alem Berihu	Marc Ciosi	Wolfgang Miller	Ikbal Agah Ince	Anne Geiger	Astan Trqore
Mechanism of trypanosome resistance in model tsetse identified								x	x						x				X				
Comparative genomics of different Glossina species and outgroups related to immunity									x		x	x					x			x			
Comparative transcriptomics of *Glossinia* in response to trypanosomes								x	x											x			
Role of symbionts in trypanosome transmission		x	X		x		x	x	x	x					x	x		x	x	X	x	x	x
Male accessory gland (MAG) function in reproduction (behavior)									x		x	x	x								x		
Role of symbionts in MAG function (mating behavior)									x		x	x	x								x		
Symbiome of infected and wild type (WT) lab and field flies	x		x		x	x	x		x	x		x	x			x	x	x	x	x	x	x	x
Symbiome parasite interaction in lab, WT and hybrid lines							x	x	x		x			x				X		x			
SGHV and microbiome interaction (pathology)	x	X		x		x			x			x	x							x			
SGHV functional genomics and diversity studies	x				x	x												x		x			
Effect of radiation on tsetse and partners							x				x			x			x			x			
Mutagenic effect on paratransgenesis								x													x		
Wolbachia CI identification									x	x		x	x							x		x	
Wolbachia impact on mating behavior									x			x	x	x						x			
Antityrpanosomal products			x			x									X						x		
Paratransgenic expression						x									X						x		
Symbiont transmission mechanism								x	x		x	x	x			x		x		x	x	x	X
Repository of reference materials															X				x				
Comply with export regulations																							

NICETT (National Institute for Controlling and Eradication of Tsetse and Trypanosomosis)
Alem Berihu
Collaborators: Solomon Mekonnen, S. Aksoy, A. Malacrida, A. Geiger, F. Njiolou, and I. Malele and Seib

Within the tsetse flies, except for the three well known symbionts, the presence of other bacteria in their gut is poorly documented. Acquiring a better understanding of other microbes present in the tsetse vector, and the physiology that underlies their interactions may allow us to manipulate tsetse flies so that they exhibit increased refractoriness to trypanosome infection. By utilizing different microbiological and molecular methods, our research will identify and characterize microbes associated with G. *pallidipes* and G. *fuscipes* and their impact on the performance of the flies in colony development in the Kality mass rearing center, Ethiopia. *G. pallidipes* and *G. fuscipes* colonies will be collected from Kality insectary. Flies will kept at 23–25°C with 75%-80% RH and fed on defibrinated and irradiated bovine blood. The flies will be chilled and then sterilized (once with 5% sodium hypochlorite and twice with 70% ethanol). The midguts of each fly will be dissected. The microbiological and PCR techniques will be conducted in the National Animal Health Diagnostic and Investigation Center (NAHDIC), which is a National and East African referral laboratory.

Prince Leopold Institute of Tropical Medicine, Belgium
Jan Van Den Abbeele
Collaborators: S. Kelm, J. Vlak, Seibersdorf lab, S. Aksoy, W. Miller, Seibersdorf lab.

The current *Sodalis* expression system will be optimized in order to obtain higher levels of active nanobodies (Nbs), especially in the tsetse fly midgut. The selected anti-*Tbb* procyclic Nbs will be evaluated for their activity against *T. brucei* procyclics in the tsetse fly midgut. The *Sodalis* expression system will also be used to express *T. congolense* trans-sialidase and its variants, candidate proteins to interfere with trypanosome development in the tsetse fly midgut. Moreover, the existing anti-*T. brucei* procyclic Nb-library will be screened for Nbs that bind to recombinant *T. brucei* trans-sialidases. Since an efficient mother-to-offspring transmission of the transformed *Sodalis* is a key issue to establish a sustainable paratransgenic tsetse fly colony, we will continue to optimize this transmission and study the biological mechanism that is driving this vertical transfer of *Sodalis*. The effect of irradiation on transformed GFP-*Sodalis* in the tsetse fly (bacterial survival, GFP expression levels) will be assessed. We will evaluate the ability of hybrid (*GmmxGmc*) flies and paratransgenic tsetse (expressing anti-procyclic Nbs) to establish a *T. brucei* infection. We will continue our study on the impact of a *T. brucei* infection on the salivary gland environment through transcriptomic and proteomic comparison of infected versus uninfected flies. An anti-*T. congolense* procyclic Nb-library will be generated and nanobodies will be selected for their binding affinity and lytic activity towards the parasite. If feasible, a Nb-library will be generated against the different symbionts (*Wolbachia*, *Sodalis*, *Wigglesworthia* and SGHV). The collaboration with J. Vlak will be continued in regarding the role of RNAi in the control of GpSGHV expression.

Laboratory of Virology, Wageningen University, The Netherlands
Just M. Vlak
Collaborators: (i) H. Kariithi, I.A. Ince, J. van den Abbeele, D. Boucias, S. Aksoy, Seibersdorf lab. (ii) I. Malele, F. Wamwiri, O. Koekemoer, F. Njioku and T. Astan, Seiberdorf lab.

The major objectives are: (i) to understand the control mechanism of GpSGHV latency, replication and expression in symptomatic and asymptomatic tsetse flies, and (ii) to understand the genetic diversity of SGHV in *Glossina* spp. over Africa.

(i) GpSGHV seems to be present in many Glossina spp. in an asymptomatic state, i.e. the virus is there either, integrated in the host genome, as an episome in the host nucleus, or as a low-replicating virus. The *Glossina pallipides* genomes will be screened for the presence of hytrosavirus(-like) genes. Tissues (salivary glands, milk glands, ovaries, fat bodies) of symptomatic and asymptomatic *G. morsitans morsitans* and *G. pallidipes* flies will be screened for GpSHGV expression (transcription) by (deep) RNA sequencing. The role of RNAi in the control of GpSGHV expression will be further investigated, including revealing the counter-defense mechanism of the virus. Asymptomatic flies (both model species) will be treated with RNAi (Dicer-2, Ago-2) in the F0 and F1 to block the RNAi response and GpSGHV replication, expression and symptom formation will be monitored. These experiments would indicate whether RNAi is involved in the asymptomatic state of GpSGHV in. Alternatively, if RNAi is not involved, cues may emerge from the transcript analysis whether GpSGHV and host genes are involved in maintaining the virus in an asymptomatic state..

(ii) The diversity of GpSGHV in the field is important for evaluating the potential for GpSGHV evolution and its contribution to refractoriness for trypanosomes. The first task is to identify and characterize SGHVs from the various *Glossina* species, initially using the sequence of the standard PCR product, if need be variable genes or repeat regions (output: paper). The second task is to select, on the basis of comparison of Uganda and Ethiopia SGHV genome, more divergent genes (as opposed to the very conserved genes used previously) and develop a nested PCR and sequence analysis to detect genetic variation. Sequences from as many *Glossina pallidipes* throughout Africa will be screened for the presence of GpSGHV and the diversity determined (output: paper). When the different *Glossina* spp. represent separate GpSGHV lineages, the analyses would reveal the overall genetic variation and possible evolution of GpSGHV in tsetse flies in Africa.

Vector & Vector Borne Diseases Research Institute (VVBDRI) – Tanga, Tanzania
Imna Malele
Collaborator: F. Wamwiri, Marc Ciosi, A. Geiger, A. Malacrida, S. Aksoy, J. Maniania, P. Takac and Seiberdorf.

At VVBDRI the following will be conducted in relation to Enhancing tsetse fly refractoriness to trypanosome infection within the 18-month period. First, we will investigate on gut microbiota and trypanosomes interaction and their role in the epidemiology of trypanosomiasis in wild tsetse species. We will continue with research on the interaction of SGHV with other gut microbiota from *Glossina pallidipes* and *G. m. morsitans* and from any other sympatric tsetse species found in the trapping area. This time we will also focus on sequencing the symbionts, the viruses and other gut microbiota isolated from field populations. These studies will aim at comparing the gut microbiota, viruses and trypanosomes from HAT and non-HAT endemic areas. Lastly, we will investigate on the use of entomopathogenic fungi (*Metarhizium anisopliae* isolate ICIPE 30) as a tool for tsetse and trypanosomes control.

ICIPE, Kenya
Fathiya Khamis
Collaborators: N.K. Maniania, J. van den Abbeele, P. Takac and I. I. Malele.

Entomopathogenic fungi (EPF) infect their target organisms through the cuticle and are being considered as potential biological control agents of tsetse flies. In addition to killing the host, sublethal doses of EPF can interfere with feeding and reproduction behaviours. In addition, EPF can affect the development and transmission of the malaria parasite in mosquitoes, and reduce replication and transmission of Iris Yellow Spot Virus in (IYSV) *Thrips tabaci*. We therefore hypothesize that infection of tsetse fly by EPF may reduce parasite acquisition, inhibiting its

multiplication and development, and transmission by the vector. In the first year, we intend to investigate the effect of infection of *G. fuscipes*/*G. pallidipes* by *Metarhizium anisopliae* on the development of *Trypanosoma brucei* and *T. congolense* in fly. Colonies of *G. fuscipes* and *G. pallidipes* at ICIPE will be boost. In case the number is not adequate, arrangement will be made with Dr. Peter Takac for regular supply of flies. *T. congolense* and *T. brucei* procyclics will be cultured in vitro as described by Bosompem et al. (1996) or on mice. In order to optimize infection of flies by parasites, collaboration is sought with Ian who will provide us with glutathione (antioxidant). *M. anisopliae* isolate ICIPE 30 will be used in this study. Trypanosome infected flies will be exposed to fungal infection and at 3, 5, 7 and 14 days post fungal infection, flies will checked for the developmental stages of the parasite. In addition to microscopic examination, qPCR will be used. Process slides prepared for fluorescence microscopy to determine whether there is coexistence between *Metarhizium* hyphal bodies and *Trypanosoma* in the hemolymph of infected flies to elucidate mechanisms underlying the interactions between the fungal pathogen, the vector and the parasites. Carry out experiments on the effect of fungal infection by *M. anisopliae* on the development of *T. congolense* in *G. pallidipes* and the refractoriness of fungus-infected flies to acquire and transmit the parasite. Investigation will also be carried out to identify potential antibodies that can be used for fungal propagules in fly.

University of Glasgow, UK
Marc Ciosi
Collaborators: I. Malele, F. Wamwiri, Seib

We will complete our investigation of potential correlations in the presence/absence of several trypanosome species (*T. brucei, T. congolense, T. vivax*) and clades of *T. congolense* (Savannah and Kilifi) in sympatric populations of tsetse of multiple species. This interspecies comparison will elucidate the extent to which particular species/clades of trypanosomes are differentially transmitted by particular species of tsetse. Trypanosomes will be identified to species level by ITS1 PCR, and positive samples then allocated to clade with clade-specific PCRs. As part of this study we will:
-      Perform blood meal analysis on trypanosome infected and uninfected flies to investigate the correlations between trypanosome presence, tsetse species and the host species available.
-      Investigate the correlations of trypanosome presence/absence with that of the symbiont *Sodalis glossinidius*.

A within-species analysis of genetic diversity will complement this between-species comparison. We will sequence some of the trypanosome ITS1 bands obtained to confirm the infection of the flies by trypanosomes and better characterize the trypanosome genetic diversity.
To evaluate intraspecific diversity we will also complete the development of multiplex microsatellite PCRs to genotype *T. congolense savannah*.

Laboratoire Central Vétérinaire de Bamako , Mali

Mme Astan Traore
Collaborators: Dr Bass, Ouédraogo **Gésèl Sanou**, Adly Abda A., D.G. Boucias.

The proposed research aims to decipher the relationships between tsetse and pathogen as a component of the sterile male release technique program. To date, sampling has been conducted in Mali where trypanosomes are prevalent. A total of 307 flies have been collected from 3 locations of Mali. The flies screened of *Glossina palpalis gambiensis* indicate an average prevalence of SGHV virus at 4.88% (15/307). The average prevalence of *Wolbachia* was 3.58% and the average prevalence of *Trypanosoma* spp was 4.23%

We will extend tsetse collections to include others regions of Mali. Collect flies along streams, using biconical traps (Charlier - Laveissiere). Each fly will be identified by sex and by species. After counting and diagnosis, all fresh tsetse be dissected the different organs (proboscis, salivary glands, midgut) will be examined under a microscope to search for possible infections of SGHV and *Trypanosoma spp*. Additional flies will be sorted by species and subsamples of 50 flies will be transferred into tube containing 15-ml of 95% ethanol for the future analysis.All samples collected will be analysis using the PCR specific primers to indicate the prevalence of SGHV*, Trypanosoma sp, Wolbachia* and *Sodalis*.The DNA will be extracted from caught flies using the method of Bender and al. (1983). PCR reactions will be performed and PCR product will be detected with agarose (2%) gel electrophoresis and ethidium bromide staining.

The specific activities planned in the next 16 months are:
- a. Collect samples:
- b. Determine the prevalence of *Trypanosoma spp* and SGHV by microscopy;
- c. Determine the prevalence of *Trypanosoma spp* and SGHV and others symbionts by PCR.
- d. Correlate the symbiont levels with presence/absence of *Trypanosoma*.

KALRO - Biotechnology Research Institute
F. Wamwiri
Collaborators: Aksoy, S., M. Ciosi and **Seibersdorf Laboratory**

We shall continue to investigate the effect of *Sodalis* infection on trypanosome establishment in both field and laboratory populations. Laboratory infections shall be performed using *G. morsitans* and *G. pallidipes* and different trypanosome isolates. Further, sequencing of *Sodalis* strains in trypanosome-infected flies shall be done. In our current work, we have recorded about 35% *Sodalis* infection in the *G. pallidipes* colony. To assess the possibility of low density infections that cannot be detected by our standard PCR assay, we shall conduct quantitative assays on these flies. We shall also assess the age structure of wild flies infected by *Sodalis*.

University of Florida, Gainesville, Florida, USA
D. Boucias
Collaborators: Abd-Alla, A, Geden, C. Vlak,J., İkbal Agah İNCE

The overall goals of our research is to further understanding of the SGHV pathology and the impact of infection on host fitness. Specific experiments will include the following topics:
1. Continue histological examination (TEM) of *G. pallipides* tissues dissected from asymptomatic and symptomatic flies in order to localize and image the interaction occurring between the viral and bacterial symbionts.
2. RNAseq data set generated from SGHV infected *Musca domestica* will be examined for additional functional assays targeting up- and down-regulated pathways. Emphasis will be placed on the significant alterations in the innate defence systems, and the down regulation of the pathways associated with oogenesis.
3. Conduct additional RNAseq analysis on symptomatic and asymptomatic *G. pallipides* to develop a comparative analysis of the impacts of SGHV on the tsetse versus housefly transcriptomes.
4. Assess the impacts of antiviral drugs, shown to be active on the GpSGHV infection, on the MdSGHV replication in *M. domestica*.
5. Based on present transcriptomic data we will examine the cuticle hydrocarbon profiles of healthy verses infected flies with an emphasis on those chemistries that serve as contact pheromones.

Department of Medical Microbiology, School of Medicine, Acibadem University, İstanbul, Turkey
İkbal Agah İNCE
Collaborators: H. Kariithi, M. Bergoin, J. van den Abbeele, D. Boucias, S. Aksoy and Seibersdorf laboratory.

The identification of expressed genes of viral entities is the main issue in recent genomic era. Expressomics, an integrated approach applying transcriptome and proteome outputs for data interpretation for *Glossina* hytrosavirus (GpSGHV) pathobiology will be applied. Determination of the 3-prime untranslated regions (UTRs) have been performed following to this progress exploring the conserved mechanisms related to viral pathogenesis will be undertaken. In addition, an integrated approach using transcriptome and proteome of infected cells performed to further knowledge on interactions between different tsetse isolates and the GpSGHV isolates are comparatively analyzing currently by generation of genome and proteome maps. Knockdown of host protein factors related to viral infection will be investigated. As there is no available genetic recombination system for host as well as for virus (e.g. bacmid), *in vitro* dsRNA production will be used for generating knockdowns of target genes for clarifying the transcriptional regulation of this new group of virus (Hytrosavirus). Production of genetic recombination systems as bacmids, will greatly enhance progress of this research proposal. The ultimate aim is to unravel the key virus-host factors responsible for viral infection and latency. This research will serve for the establishment of the effective mitigation strategies for tsetse rearing factories.

Centre for Biomolecular Interactions, Faculty for Biology and Chemistry University Bremen:
Soerge Kelm, Jonathan Andrew Nok
Collaborators: Jan Van Den Abbeele, all groups sampling flies and groups analysing symbiome

The present research aims a better understanding of the role of trans-sialylation in the transmission and colonisation of trypanosomes between insect vector and mammalian host. With in the CRP the focus will be on evaluating the potential of sialidases expressed in the symbiome to prevent and/ or eradicate trypanosomal colonisation and maturation in tsetse flies.
The following activities are planned for the next 18 months:
- screen field tsetse fly midgut samples collected for sialidase activity using the enzyme assay established in the first part of the CRP
- provide samples for virus and symbiont diversity studies in CRP
- screen existing bacterial isolates from tsetse midguts for sialidase activity
- develop expression system for trans-sialidase variants in *Sodalis*, evaluate the secretion of active enzyme
- investigate the impact of TS expression on *Sodalis* biology in culture and in Tsetse flies
- analyse trans-sialidase diversity in field samples
- explore the potential of TS genes to identify *T. vivax* in field samples
- prepare TS as antigens for nanobody screening and characterize epitopes for nanobodies obtained

University of Patras, Agrinio, Greece
Eva Dionyssopoulou and George Tsiamis
Collaborators: P. Takac, V. Michalkova, S. Aksoy and Seib

We will be using populations from *Glossina morsitans morsitans*, *Glossina pallidipes*, *G. fuscipes fuscipes*, and *G. palpalis gambiensis* to detect and characterize reproductive parasites like *Spiroplamsa*, *Rickettsia*, *Arsenophonus* and *Cardinium*. The symbiotic bacterial diversity of the gastrointestinal tract (GT) and of the reproductive tissue (ovaries and testes) will be characterized by adopting a 16S pyrotagging approach, using mass rearing and natural populations of *G. m. morsitans*, *G. pallidipes*, *G. f. fuscipes* and *G. p. gambiensis*. The location of *Spiroplasma* at different stages of insect development will be determined using in situ Hybridization (FISH). A culture-dependent approach will be used in order to isolate the *Spiroplasma* strain found in *G. fuscipes fuscipes*, *G. tachinoides*, and *G. p. palpalis*. Finally, *in silico* analysis will be deployed to: (a) assembly the *Spiroplasma* genome found in *Gff*, and (b) examine Horizontal Gene Transfer events between in different *Glossina* species and outgroups.

Laboratoire de Recherches et de Coordination sur les Trypanosomoses, France
Anne Geiger
Collaboration:F. Njiokou and Seib.

Tsetse flies transmit African trypanosomes responsible for sleeping sickness in humans and Nagana in animals. This disease, fatal when untreated, affects many people with considerable impact on public health and economy in sub-Saharan Africa. The available drugs are inefficient and have induced trypanosome resistance. Therefore the investigations for novel strategies must continue, among them are alternative vector-based strategies such as the engineering of insects capable of blocking the transmission of the parasite. Field-captured flies are colonized with symbionts and a taxonomically complex microbiome acquired from their environment. In other insects, such as *Anopheles* sp., the gut microbiome was shown to prevent establishment and/or transmission of pathogens.

In the light of the new IAEA-CRP, our project will aim at bringing knowledge regarding the interactions between the tsetse fly *G. m. submorsitans*, trypanosomes and symbionts (*Sodalis* and *Wolbachia*) and microbiome going on in African Trypanosomiasis focus, Adamaoua, in Cameroon.

Practically, this project will involve entomological survey, in endemic area of Trypanosomiasis in Cameroon, with specific PCR identification of trypanosomes, symbionts and microbiome. We have first screened limited number of samples and identified high bacteria diversity using metataxogenomic analyses. We have also isolated the bacteria using hungate tubes
Planning :
      Phase 1 : larger entomological prospections and tsetse fly sampling
      Phase 2 : identification of the *Glossina* species; evaluation of trypanosome prevalence
      Phase 3 : complementary entomological prospections and sampling
      Phase 4 : bacteria isolation in Hungate tubes for the next samples
      Phase 5 : characterization of the bacteriome of the next samples using metataxogenomic analysis

Max Planck Institute for Chemical Ecology, Research Group Insect Symbiosis, Jena, Germany
M. Kaltenpoth and T. Engl
Collaborators: S. Aksoy, J. Van Den Abbeele, W. Miller, A. Malacrida, V. Michalkova, P. Takáč, and Seibersdorf laboratory

In the proposed project, we aim to investigate the impact of the bacterial symbionts *Wigglesworthia, Sodalis*, and *Wolbachia* as well as the trypanosome parasites on cuticle hydrocarbons (CHC) profiles of *Glossina morsitans morsitans* and their possible influence on sexual selection and mate

choice. We will use gas chromatography coupled to mass spectrometry (GC-MS) as a rapid tool to analyze CHC profiles of tsetse flies after artificial perturbations of the host-symbiont-parasite equilibrium. Our collaborators at Yale University, Serap Aksoy, Veronika Michalkova and Peter Takáč have established procedures to cure flies of *Wigglesworthia* only (by ampicillin treatment) or of the complete symbiotic bacterial community (by tetracycline treatment) and subsequently rear the flies successfully on artificial diets. Jan Van Den Abbeele (Institute of Tropical Medicine, Antwerp) can experimentally manipulate *Sodalis* and *Trypanosoma* infection in *G. m. morsitans*, Wolfgang Miller and Daniela Schneider (Medical University of Vienna) have established procedures to manipulate *Wolbachia* titers via depletion by antibiotics or overreplication by hybrid introgression, and Adly Abd-Alla can provide SGHV-symptomatic and –asypmtomatic as well as irradiated flies. We plan to characterize CHC profiles of male and/or female *G. m. morsitans* with different symbiont/parasite/SGHV infection status to assess whether the microbial community affects chemical profiles in *G. m. morsitans*. These experiments will allow us to get first insights on whether symbionts and/or parasites via changes in chemical profiles may influence male mating behaviour in *G. m. morsitans*, or female mate choice decisions based on male CHCs, and they will thereby provide a baseline for behavioural analyses of the mating behaviour and sexual selection in this species.

Department of Biology and Biotechnology, University of Pavia, Pavia, Italy
Anna Malacrida
Collaborators: S. Aksoy, I. A. Ince, A. Heddi, P. Takac, V. Michalkova, Seibersdorf laboratories

Our research activities will center on the analyses of the impact of *Wolbachia* and SGH virus on the reproductive behaviour of *Glossina* male. In particular, we are interested in the analyses of the impact of *Wolbachia* and SGH virus on the molecular machinery controlling accessory gland proteins (MAG) and sperm formation and transfer. These analyses will be important to understand the effect of *Wolbachia* on the CI expression and SGH on male sterility. .Presently, we have generated six libraries from testes, accessory glands from *Wolbachia* infected males from *Glossina morsitans morsitans*: teneral, 3 day old virgin /mated males with the help of IAEA support. We have data on the categories of transcripts that are responsive to sexual maturation and post mating. We will perform proteomic analysis of spermatophora to detect the proteins which are transferred to the females. We will create libraries from testes and accessory glands from *Wolbachia* free *Glossina morsitans* males. This will allow a comparative analyses in relation to the transcripts /proteins which are present/absent in *Wolbachia* infected male MAGs. We will similarly create libraries from MAGs from SGH males. We will study the mating behaviour of *Wolbachia* infected/non infected males. We will analyse the tissue localization of *Wolbachia* in testes and accessory glands from males (different ages) and in sperm storage organs of females. We will investigate the effect of polyandry and migration rate on the persistence and spreading of *Wolbachia* in *G. pallidipes* populations. Collectively our results can also provide species-specific genetic markers as well as Y-specific markers.

Institut National des Sciences Appliquées de Lyon (INSA-Lyon), Villeurbanne, France
Abdelaziz Heddi
Collaborators: Aksoy, W. Miller, A. Malacrida, Adly Abd-Alla, and Seib

The Tsetse flies harbour three symbiotic bacteria namely *Wigglesworthia*, *Wolbachia* and *Sodalis*. Their mode of transmission and their location in the insect tissues were investigated during the last CRP program (Balmand et al., 2013, JIP), but several features are unresolved. Significantly, how

these three symbiotic bacteria are perceived and controlled by the host immune system remains questionable, although S. Aksoy lab has made impressive advance on this field.

The purpose for the next 18 months is:

- To pursue the precise location of the tsetse endosymbionts in the tsetse somatic and germ tissues at different stages of insect development, by using *in situ* Hybridization (FISH) methodology that allows the specific screening of bacteria through the design of specific 16S rDNA probes,
- To pursue symbiont "behaviour" following insect irradiation,
- To screen symbiont location in natural and lab tsetse flies, including Hybrid colonies,
- To perform comparative immunity between *Sitophilus* weevils and *Glossina* tsetse.

Yale School of Public Health,
S. Aksoy
Collaborators: A. Malacrida, M. Kaltenpoth, G. Tsiamis, J. Van Den Abbeele, I. A. Ince, F. Wamwiri, I. Malele, P. Takac, A. Heddi and Seib

We will work on three fronts related to the CRP goals: microbiota, parasite resistance mechanisms and comparative genomics. To date, it has been shown that insects have restricted diversity in their gut microbiomes and that the laboratory insect lines have divergent microbiota in comparison to field flies. The complexity of the microbiota of flies can further be impacted by the presence of the trypanosome parasite. We will work on the identification of the microbiota from field populations of different tsetse species infected by trypanosomes and compared with uninfected counterparts using next gen methodologies. A number of genetic barriers have been identified in tsetse (*Glossina morsitans morsitans*) that impact the transmission of trypanosomes. We will focus on understanding the role of the gut peritrophic matrix (PM) and the role of the microbiota on PM integrity as they impact parasite transmission. Finally as the genomics data from different tsetse species become available, we will work on comparative analysis on reproductive and immune system related genes. We will participate in experiments that aim to understand the male accessory gland physiology and also role of symbionts on host behaviour.

University of Yaounde I, Cameroon
Flobert NJIOKOU
Collaboration: A. Geiger, A. Berihu and Seib.

Tsetse flies transmit African trypanosomes responsible for sleeping sickness in humans and Nagana in animals. This disease, fatal when untreated, affects many people with considerable impact on public health and economy in sub-Saharan Africa. The available drugs are inefficient and have induced trypanosome resistance. Therefore, the investigations for novel strategies must continue, among them are alternative vector-based strategies such as the engineering of insects capable of blocking the transmission of the parasite. Field-captured flies are colonized with symbionts and a taxonomically complex microbiome acquired from their environment. In other insects, such as *Anopheles* sp., the gut microbiome was shown to prevent establishment and/or transmission of pathogens.

In the light of the new IAEA-CRP, our project will aim at bringing knowledge regarding the interactions between the tsetse fly *G. m. submorsitans*, trypanosomes and symbionts (*Sodalis* and *Wolbachia*) and microbiome going on in African Trypanosomiasis focus, Adamaoua, in Cameroon.

Practically, this project will involve entomological survey, in endemic area of Trypanosomiasis in Cameroon, with specific PCR identification of trypanosomes, symbionts and microbiome.

Preliminary results:
An entomological prospection was done in the Faro and Deo animal trypanosomiasis focus. *Glossina morstians submorsitans* individuals were caught. *Sodalis and Wolbachia* were identified. *Trypanosoma brucei* sl, *T. congolense* forest type and *T.congolense* savannah type were identified with very low prevalences. No association was observed between trypanosome infections and presence of the symbionts.
<ins>**Planning for next 18 months:**</ins>
- Genotyping of the identified symbionts
- Entomological prospection in others villages of the Faro and Deo focus
- Identifications and genotyping of Symbionts (*Sodalis and Wolbachia*)
- Identification of trypanosomes
- Testing the association between the symbionts, some genotypes and the trypanosomes

Medical University, Vienna, Austria
Wolfgang J. Miller
Collaborator: A. Heddi, F. Ponton, M. Kaltenpoth, S. Aksoy, V. Michalkova, P. Takac and Seib

Maintaining homeostasis between all symbiont players and tsetse flies is essential for tsetse symbiosis. Quantitative and qualitative interactions between tsetse and bacterial symbionts will be elucidated that restrict symbiont titer and tissue tropism during development and in adults. Symbiont gain- and loss-of-function strategies will be applied for artificially disturbing native host-symbiont equilibrium *in vivo*, i.e. in mixed genetic host backgrounds of inter-species hybrids, upon treatment with antibiotics and gamma-irradiation. Finally, we plan to assay potential alterations in composition of gut symbionts upon such artificial perturbations and their potential impact on host pheromonal profiles and mating success.

Specific aims within the next 18 months:
1. Monitoring and quantifying symbiont titer and tropism dynamics of *Sodalis, Wigglesworthia* and *Wolbachia* by qRT-PCR and FISH, in different age classes of (*i*) wildtype, (*ii*) antibiotic-treated, (*iii*), gamma-irradiated tsetse flies, plus (*iv*) inter-species hybrids (A. Heddi).
2. Profiling gut microbiota of (*i*) wildtype, (*ii*) antibiotic-treated, (*iii*) gamma-irradiated tsetse flies, plus (*iv*) inter-species hybrids (F. Ponton).
3. Monitoring CHC signatures of (i) wildtype, (*ii*) antibiotic-treated, (*iii*) gamma-irradiated tsetse flies, plus (*iv*) inter-species hybrids (M. Kaltenpoth).
4. Generating additional rescued hybrid colonies and analyzing dynamics and tropism of their symbionts (AH), gut microbes (FP), CHC profiles (MK), plus their mating behavior (S. Aksoy, V. Michalkova, P. Takac).
5. Generating isofemale lines from stabilized hybrid colonies (Seib) and consequently select for candidate lines, which lack assortative mating (SA, VM, PT) but induce complete postmating isolation (Seib) against parental and wildtype females.

Institute of Zoology, Slovak Academy of Sciences
Peter Takáč and Veronika Michalková
Collaborators: S. Aksoy, G. Tsiamis, A. Malacrida, M. Kaltenpoth and Seib.

The aim is to reveal maternal transmission of endosymbiotic bacteria to progeny during the larval intrauterine development. The study is focused on the bacteria and progeny molecular cross-talk, bacterial transcriptome changes in the adult female and in larvae throughout development. Bacterial gene expression during development will be related to the expression of host molecules that may modulate transmission of trypanosomes in the emerging progeny, particularly those that with anti-

trypanosomal activity. The first step is to investigate maternal transmission of symbionts to the progeny in cooperation with the other members of CRP. We will study the molecular aspects of host-symbiont dialogue during colonization and establishment in host tissues, knowledge critical for paratransgenic applications.

Simultaneously we will continue to evaluate impact of different kind of diets as substitutes for the symbionts and investigate the nutritional influence on endosymbionts. The impact of different kinds of diet (mostly different bacterial extracts and B-complex vitamins) on the gut symbiotic flora, fitness and fecundity of the flies, related also to trypanosomiasis; will be studied and compared. The study will be extended also on other species except *G. m. morsitans*.

As a mass rearing insectary facility with laboratory colony of *Glossina morsitans morsitans, G. pallidipes, G. p. gambiensis* and *G. fuscipes*; we will provide material and samples for the other CRP collaborators.

Agricultural Research Council - Onderstepoort Veterinary Institute, South Africa
Makhosazana (Khosi) Y. Motloang
Collaborators: S. Aksoy, George Tsiamis, Anna Geiger and Seib.

Glossina austeni and *G. brevipalpis* co-exist in the north-eastern parts of KwaZulu-Natal (KZN) Province of South Africa (SA). Their distribution covers an estimated area of about 18 000 km^2 and they are suspected to be the main vectors of livestock trypanosomosis in this area. From literature, it has been shown that different tsetse species show differential abilities to transmit trypanosomes. Limited data is available that suggests high vectorial capacity in *G. austeni* species compared to *G. brevipalpi* in SA. The present project aims to identify tsetse symbiomes and other microbial fauna in colonies and wild tsetse populations of South Africa, and to determine the correlation between trypanosome and microbial infections in wild populations. Colonies of *G. austeni* and *G. brevipalpis* reared at Onderstepoort Veterinary Institute (OVI) of the Agricultural Research Council (ARC), South Africa, and wild populations of the same species will be used. Field collections, dissections of colony and field flies and preservation of organs for DNA analysis will be carried out at OVI. Use of the protocol on isolation of bacteria in Hungate tubes will be discussed with Anne Geiger. Trypanosome infections will be determined using KIN and species-specific PCR. The symbiotic bacterial diversity of the gastrointestinal tract (GT) and of the reproductive tissue (ovaries and testes) will be characterized by adopting a 16S pyrotagging approach, using mass rearing and natural populations. Statistical analysis will be conducted to compare symbionts and microbial communities between colony and trypanosome-infected wild flies and their uninfected counterparts. This data, if adequate, could be used to determine if any species of bacteria can inhibit natural infections of *G. austeni* and *G. brevipalpis* with trypanosomes.

4. Recommendations

1- Specify standardized markers to detect and quantify the symbiome components.

2- Develop a website to provide protocols, data sets, and published material, and to facilitate communication among CRP participants.

3- Develop standard protocols for the sample collection, handling, and processing.

5. AGENDA

SECOND RESEARCH CO-ORDINATION MEETING

JOINT FAO/IAEA DIVISION OF NUCLEAR TECHNIQUES IN FOOD AND AGRICULTURE

"Enhancing Vector Refractoriness to Trypanosome Infection"

Addis Ababa, Ethiopia

1 -5 December, 2014. Nexus hotel

AGENDA

Monday, 1st December, 2014

SESSION 1

08.00-09. 00   Registration

09.00-09.10   **Thomas Cherenet:** Welcome speech

09.10-09.20   **Adly Abd-Alla:** Introduction

09.20-09.30   Administrative details

09.30-10.00   **A. Abd-Alla**: Impact of irradiation treatment and *Glossina pallidipes* Salivary gland hypertrophy virus infection on tsetse symbiont prevalence.

10.00-10.15   COFFEE

SESSION 2

10.15-10.45   **Jan Van Den Abbeele:** Update on the *Sodalis* expression of anti-trypanosoom nanobodies

10.45-11.15   **Sorge Kelm.**: Sialic acid, trans-sialidase and sialidase in the midgut of tsetse flies

11.15-11.45   **Njiokou F**., Kame Ngasse G., Nana-Djeunga H., Melachio-Tanekou T. & Geiger A: Relation between the symbionts *Sodalis glossinidius*,

Wolbachia sp and trypanosomes hosted by *G. m. submorsitans* in the animal trypanosomiasis focus of Faro-Deo in Cameroon

11.45-12.15   Njiokou F., Jacob F., Fardeau M-L., Melachio T., Nitchouang G-R., Ollivier B., **Geiger A**.: Bacteria diversity in tsetse flies from sleeping sickness foci in Cameroon

12.15-13.00

LUNCH

SESSION 3

13.00-13.30   **Motloang M. :** Microbial diversity in tsetse in SA and its influence on vector capacity

13.30-14.00   **George Tsiamis,** Evangelos Doudoumis, Antonios A. Augustinos, Eva Dionyssopoulou, Peter Takac, Adly M.M. Abd-Alla and Kostas Bourtzis: Detection and Characterization of new bacterial symbionts in tsetse flies

14:00- 14:30   **Marc Ciosi,** Imna I. Malele, Winnie A. Okeyo, James Buckley, Barbara K. Mable, Charles M.R. Turner and Daniel K. Masiga**.**: Genetic bottlenecks in natural populations of the tsetse fly *Glossina pallidipes*: impact of vector control.

14.30-14.45

COFFEE

SESSION 4

14.45-15.15   **Solomon Meknnen:** The Status of Kaliti Colony Establishment for SIT Application

15.15-15.45

Martin Kaltenpoth, Tobias Engl, Veronika Michalkova, Brian Weiss, Daniela Schneider, Wolfgang Miller, Serap Aksoy: Influence of symbiotic bacteria on cuticular hydrocarbon profiles in tsetse flies (*Glossina m. morsitans*)

15.45-16.15

Wolfgang J. Miller and **Daniela Schneider**: Symbiont-triggered Speciation - Lessons from Tsetse Fly Hybrids

16.15-17.00 **General discussion**

Tuesday, 2nd December, 2014

SESSION 5

08:30- 09:00 **Abdelaziz Heddi**: Evolutionary and Immune Processes in Insect Endosymbiosis

09.00-09.30 **S. Aksoy**: The functional role of *Wigglesworthia* in the tsetse host

09.30-10.00 Scolari F, Benoit JB, Michalkova V, Aksoy E, Takac P, Abd-Alla A, **Malacrida AR**, Aksoy S, Attardo GM: Unravelling tsetse male reproductive machinery: the contribution of Male Accessory Glands and Testes to the spermatophore.

10.00-10.15 **COFFEE**

SESSION 6

10.15-10.45 Veronika Michalková; Joshua B. Benoit; Brian L. Weiss; Geoffry M. Attardo; Serap Aksoy; **Peter Takac**: Vitamin B6 generated by symbionts is necessary for tsetse fly proline homeostasis and fecundity.

10:45- 11:15 **Alem Berihu,** Solomon Mekonnen**:** Bacterial diversity in the Midgut of *G.pallidipes* and *G.fuscipes* from Kality Mass Rearing Center and Wild flies

11.15-11.45 **Florence N. Wamwiri,** Kariuki Ndungu, Paul C. Thande, Daniel K. Thungu, Joanna E. Auma and Raphae M. Ngure**:** Infection with the secondary tsetse endosymbiont *Sodalis glossinidius* may influence parasitism in the tsetse fly *Glossina pallidipes.*

11.45-12.15   **Fathiya Khamis and N. K. Maniania**: Effects of fungal infection on the *Trypanasoma congolense* load in *Glossina fuscipes fuscipes*'.

12.15-13.00   **LUNCH**

SESSION 7

13.00-13.30   Hamisi Nyingilili, Eugene Lyaruu**, Imna Malele**.: Micro-fauna diversity, interaction and their effects on trypanosome infection in wild savannah tsetse.

13.30-14.00   **Drion Boucias,** C.Geden and D. Nayduch **:** Studies on the Pathology of the Hytrosaviruses

14.00-14.30   **Ouédraogo / Sanon G.M. Sophie**: Prevalence of salivary gland hypertrophy virus Wolbachia and trypanosome in wild population of tsetse flies from West Africa

14.30-15.00   **Astan Traore:** Prevalence of salivary gland hypertrophy virus Wolbachia and trypanosome in wild population of tsetse flies from Mali.

15.00-15.15   **COFFEE**

SESSION 8

15.15-15.45   **Henry M. Kariithi;** Sjef Boeren; İkbal Agah İnce; Jan Van Den Abbeele; Just M. Vlak and Adly M. M. Abd-Alla : Invasion of Tsetse Salivary Glands by Trypanosomes and Hytrosavirus: Prerequisites and Control Strategies

15.45-16.15   **İkbal Agah İnce,** Adly Abd-Alla, Henry M. Kariithi: *Infectomics of Glossina hytrosavirus*

16.15-16.45   **Just Vlak,** Irene Meki, Karin de Ridder, Henry Kariithi, Jan van den Abbeele and Adly Abd-Alla: On the role of innate immunity responses on Glossina Salivary Gland Hypertrophy Virus replication in tsetse flies

16.45-17.00   **General discussion**

Wednesday 3rd December, 2014

SESSION 9

08.30-10.00

General Discussion of the Logical Framework and CRP evaluation documents

10.00-10.15

COFFEE

10.15-11.15   **General Discussion of the Logical Framework**

11.15-12.00

Working Group Discussions

12.00-13.00

LUNCH

13.00-17.00

Excursion to STEP project tsetse mass rearing facility

17:00- 21:00

Group Dinner

SESSION 10

08.30-10.00

Working Group Discussions

10.00-10.15   **COFFEE**

10.15-12.00

Working Group Discussions

12.00-13.00

LUNCH

13.00-15.00

Drafting Report

15.00-15.15   **COFFEE**

15.15-17.00

Drafting Report

Friday 5th December, 2014

SESSION 11

08.30-10.00   **Reports of Working Groups and CRP evaluation**

10.00-10.15   COFFEE

11.15-12.00   **Drafting of CRP evaluation**

12.00-13.00   LUNCH

13.00-14.30   **General Discussion**

Closing Remark by Host Country representative

Working Group 1: Symbionts
Aksoy, İnce , Heddi, Geiger, Kaltenpoth, Malacrida, Miller, Njiokou, Tsiamis, Takac, Fleur, Martin, Berihu, Khosi

Working Group 2: Pathogens
Boucias, İnce, Kelm, Malele, Khamis, Ouedraogo, Ciosi, Vlak, Van Den Abbeele, Wamwiri, Henry

6. LIST OF PARTICIPANTS

**LIST OF PARTICIPANTS TO THE SECOND RCM
ON ENHANCING VECTOR REFRACTORINESS TO TRYPANOSOME INFECTION
From Dec 1-5, 2014, Addis Ababa, Ethiopia**

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Participant did not attend the 2nd RCM in Addis Ababa, Ethiopia

7. NEXT MEETING

Location: Lyon, France -

Period: 6-10 June 2016

8. WORKSHOP

Tasks:

1) Bioinformatics approaches for microbiota profiling based on amplicon sequencing data. Proposed organiser: Georges Tsiamis and Geoffrey Attardo

2) Tissue localisation and analysis of insect endosymbionts by fluorescence microscopy: Theoretical background and practical applications. Proposed organisers: Abdelaziz Heddi, Severine Balmand, Martin Kaltenpoth

Proposed Location: Lyon, France

Date: In conjunction with the 3rd RCM from 30 May to 04 June 2016.

Numbers of participants: 8-10 participants from Africa

ANNEX I: WORKING PAPERS

Title: Evolutionary and Immune Processes in Insect Endosymbiosis

Abdelaziz Heddi
Université de Lyon, INRA, INSA-Lyon, IFR-41, UMR203 BF2I, Biologie Fonctionnelle Insectes et Interactions, F-69621 Villeurbanne, France.

Abstract

In insect, most species that have succeeded to develop exclusively on nutritionally poor and unbalanced mediums house integrated intracellular bacteria (endosymbionts) that transmit to the offspring vertically for million years and supplement the insect diet with several products. However, maintaining the beneficial nature of this long-term relationship constrains both the host and the symbiont to adaptive changes in their interactions.

Genomic and evolutionary data have shown that insect endosymbionts experience drastic deletions and mutations of genes, some of which are involved in bacterial virulence and host tolerance. We have recently demonstrated on the cereal weevil association with *Sodalis pierantonius* that genomic shrinkage occurs very rapid at early stages symbiogenesis (Oakeson et al., 2014). An extensive IS element expansion was unraveled in the bacterial genome and appears to have mediated numerous genome rearrangements, deletions, and duplications that might be beneficial to the association.

At the host side, we have shown that insects have selected an "endosymbiont compartmentalization" strategy and have shaped immune responses to maintain and control endosymbionts, but also to adjust their number according to the host physiological requirement at different stage of development (Login et al., 2011; Vigneron et al., 2014).

Introduction

Interspecific associations are widespread in nature and occur at different level of organism complexity. They imply monocellular associations ranging from bacteria to protists, as well as heteroclite species combinations forming, for examples, the soil ecosystem and all pathogenic and mutualistic relationships involving prokaryotic and eukaryotic cells (Heddi et al., 2001). Symbiosis is currently believed to take part in evolution either by improving the partner's fitness and adaptation through integrated nutritional endosymbioses, or by causing reproductive isolation and subsequent host speciation, such as in the *Wolbachia* symbioses. At the macroevolutive scale, it is now admitted that symbiosis has significantly contributed to eukaryote cell complexity and compartmentalization through the serial integration of cellular organelles.

Associations between insects and bacteria are common in nature and include a wide type of interactions ranging from parasitism to mutualism. In insects subsisting on nutritionally-deficient habitats symbiosis involves intracellular bacteria (endosymbiont) living within specialized host cells (the bacteriocytes) that sometimes form an organ, the bacteriome. Endosymbionts are thought to provide limiting components to insects, improving thereby their *fitness* and their invasive power. However, unlike plant-root symbiosis, where molecular processes and coordinated events triggering the development of nodules are well documented, the mechanisms leading to insect bacteriocyte cell differentiation and to bacterial maintenance and control remain less understood.

Insect endosymbionts often transmit vertically to insect offsprings for many of generations, resulting in the co-speciation between the bacteria and the insect. This way of symbiont transmission results in peculiar symbiont DNA evolution. Most striking features are the tendency to genome size reduction, a global enrichment in AT nucleic bases and fast nucleotide substitution rates. Selective constraints favor the deletion of genes becoming "unnecessary" to the new association. Among them, virulence genes and genes encoding the bacterial cell wall elements have been prone to serial gene deletions, as evidenced from insect endosymbiont genomes sequenced so far. Hence, host-symbiont interactional features may rely not only on the host functions but also on the level of bacterial genomic alteration and, thereby, on the age of the association. More recent associations have fewer bacterial genomic alterations when they are compared with free-living relatives. In this evolutionary context, recent associations with less deleted bacterial genomes should provide insight into the early interplay between host immunity and bacterial virulence. Among insect endosymbioses, the *Sitophilus* spp. (Dryophthoridae) associations provide an excellent model for studying early molecular and cellular aspects involved in symbiosis establishment and maintenance, because the association relatively recent (less than 25 MY ago) endosymbiont genomes have not experienced severe gene deletion, and they encode, as *Sodalis* endosymbionts, functional virulence genes and secretion systems.

To approach the role of the innate immune system on endosymbiont perception and maintenance we have identified in *Sitophilus zeamais* by suppressive subtractive hybridization (SSH) host immune-relevant genes and we have quantified their steady state levels in the bacteriocyte cells. We show a modulated but effective immune response to endosymbionts along with the overexpression of one antibacterial peptide and of a negative regulator of the Toll pathway (*Tollip* gene), in addition to the previously described *wPGRP* gene (Heddi, 2005; Anselme et al., 2006). The upregulation of these three genes in the bacteriocytes uncovers a local and specific immune response, and provides the first indication into how insects may modulate their immune response in the bacteriocytes to prevent both endosymbiont clearance and insect tissue invasion.

Result and discussion
Adaptive changes taking place early in the evolution of insect endosymbionts
We have investigated in 2002 the occurrence of bacterial intracellular symbiosis through the analysis of 19 Dryophthoridae species. All except *Sitophilus linearis* were shown to bear symbiotic bacteria within the bacteriome structure (Nardon et al., 2002). Phylogenetically, three endosymbiotic clades, named R, S and D, were defined (Lefèvre et al. 2004). The R clade exhibits the much higher relative rate of substitution, has a relative high A+T bias (40.5% G+C on the 16S rDNA) and contains 6 out of 9 genera studied including the putative ancestral species *Yaccaborus frontalis*. It was therefore assigned as the oldest clade, on which the ancestral bacterial infection occurred around 100 M.Y. ago. The genus name *Candidatus* Nardonella was ascribed to this clade of endosymbionts (Lefèvre *et al.*, 2004). Conversely, the S and the D clades are restricted to only 1 and 2 genera, respectively. Furthermore, these clades do not show any A+T bias nor significant high rate of evolution. These determinations, along with the dating estimation (O'Meara and Farrell, personal communication), suggest that the S and the D clade endosymbionts established symbiosis with the Dryophthoridae recently, probably by endosymbiont replacement. This symbiont replacement hypothesis was strongly

supported recently by studying two endosymbiont belonging to the *Hylobius* genus of Molytinae family. Endosymbionts from this family were shown to be monophyletic with *Candidatus* Nardonella and distanced from *Sitophilus* clade (Conord et al., 2008).

To get insight into the early molecular rearrangement within endosymbiont genome, we have proceeded, in collaboration with the groups of C. Dale, A. Latorre and A. Moya, to the sequencing and analyzing of *Sodalis piearantonius*, the endosymbiont of the S Clade. Structural, functional, and evolutionary analyses showed that *S. pierantonius* has undergone extensive adaptation toward an insect-associated lifestyle in a very short time period (Oakeson et al., 2014). The genome of *S. pierantonius* is large in size when compared with many ancient bacterial symbionts; however, almost half of the protein-coding genes in *S. pierantonius* are pseudogenes. There is also evidence for relaxed selection on the remaining intact protein-coding genes. Genomic analysis highlighted numerous genomic rearrangements, duplications, and deletions facilitated by a recent expansion of insertions sequence elements, some of which appear to have catalyzed adaptive changes. Functional metabolic predictions suggest that *S. pierantonius* has lost the ability to synthesize several essential amino acids and vitamins (Oakeson et al., 2014). Analyses of the bacterial cell envelope and genes encoding secretion systems suggest that these structures and elements have become simplified in the transition to a mutualistic association.

Bacteriocyte cells express a modulated but effective immune response for symbiont maintenance and control

The bacteriocytes are of peculiar importance in the insect world as, in addition to the germ cells, they are the only cells able to tolerate the bacteria and to limit their space of infection. To approach bacteriocyte cellular features, we have analyzed in 2008 the amount transcripts of the immune genes described above in the larval bacteriome (Anselme et al., 2008). We show that all genes with potential bacteriolytic activity, such as AMPs peptides, lysozymes and phenol oxydase pathway are not (or slightly) expressed in the bacteriome with the notable exception of one antibacterial peptide gene, the coleoptericin-A (ColA). Recently, we have reported that ColA selectively targets endosymbionts within the bacteriocytes and regulates their growth through the inhibition of cell division (Login et al., 2011). Silencing the *colA* gene with RNA interference resulted in a decrease in size of the giant filamentous endosymbionts, which escaped from the bacteriocytes and spread into insect tissues. Although this family of peptides is commonly linked with microbe clearance, this work shows that endosymbiosis benefits from ColA, suggesting that long-term host-symbiont coevolution might have shaped immune effectors for symbiont maintenance.

Adult weevils recycle endosymbionts following the cuticle synthesis

While intracellular symbiosis is known to improve host physiology and performance, little is known about how hosts balance the costs and benefits of having endosymbionts, and whether and how they adjust symbiont load to their physiological needs. By investigating the cereal weevil *Sitophilus* association with the *Sodalis pierantonius* endosymbiont, we discovered that endosymbiont populations intensively multiply in young adults, before being rapidly eliminated within few days (Vigneron et al., 2014). We show that young adults strongly depend on endosymbionts and that endosymbiont proliferation after metamorphosis matches a drastic host physiological

need for the tyrosine (Tyr) and phenylalanine (Phe) amino acids to rapidly build their protective exoskeleton. Tyr and Phe are precursors of the dihydroxyphenylalanine (DOPA) molecule that is an essential component for the cuticle synthesis. Once the cuticle is achieved, DOPA reaches high amounts in insects, which triggers endosymbiont elimination. This elimination relies on apoptosis and autophagy activation, allowing digestion and recycling of the endosymbiont material. Thus, the weevil-endosymbiont association reveals an adaptive interplay between metabolic and cellular functions that minimizes the cost of symbiosis and speeds up the exoskeleton formation during a critical phase when emerging adults are especially vulnerable (Vigneron et al., 2014).

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<u>Title of Project</u>: - Identification and Characterization of Microbes in Mid Gut of *G.*pallidipes and *G.*fuscipes in Kality Mass Rearing Center, Addis Ababa Ethiopia.

<u>Abstract</u>:-

G.*pallidipes* and G.*fuscipes* colonies were collected from Kality insectary, Addis Ababa and kept under Laboratory conditions (at 23-25^{0}c and 75-80RH) and feed on Irradiated fresh bovine blood. Then flies were chilled and surface-sterilized (once with 5% sodium hypochlorite and twice with 70% ethanol), Surface sterilization of the safety cabinet was made using 70% ethyl alcohol. Once the flies brought in to the laboratory they were entered in to the safety cabinet so as to avoid environmental contamination in the middle of the dissection, the midgut of each fly was sterilely dissected and grounded with a pestle.

Media preparation, bacterial culturing, and Gram staining to identify gram positive and gram negative bacteria were performed according to Microbiological Laboratory procedures. Now we only dissected 50 flies, we are hoping to dissect other 250 flies both from insectery and field so as to make a good comparison. Accordingly, 15 gram positive and 35 gram negative bacteria were observed under the oil immersion using binocular microscope.

In Ethiopia, there is shortage of data regarding any microbes isolated from both the laboratory and field collected tsetse flies. Therefore the present study is aimed to characterize microbes that are harbored in the flies gut and thus to broaden the current knowledge on the composition of the gut bacterial flora of tsetse flies. Recording such data is important not only because it may increase our knowledge on the bacterial diversity that is associated with insects, but also because it provides information on microbial species potentially of ecological interest or possibly involved in biological interactions with tsetse flies and/or with the parasite they transmit.

Working Paper

Looking inside *Glossina* male reproductive behaviour

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Background

The fitness of *Glossina* males, like other insects, is shaped through its reproductive behaviour; the production, quantity and management of sperm, the ability to acquire mates by competing with other males, female choice, the number of matings, the means of delivering the sperm to female and the investment in offspring in terms of fertilization.

The presence of symbionts such as *Wolbachia* may have a strong impact in manipulating these different aspects of mating behaviour, affecting multiple mating, the male molecular machinery controlling seminal fluid and sperm production and transfer, sperm use and fertilization [Lewis Z et al. (2011) Biol Lett 7: 187-189].

Knowledge of these different aspects is of fundamental importance for the improvement of the SIT against *Glossina* vector species such as *G. morsitans morsitans*, *Glossina fuscipes fuscipes* and *Glossina pallidipes*.

Given this background, the aims of our Research Unit are to investigate the impact of *Wolbachia* on:

1) mating/remating behaviour

2) the male machinery controlling seminal fluid, sperm production and transfer.

Effect of *Wolbachia* on mating and remating behaviour

Using microsatellite markers to genotype sperm stored in the spermathecae of wild females from two *Glossina fuscipes fuscipes* populations from Uganda: Buvuma Island and Kabukanga in 2008, we estimated the minimum number of males that were able to transfer sperm to a female's spermathecae. We provided evidence that remating is a common event and, most important, females store sperm from multiple males which may potentially be used for insemination. On these bases we are analysing 1) how the females use these sperm, 2) whether sperm competition is present, 3) the possibility that *Wolbachia* infection promotes polyandry and impacts sperm use. To answer these questions, in October/ December 2013, we collected additional flies from Buvuma Island and females were allowed to deposit their progeny in the lab. Using several SSR markers that mark both the sex chromosomes and the autosomes we are genotyping these females, the sperm that are present in their spermathecae and their offspring. The genotyping of the offspring can reveal the minimum number of males that sire a brood, but not necessarily the number of males with which a female had mated, as females may bias

paternity towards one or a few of their mates, as a result of differential sperm use (paternity skew).

With the sperm genotyping we are also collecting data on the number of mates for each female to obtain an estimate of polyandry. We are also screening the females also for the presence of *Wolbachia*.

All these data are relevant for interpreting the reproductive biology of the species, but they may also have an immediate impact on the strategy to be employed for eradication success.

Impact of *Wolbachia* on the male machinery controlling seminal fluid and sperm production and transfer

Insect seminal fluid is a complex mixture of proteins, carbohydrates and lipids, produced in the male reproductive tract and transferred to the female along with spermatozoa upon mating. Seminal fluid proteins are known to play key roles in inducing post-mating responses in the female, regulating sperm storage, motility and competition, female sexual receptivity, egg production, ovulation, feeding and flight behaviour, and other important biological aspects.

Information relative to the reproductive biology of the tsetse fly, *Glossina morsitans morsitans* is increasingly becoming available. However, the molecular identity and functional roles of male seminal secretions remain unstudied. Moreover no information are available on the impact of *Wolbachia* on these proteins.

The male reproductive organs of tsetse are similar to those of other insects in that they consist of a pair of testes, accessory glands (MAGs), and vas deferens opening into a common ejaculatory duct [Itard J (1979), Rev Elev Med Vet Pays Trop 23:57-81, Tobe SS, Annu Rev Entomol 23: 283-307 (1978)]. It has been suggested that the spermatophore is primarily derived from these glands [Pollock J (1970) Nature 225: 1063-1064]. Moreover, DNA virus infection affects the function of the MAGs and it has been observed that the majority of virus-infected males fail to produce a complete spermatophora and do not inseminate the female (Sang RC et al. (1999) Current Microbiology 38: 349-354]

Thus our aims are the identification and characterization of 1) the proteins and the components of male seminal fluid, 2) the organs, testes and MAGs, from which these components are secreted, 3) the proteins from the spermatophore. We have used a combination of transcriptomics and proteomics and we have made morphological observations to determining the timing of the presence of spermatophore in the uterus of female.

Spermatophore in the Uterus of Female:

The Spermathophore is evident in the uterus towards the end of mating (around 100 minutes from the beginning of mating) placed with the opening towards the spermathecal ducts in such a way that the sperm can flow directly to the spermathecae (within 24 hr after the initiation of mating). The spermatophore is composed of a barrier containing the sperm.

To this aim, we developed transcriptomes of the accessory glands and proteome analyses of the spermatophore. This analysis was also supported by the availability of an annotated genome (International Glossina Genome Initiative (2014), Science 344: 380-386).

Spermatophore proteome

We identified 479 proteins in the spermatophore. Of these, 95% (454) produced best hits against the nr database using BLASTX. The remaining 5% (25) shared no sequence similarity to genes present in the GenBank database. These deserve particular attention as they may represent novel tsetse specific proteins or rapidly evolving candidates. In this case these proteins may be taxonomically very useful to resolve the presence of subspecific entities within *Glossina*.

The 454 proteins that gave significant hits to the nr database were assigned to different GO functional classes (GO terms for Molecular functions). This approach allowed us to quantify the distribution of the 454 spermatophora proteins in the different functional classes in order to infer their possible functional roles.

Transcriptomes from accessory glands and from testes

For each tissue we developed three libraries (from teneral, 3 day old mature, 6/8 hours post mating) to cover all male physiological states. A total of 131 million and 128 million reads were obtained from testes and MAGs dataset, respectively. Removal of low quality reads after trimming resulted in removal of less than 3% of the reads in each library.

Tissue of origin of spermatophora proteins

By cross-referencing the testes and MAG transcriptomes and the spermatophora proteome we found that among the 479 spermatophora proteins, a large amount is derived from a relatively small number of proteins produced by the MAGs and a more diverse array of less abundant sperm-associated proteins is derived from the testes. This implies that both organs contribute to the spermatophora formation, but at different levels. In particular, 103 transcripts (21%) had similar expression levels in the testes and MAGs. 344 transcripts (72%) were enriched in the testes, while 32% (7%) were enriched in the MAGs.

Conclusions and perspectives

Using a *Wolbachia* infected strain of *Glossina morsitans morsitans* we have developed a data set of genes that are transcribed in testes and in the MAGs. We have identified the semen proteins that are transferred to the female and that form the spermatophore.

The most abundant class of transferred proteins we identified includes 25 tsetse-specific accessory gland proteins, as determined by cross-referencing our tissue specific transcriptomes and the proteome. Three of these novel proteins are the most abundant in the spermatophore and form a novel tsetse-specific family that appear to have arisen through tandem gene duplication events. A phylogenetic analysis revealed the conservation of this family among *Glossina* species, an exception being *G. brevipalpis*, which appears to have generated a fourth duplicate of this gene family that is not found in the other species. Interestingly, the genes encoding the majority of the most abundant proteins displayed male-specific or male-enriched expression. Moreover, a limited number of spermatophore components belonging to the classes of enzyme inhibitors, peptidase regulators and odorant binding proteins are among the most represented in tsetse seminal fluid and are also mostly produced by the accessory glands.

Our future aims include: 1) the functional characterization of spermatophore proteins, 2) the production of RNAseq data from accessory glands and testes using a aposymbiont strain and to compare the transcript abundances with those of the *Wolbachia*-infected

strain, 3) the production of accessory gland and testes RNAseq data using a virus-infected strain, in collaboration with the Siebersdorf laboratory, 4) morphological analysis of the spermatophore both in aposymbiont and virus-infected strains, 5) proteomic analyses of the spermatophore of aposymbiont and virus-infected strains.

Bacteria diversity in tsetse flies from sleeping sickness foci in Cameroon

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Introduction

Tsetse flies transmit African trypanosomes responsible for sleeping sickness in humans and Nagana in animals. This disease, fatal when untreated, affects many people with considerable impact on public health and agriculture in sub-Saharan Africa. The available drugs are poorly efficient and some have induced trypanosome resistance. Therefore the investigations for novel strategies must continue, among them are alternative vector-based strategies such as the engineering of insects capable of blocking the transmission of the parasite (Rio et al., 2004). Field-captured flies are colonized with symbionts and a taxonomically complex microbiome acquired from their environment (Farikou et al., 2010; Hamidou Soumana et al., 2013). In other insects, such as *Anopheles* sp., the gut microbiome was shown to prevent establishment and/or transmission of pathogens (Reviewed in Azambuja et al., 2005). Recent investigations on the midgut microbiota composition in insectary and natural tsetse fly populations collected in HAT foci in three African countries (Angola, Cameroon, Kenya) have revealed the presence of a diversified bacterial community:

* using a culture-dependent method we have identified:

- a novel bacterium species, *Serratia glossinae*, in *G. p. gambiensis* from insectary-reared flies (Geiger et al., 2010)

- eight bacteria species in *G. p. palpalis*, one species in *G. nigrofusca* and three bacteria species in *G. pallicera,* all of them in flies collected in HAT foci in Cameroon (Geiger et al., 2011)

- three bacteria species in *G. p. palpalis* flies from Angola (Geiger et al., 2009)

* using culture-dependent and non-dependent methods, Lindh and Lehane (2011) have identified more than 10 bacteria species in *Glossina fuscipes fuscipes* from Kenya.

* recently, using molecular methods, the group of Aksoy (Aksoy et al., 2014) has identified a limited microbiota diversity in *Glossina morsitans morsitans*, *G. f. fuscipes*, and *Glossina pallidipes* from Uganda.

In the frame of the new IAEA-CRP, our project aims to pursue and extend such investigations, in order to attempt to characterize the overall diversity of the bacterial species inhabiting the tsetse flies' gut.

Materials and Methods

To achieve this objective, tsetse flies will be caught using pyramidal traps, the species of the flies will be identified and their midguts dissected. DNA will be extracted using DNeasy Blood and Tissue kit. Deep sequencing of the V4 hypervariable region of the 16S rRNA gene will be performed on fly samples from Bipindi, Campo and from Fontem, using MiSeq. The QIIME software will be used to determine the species of the bacteria.

Objective / Expected results

To identify the whole bacteriome of tsetse flies from diversified geographic origins and belonging to diverse *Glossina* species.

To characterize the bacteria diversity on a larger number of flies

To estimate the diversity rate within the bacteria metacommunity of flies associated with the diversity of their geographic origin.

To evaluate the possible association between fly susceptibility/refractoriness to trypanosome infection and the composition of the harbored bacteriome.

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Impacts of the Hytrosavirus on the Transcriptome of the House fly *Musca domestica*

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Introduction

The hytrosaviruses associated with *Glossina pallipides* and *Musca domestica* are unique (Garcia-Maruniak et al., 2009) exhibit distinct pathologies in their homologous hosts (Lietze et al. 2011). The GpSGHV existing in an asymptomatic state in *G. pallipides* will, under certain conditions, infect progeny and stimulate the symptomatic expression of salivary gland hypertrophy. Alternatively, the MdSGHV in *M. domestica* exists only in the symptomatic state, expressing the SGH symptoms resulting in complete female sterility. Using the MdSGHV and its host *M. domestica* we conducted a RNAseq analysis to determine the global impact of this virus on the host transcriptome. The data generated from this study is presently being compiled and will be submitted for publication in spring 2015.

Materials and Methods

Virus challenge of *Musca domestica*

House fly pupae, obtained from colonies maintained at the USDA Center for Medical, Agricultural and Veterinary Entomology (CMAVE, Gainesville, FL), were placed in rearing cages, provided with deionized water, and incubated at 26 °C, a 12:12 h light: dark photoperiod, and 40% relative humidity until adult emergence. Experiments were conducted with MdHV03, the Florida type strain collected in 2005 and subsequently sequenced (Prompiboon et al., 2010, Garcia-Maruniak et al., 2008). To produce cohorts of synchronously infected house flies for RNA-seq assays, newly emerged females were injected with a filter-sterilized viremic salivary gland homogenate as described in Lietze et al. (2007). This treatment guarantees symptomatic infection in 100% of the injected flies (Lietze et al., 2007). Control flies were injected with sterile phosphate buffered saline (PBS). Both PBS- and virus-injected flies were maintained in separate groups at constant conditions (26 °C, 12L: 12D photoperiod, 40% relative humidity) and provided with food and water *ad libitum* until used for sample preparation.

RNA extraction and quantitation

At 48 h post-challenge, a series of PBS- and virus-injected females were placed individually in tubes containing aliquots of 1 ml of Tri-Reagent (Sigma-Aldrich). Each sample was homogenized by adding approximately twenty zirconium beads (BioSpec Products, Bartlesville, OK) followed

by vigorous shaking in a bead-homogenizer (FastPrep® Instrument, Qbiogene, Carlsbad, CA) for 30s. Total RNA were extracted according to the TriReagent protocol. Ethanol precipitated RNA pellets were suspended in 100µl DEPC-treated water and subjected to treatment with RNase-free DNase (Qiagen). Total RNA preparations precipitated with isopropanol were subjected to an additional extraction step using the SV Total RNA Isolation kit (Promega, Fitchburg, WI, USA) following the manufacturer's protocol. RNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The absence of contaminating DNA in RNA samples was verified using conventional PCR amplification primers targeting the 28S gene (Salem et al., 2009).

RNA-seq and data analysis

Preparation and sequencing of libraries were performed by ICBR/UF (Gainesville, FL USA) according to the manufacturer's instructions (Illumina, Inc.) using the NextSeq500 platform platform. Briefly, the mRNA was enriched from 1ug of total RNA per sample using oligo-dT attached magnetic beads and then subjected to thermal fragmentation using the elute, prime, and fragmentation mix from Illumina TruSeq™ v2 RNA sample preparation kit. RNA fragments were then converted to double stranded cDNA using reverse transcriptase and random primers provided in TruSeq RNA sample preparation kit. The double stranded cDNA fragments were end repaired by enzymatic polishing with T4 DNA polymerase and *E.coli* DNA polymerase 1 Klenow fragment. A single non-templated dA-tail was added to 3'end of the repaired fragments and then ligated to NEB adaptors (NEBNext®Utra RNA libraray prep kit) according to manufacture's instructions (NEB). The required fragments were purified by AMPure beads (Agencourt; PN A63881) and enriched by PCR amplification. The amplified libraries were purified and quantified using the Agilent DNA high-sensitivity kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc) and qPCR. Based on the calculated values, the libraries were pooled in equimolar ratios into one pool and sequenced for 2X150 bp reads on Illumina NextSeq 500 platform. Image analysis and base calling were performed using the Illumina Pipeline, where sequence tags were obtained after purity filtering.

Reads acquired from the illumina NextSeq 500 sequencing platform were cleaned up with the Cutadapt program to trim off sequencing adaptors and low quality bases with a quality phred-like score<20.   Reads <40 bases were excluded from RNA-seq analysis. The reference sequences of *Musca domestica* (38,323 sequences) were used as reference sequences for RNAseq analysis. The Cleaned reads of each sample were mapped independently to the reference sequences using the mapper of bowtie2 with a maximum of 3 mismatches for each read. The mapping results were processed with the samtools and scripts developed in house at ICBR to remove potential PCR duplicates and select uniquely mapping reads for gene expression estimation. Digital gene expression is determined as follows. The number of mapped reads for each individual gene were counted using the scripts developed in house at ICBR and analyzed by the DEB application. Significant up- and downregulated genes were selected using the adjusted P-value, fold-change, or both for downstream analysis.

RTqPCR validation

RNA samples from buffer and virus injected female flies were subjected to a one-step RT-qPCR to quantitate the relative transcript abundance of twenty-five genes selected from the RNAseq data. Primer sets were designed using Primer3 Plus to amplify 125-200 bp from *M. domestica* genes predicted from the RNAseq reads . In addition to host gene targets, additional primers designed to amplify MdSGHV ORFs 001, 010, and 108 were used to confirm the infection status of the virus-injected samples. The *M. domestica* ribosomal 28S gene (GenBank accession number DQ656974) served as a reference gene and internal positive control. Using the iTaq™ Universal SYBR® Green One –Step kit (BioRad, CA), each 20-µl reaction contained 50 ng of DNase-treated total RNA, 5 pmol of each gene-specific forward and reverse primer, and the reaction mix with iScript® reverse transcriptase. The one-step RT-PCR program was 50°C for 10 min, 1 min and 95°C for 3 min, then 40 cycles of 95 °C for10 sec, 60°C for 30 sec and melt curve analysis of 65-95 °C at 0.5°C increments (5 sec/step). The melting peaks were inspected to confirm the presence of single amplification product for each reaction.

Results

MdSGHV reads

Mapping the RNAseq data onto the MdSGHV genome (gi|187903037|ref|NC_010671.1|) demonstrated that the buffer-injected RNA pools had only 98, 601, and 849 reads mapped onto the MdSGHV ORFS. In general, the limited reads detected in the buffer-injected samples were mapped mainly onto the highly expressed MdSGHV ORFS. The three virus-infected RNA pools contained 4.49×10^6, 4.24×10^6 and 1.95×10^6 reads that were mapped onto 108 putative viral ORFs (Garcia-Maruniak et al., 2009).
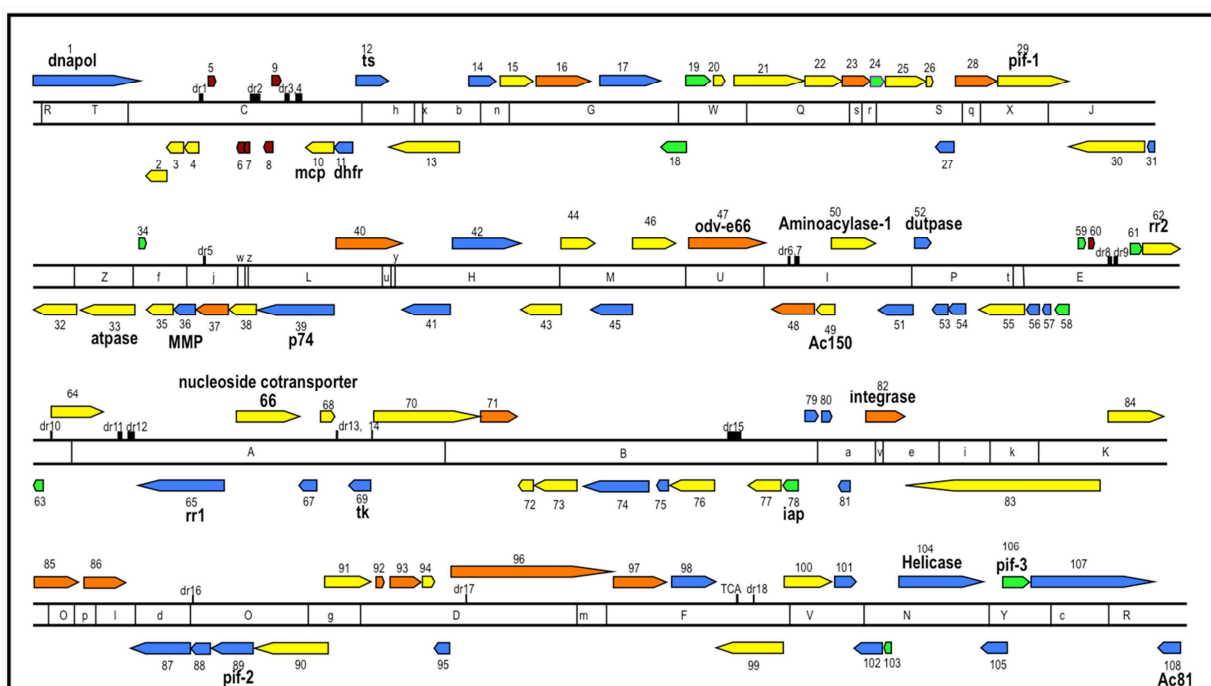
Fig. 1. Overlay of the RNA seq reads onto the linear representation of the MdSGHV genome and *Eco*RI physical map Garcia et al., 2009). The genome location, relative size and transcriptional direction of each putative ORF is indicated by the arrows. ORFs (15) having >50,000 reads; ORFS having <50,000 but >10,000 reads; ORFS having <10,000 but >2,000 reads; ORFs having <2000 but >100 reads; and ORFs having <100 but > 2 reads.

Overall, the number of reads were not correlated ($R^2 = 0.056$) to size of the ORFs, it should be noted the six ORFs having the least number of reads were less then 300 bp in length (Figs.2).

Fig 2. Regression analysis of transcript length versus number of reads from RNAseq database.

Based on read frequency viral transcription appears to be highly regulated; 16 ORFs (highly abundant) had more then 5.0×10^4 reads/library, 38 ORFs (abundant) had between $1.0 - 5.0 \times 10^4$ reads/lib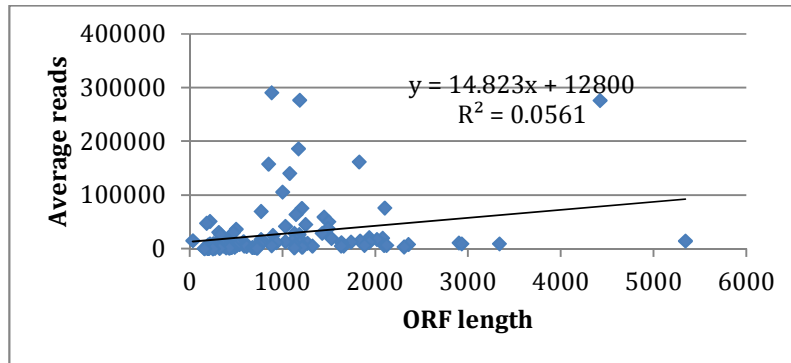rary, 36 ORFs (moderate) had between $10.0 - 2.0 \times 10^3$ reads/library, 11 ORFs (low) had between $20.0 - 2.0 \times 10^2$ reads /library (Fig. 3). Ten of the top fifteen ORFs (red) represent structural genes implicating an that viral morphogenesis was well underway at 48h pi.
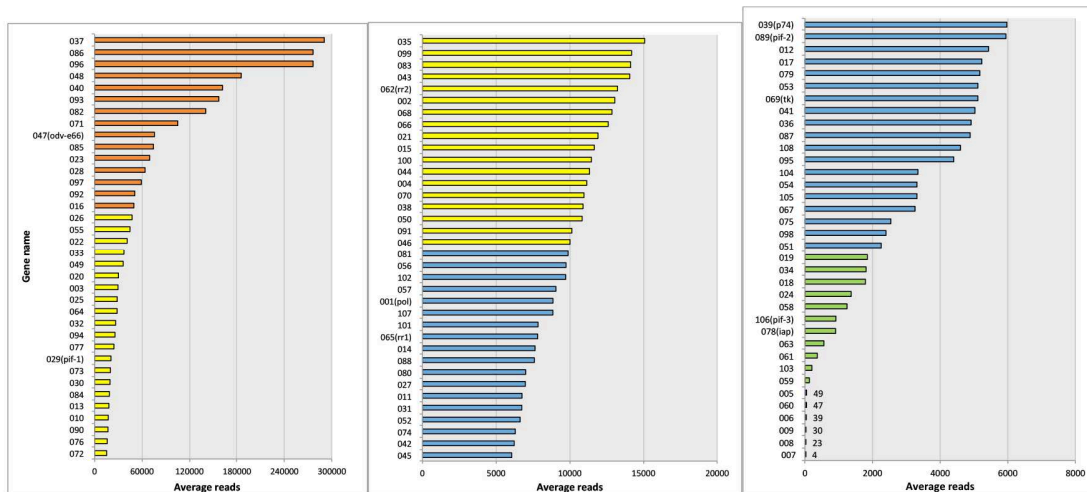
Fig 3 Histogram of the relative abundance of viral transcripts at 48h pi. Relative numbers for the MdSGHV ORF is indicated by the arrows. ORFs (15) having >50,000 reads; ORFS having <50,000 but >10,000 reads; ORFS having <10,000 but >2,000 reads; ORFs having <2000 but >100 reads; and ORFs having <100 but > 2 reads.

Musca domestica Reads

The summary statistics on the RNAseq data on the six libraries is presented in Table 1. In general, the control RNA library produced more *M. domestica* reads than the infected (48h pi) libraries. The transcriptional machinery in some of the host cells was redirected to produce the viral

transcripts (see above). The short RNAseq reads were mapped onto 17,035 *M. domestica* transcripts. Significant up- and downregulated genes, selected using the adjusted P-value and fold-change identified 5,494 genes that were were impacted SGHV infection.

The up and down regulation was validated by qPCR conducted on selected genes. Using calculated CT values the relative degree of transcription of the RTqPCR data was compared to the RNA seq data (Fig. 4). Analysis demonstrated that the two datasets were correlated (R^2 value=0.80034).
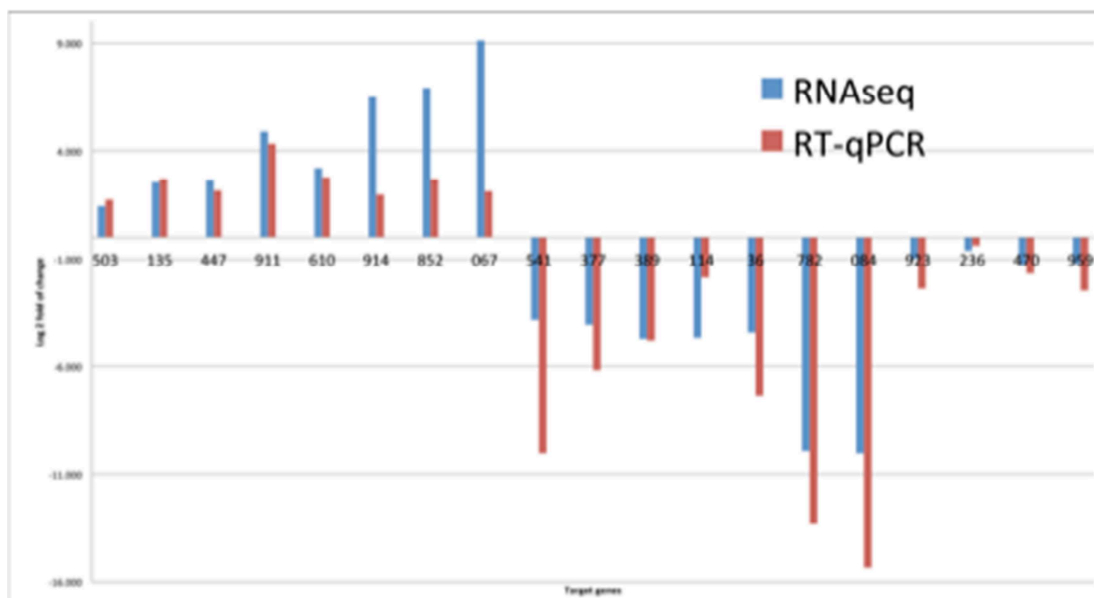
Fig 4. Log2 fold data from the RTqPCR reactions conducted on selected *M. domestica* transcripts were overlayed with the up- and downregulation calculated from the RNAseq data.

In addition to the *M. domestica* genes (shown in above histograms), RTqPCR was also conducted on select viral ORFs 010, 001,108, no significant amplification of these genes were observed in control RNA libraries whereas the 48h pi infected libraries had a calculated $10\text{-}50\times10^4$ gene copies. In summary, our RTqPCR data validated the RNAseq data set.

Reads mapped onto the *M. domestica* genes were subsequently assigned a GO id and placed into one of the categories. In total 2,271, 1,980, and 2,462 of the differentially expressed genes could be placed into either the Biological, Cellular or Molecular Processes, respectively. Within each general category the number down regulated host genes were significantly greater then the upregulated genes. A good example of this difference can be observed in the subcategory reproductive process, within this category166 genes are down regulated whereas only 51 genes

are upregulated. More importantly one observes that fold change in down regulation of the reproductive genes is much greater then that observed in those genes that are upregulated by MdSGHV infection. An exception to the MDSGHV induced downregulation would be the immune system process. Within this subcategory 51 genes are strongly upregulated whereas only 28 have been identified as being downregulated in the infected flies. Included within this category are components of both the IMD and TOLL pathway resulting in the upregulation of the *sarcotoxin, attacin, defensin* antimicrobial peptides. Whether or not this upregulation leads to higher translation rates and alterations in the fly microbiome is being examined. In addition to these pathways, components of the RNAi system (*Argonaut* and *Dicer*) are also upregulated suggesting these may paly a role in the differential transcription of viral ORFs.

In summary, during the past 12 months our lab has generated an RNAseq data base on both infected and control transcriptomes. The resulting 600,000,000 short reads have been processed and mapped onto either the *M. domestica* or MdSGHV genomes. Presently, this information in combination with targeted functional data are being compiled for publication

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Table xxx. List of *Musca domestica* and MdSGHV genes targeted for validation by RT-qPCR reactions conducted on replicate buffer- and virus-injected RNA pools.

IDENTITY	GENE PREDICTION	RNA-seq LOG2 FOLD	P-ADJ	RT-qPCR
LOC101892105	argonaute-2	3.84	3.3 E-7	
LOC101890924	argonaute-2	2.06	1.3 E-4	
LOC101893656	endoribonuclease dicer-1	2.89	0.02	
LOC101897503	CD63 antigen	2.70	3.5 E-6	2.3
LOC101889135	peritrophin-1	5.83	5.2E-15	4.47
LOC101900447	mucin-19	6.22	2.7E-14	3.08
LOC101891911	multidrug resistance-associated protein 4	4.90	1.0E-57	13.96
LOC101890610	proline-rich protein 4-like transcript variant X1	9.16	6.8 E-6	4.7
LOC101890093	proclotting enzyme	5.09	1.5 E-4	N/A
LOC101896914	attacin-A	6.53	2.8E-11	2.7
LOC101893852	sarcotoxin II-1	6.94	2.9E-12	4.44
LOC101897067	diptericin-D	9.13	1.1 E-7	3.02
LOC101888541	protein bicaudal C	-3.84	1.6E-26	6.61E-04
LOC101894377	tenascin	-4.06	1.9E-31	9.48E-03
LOC101900389	alpha-amylase A	-4.72	2.7E-46	2.51E-02
LOC101891114	meiosis arrest female protein	-4.66	7.2E-41	1.90E-01
LOC101894036	chorion peroxidase	-4.42	1.4E-50	4.23E-03
LOC101888782	lectin subunit alpha	-9.93	2.7E-69	6.79E-05
LOC101891084	vitellogenin-1	-10.04	4.2E-198	1.67E-05
LOC101900923	protein will die slowly	-0.99	0.01	0.13
LOC101890236	importin subunit alpha-4	0.62	0.05	0.53
LOC101900470	cold shock domain-containing protein E1	-1.28	8.0 E-6	0.22
gi_558515959	armadillo segment polarity protein	1.22	5.4 E-6	0.13
ACD03469.1	MdSGHV ORF10	NA	NA	48253.08
ACD03460.1	MdSGHV ORF001	NA	NA	7272.99

Improving knowledge of the interactions between *Glossina morsitans submorsitans*, its symbionts *Sodalis glosinidus, Wolbachia*, its microbiome and its parasites *Trypanosoma* sp in the African Animal Trypanosomiasis foci of Adamaoua, northern Cameroon

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Introduction

Control of trypanosomiasis is possible by detection and treatment of human and animal cases. Drugs are available but difficult to administer (Barret, 2006), and resistance also occurs. Resistance to insecticides used for vector control exists too. So, strategies to enhance tsetse fly refractoriness to trypanosome infection are welcome.

There is an obligate intestinal development phase for many trypanosome species. The capacity of intestinal establishment and development of mature infection (vectoriel competence) depends on trypanosome species and tsetse fly symbionts. Three species of symbiont belonging to the Family Enterobacteriacae was defined (Aksoy, 2000). *Sodalis* is suspected to be involved in the vectoriel competence. The ability of *T. b. gambiense* and *T. b. brucei* to establish in *G. p. gambiensis* midgut was statistically linked to the presence of

specific genotypes of *S. glossinidius* (Geiger et al., 2007). In three Cameroon HAT foci, statistical significant associations were found between the presence of *S. glossinidius* and trypanosome infections in *G. p. palpalis*. *Wolbachia* causes a number of reproductive phenotypes (cytoplasmic incompatibility, male killing ...) in many arthropods. Tsetse flies were shown to naturally harbour *Wolbachia* strains.

Preliminary studies in the Faro and Deo animal trypanosomiasis focus have identified *G. m. submorsitans* as the tsetse fly vector of animal trypanosomes. This fly habours *Sodalis* and *Wolbachia* but the rate of animal trypanosomes infections is very low. So the objective of the next step of the studiy is to identify another animal trypanosomiasis villages, to trap tsetse flies, to identify the symbionts and animal trypanosomes, to genotype the symbionts and to highlight the relation between the presence of the symbionts or some of their genotypes, and trypanosomes infections.

Materials and Methods

The work will be carried on in the Faro and Deo district which belongs to the Adamaoua Region in Cameroon. Tsetse flies will be captured using pyramidal traps. After identification of sex and teneral statue, the flies will undergo two protocols:

The first group will be dissected and the mid gut will be checked in microscope for the presence of animal trypanosomes and blood meals. In the laboratory, DNA will be extracted using the Chelex 100 method. Trypanosomes (*Trypanosoma congolense*, *T. vivax* and *T. b. brucei*), *Sodalis* and *Wolbachia* will be identified using PCR. (Farikou et al., 2010; Doudoumis et al., 2012).

The symbionts *Sodalis* and *Wolbachia* will be genotyped using microsatellites (Farikou et al., 2011) and MLST (Baldo et al, 2006) respectively.

Data analysis will be focused on the evaluation of the level of association between the presence of the symbionts (*Sodalis* and *Wolbachia* or some of their genotypes) and the trypanosomes.

Expected results

- Species of tsetse flies recorded in Faro Deo Division

- Percentage of midgut infected by trypanosomes detected by parasitological tests

- Percentage of midgut infected by different trypanosome species (*T. brucei brucei, T. congolense* and *T. vivax*) detected by PCR

Proportion of midgut harbouring *S. glossinidius*

- Different *S. glossinidius* haplotypes occurring in studied sites,

- Level of association between the symbionts or symbiont genotypes and trypanosome species.

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Infection with the secondary tsetse endosymbiont *Sodalis glossinidius* may influence parasitism in the tsetse fly *Glossina pallidipes*

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Abstract

The establishment of infection with *Trypanosoma brucei brucei* (Kinetoplastida: Trypanosomatidae), *T. b. rhodesiense* and *T. congolense* was evaluated in *Glossina pallidipes* (Diptera: Glossinidae) that either harbored or were uninfected by the endosymbiont *Sodalis glossinidius* (Enterobacteriales: Enterobacteriaceae). Temporal variation of co-infection with *T. b. rhodesiense* and *S. glossinidius* was also assessed. The results show that both *S. glossinidius* infection (χ^2 = 1.134, df = 2, p = 0.567) and trypanosome infection rate (χ^2=1.85, df=2, *p*=0.397) were comparable across the three infection groups. A significant association was observed between the presence of *S. glossinidius* and concurrent trypanosome infection with *T. b. rhodesiense* (*p*=0.0009) and *T. congolense* (*p*=0.0074*)* but not with *T. b. brucei* (*p*=0.5491). The time-series experiment revealed a slight decrease in the incidence of *S. glossinidius* infection with increasing fly age, which may infer a fitness cost associated with *Sodalis* infection. The present findings contribute to research on the feasibility of *S. glossinidius*-based paratransgenic approaches in tsetse and trypanosomosis control, in particular relating to *G. pallidipes* control.

Introduction

Glossina pallidipes (Austen) (Diptera: Glossinidae) is one of the most important tsetse fly vectors in eastern Africa due to its widespread distribution (Ouma et al., 2011). This species has been implicated in the spread of Human African Trypanosomiasis (HAT) and is a key vector for animal trypanosomes in this region (Ohaga et al., 2007; Malele et al., 2011; Peacock et al., 2012). Tsetse flies are considered to be naturally refractory to trypanosome infection and only a few of the trypanosomes introduced into a fly via an infective feed are able to overcome the immune system response and thus establish an infection (Welburn and Maudlin, 1999). The establishment and maturation of trypanosomes in the tsetse gut is dependent on many variables and involve complex interactions between the fly, endosymbionts and the parasite itself (Welburn and Maudlin, 1999). Tsetse flies harbor at least three gut endosymbionts namely; the obligate *Wigglesworthia glossinidae,* the facultative *Sodalis glossinidius* and the ricketssia-like *Wolbachia pipientis* (Cheng and Aksoy, 1999). *Wigglesworthia* and *S. glossinidius* are transmitted through maternal milk gland secretions to the intra-uterine developing larva, whereas

Wolbachia is transmitted transovarially. Symbionts are therefore present at eclosion in the teneral fly, whereas trypanosome infection is acquired mainly at the first feed in the presence of an infected blood meal source. In various arthropods, gut microbiota have been shown to increase insect immunity to pathogens such as viruses and parasites (Teixeira et al., 2008; Moreira et al., 2009; Koch and Schmid-Hempel, 2011). In the adult tsetse fly, responses to parasite infection are indirectly modulated by symbionts (Weiss et al., 2013). The possible influence of secondary symbionts on tsetse vectorial capacity has been investigated primarily using homogeneous laboratory populations of *G. m. morsitans* (Rio et al., 2006) and *G. palpalis gambiensis* (Geiger et al., 2007), whereby all individuals are *S. glossinidius* – infected. This study utilized using a naturally heterogenous population of *G. pallidipes* to establish the correlation between *S. glossinidius* infection in *G. pallidipes* and experimental infection with *T. b. brucei*, *T. b. rhodesiense* and *T. congolense*. The temporal variation of *T. b. rhodesiense* and *S. glossinidius* co-infection was also investigated.

Materials and Methods
Infection of tsetse flies: Male teneral *Glossina pallidipes* of age 0-2 day old from the Trypanosomiasis Research Centre (TRC) colony were used. Three trypanosome isolates were used: *T. b. rhodesiense*, *T. b. brucei* and *T. congolense*. **Error! Reference source not found.**The stabilates were expanded in two donor Swiss White mice which had been immune-suppressed with cyclophosphamide at a dose of 300mg/kg body weight. Disease progression in the mice was monitored by collection and microscopic examination of blood obtained through tail snipes on alternate days. At the peak of parasitaemia, the mice were euthanized using concentrated carbon dioxide. Blood from the heart was then collected by cardiac puncture into a tube containing ethylene di-amine tetra acetic acid (EDTA). The level of parasiteamia was estimated using the matching method (Herbert and Lumsden, 1976) and subsequently, an inoculum dose of 1×10^{6} trypanosomes/ml was prepared in phosphate saline glucose (PSG) pH 8.0. Two milliliters of this inoculum used to infect 12 recipient mice. At peak parasitaemia, teneral flies in 4" diameter cages were allowed to feed on the belly of the infected mice. Feeding success was confirmed by visual observation of engorged fly abdomens. Flies that did not feed were excluded from the experiment. After 10-15 minutes, feeding was interrupted and the engorged flies transferred to the insectary which is maintained at a temperature of 24 ± 1^{0}C and $70\pm5\%$ relative humidity. These flies were fed on defibrinated bovine blood on alternate days using the *in vitro* feeding system (Feldmann, 1994).

Experimental Design: Experimental flies were assigned to four infection groups (i) *T. b. rhodesiense* infection time series experiment (TBR[ts]) (ii) *T. b. rhodesiense* infection (TBR[35]) (iii) *T. b. brucei* infection (TBB) and (iv) *T. congolense* (TC) infection group were constituted as detailed in TABLE 1 below. The assays for group ii-iv were conducted after completion of the respective trypanosome maturation period.

TABLE 1: Details of the experimental groups used

Group No.	Experimental Group	n	Parasite	Dissection performed at dpi
1	TBR[ts]	100 (25, 22, 26, 27)	*T. b. rhodesiense*	7, 14, 21, 28
2	TBR[35]	18	*T. b. rhodesiense*	35
3	TBB	77	*T. b. brucei*	40
4	TC	98	*T. congolense*	30

TBR- *T. b. rhodesiense*; TBB- *T. b. brucei*; TC- *T. congolense*; dpi-days post-infection; TBR[ts]- TBR infection time series experiment; TBR[35] - TBR infection with dissection at 35dpi; numbers of n in brackets represent number of flies dissected the different time points of 7, 14, 21 and 28 dpi for the group TBR[ts]

Dissections and DNA extraction: Dissections were performed on a microscope slide using phosphate buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, pH 7.4). Following the method of Lloyd and Johnson (Lloyd et al., 1924), the mouthparts, gut and the salivary glands (in the *brucei* infection groups only) were isolated and examined microscopically. Subsequently, individual midguts from the dissected tsetse flies were placed in a 1.5ml microfuge tube. Total genomic DNA was isolated from the midgut samples using the DNeasy® Blood and Tissue Kit (Qiagen Sciences, MD, USA), with a minor modification to the manufacturer's instructions being that the final elution step was performed with 50µl instead of 100 µl of elution buffer.

PCR detection of trypanosome and S. glossinidius infections: Midgut trypanosome infection in the *T. congolense* treatment group was determined using the primers specific for *T. congolense* savannah (TCS) previously described by (Masiga et al., 1992) while infections in the *T. brucei* infection groups were determined using *T. b. rhodesiense* specific primers TBR1/2 (Moser et al., 1989). The presence of *S. glossinidius* in the gut tissues was determined using the primers *GPO1*F/R which amplify a 1.2kb product of the extra-chromosomal plasmid (Dale and Maudlin, 1999). The 20µl final PCR reaction contained 2µl of 10x PCR reaction buffer, 2.5mM $MgCl_2$, 0.5mM dNTPs, 500nM of each primer and 0.3µl of GoTaq® Flexi DNA polymerase 5units/µl (Promega, Madison, WI, USA). For each PCR run, a negative control (water) and the respective positive controls were included. After completion of the PCR run, 10µl of the amplification products was analyzed by electrophoresis in TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.0) on a 1.5% agarose gel together with a 100bp DNA ladder size standard (Invitrogen, Carlsbad, CA, USA) and visualized using ethidium bromide staining.

Statistical analysis

Fisher's exact test was used for analysis of categorical data using the online program GraphPad found at http://www.graphpad.com/quickcalcs/contingency1.cfm.

Results

S. glossinidius infection

S. glossinidius was detected in 38.9%, 36.4% and 32.7% of the TBR[35], *T. b. brucei* and *T. congolense* – group flies respectively. There was no significant difference in *S. glossinidius* prevalence among these three groups ($\chi^2 = 1.134$, df = 2, $p = 0.567$). However, *S. glossinidius* infection prevalence in the TBR[ts] was higher and ranged between 65 - 86% depending on the period after the infective bloodmeal, with an average infection rate of 76.5 ± 8.9 %.

Trypanosome infection

Dissections of TBR[ts] group flies detected three midgut infections, two of which were identified at 7 days post-infection (dpi) and one at 14 dpi (TABLE 2). No parasites were observed microscopically in the TBR[35] treatment group. In the *T. b. brucei* group, 7.8% (n=77) of the dissected flies were infected with trypanosomes in the mouthparts and/or the midgut. No salivary gland infections were observed indicating the absence of mature infections. A total of 13.3% (n=98) of *T. congolense* experimental group flies dissected harboured trypanosomes. In the latter group, out of 13 infected flies, 12 had parasites in both mouthparts and the midgut, whereas only one fly had an immature infection with no trypanosomes found in the midgut. PCR analysis detected 74% trypanosome infection rate in the TBR[ts] group, but only 50% infections at 35 dpi (TBR[35] group). Trypanosome infection was detected in 52% and 61% of the *T. b. brucei* and *T. congolense* treatment groups respectively. Comparing infection rates at trypanosome maturity, these were not significantly different between the TBR[35], TBB and TC infection groups as determined by both dissection ($\chi^2=3.62$, df=2, $p=0.163$) and PCR methods ($\chi^2=1.85$, df=2, $p=0.397$).

TABLE 2: Trypanosome infections in *G. pallidipes*

Parameter	*T. b. rhodesiense*[ts]	*T. b. rhodesiense*[35]	*T. b. brucei*	*T. congolense*
Dissection	3/100 (3)	0/18 (0)	6/77 (7.8)	13/98 (13.3)
Mature infections[a]	0/3 (0)	0/0 (0)	0/6 (0)	12/13 (92.3)
Trypanosome infection (PCR)	74/100 (76)	9/18 (50)	40/77 (51.9)	60/98 (61.2)

[a] number of mature trypanosome infections out of total infections; Percentages are in brackets; *T. b. rhodesiense*[ts]- TBR infection time series experiment; *T. b. rhodesiense*[35]- TBR infection with dissection at 35dpi.

S. glossinidius and trypanosome co-infection

T. b. rhodesiense infection group*: The temporal variation of parasite and *Sodalis* infections in the time-series TBR[ts] experiment is detailed in TABLE 3. An apparent decrease in the prevalence of *S. glossinidius* with increasing number of days post-infection (dpi) and hence with the age of the assayed flies (r = -0.56) was noted. This may infer a negative fitness cost associated with *Sodalis* infection, whereby infected flies have reduced longevity.

TABLE 3: Temporal variation of *T. b. rhodesiense* and *Sodalis* infections in *G. pallidipes* midgut

dpi	n	S+	T+	S+T-	S-T-	S+T+	S-T+	*p-value*
7	25	20 (80.0)	15 (60.0)	6 (24.0) [30]	4 (16.0)	14 (56.0) [70.0]	1 (4.0)	0.1206
14	22	19 (86.4)	16 (72.7)	4 (18.2) [21.1]	2 (9.1)	15 (68.2) [78.9]	1 (4.5)	0.1688
21	26	17 (65.4)	18 (69.2)	3 (11.5) [17.6]	5 (19.2)	14 (53.8) [82.4]	4 (15.4)	0.0781
28	27	20 (74.1)	25 (96.6)	0 (0) [0]	2 (7.4)	20 (74.1) [100]	5 (18.5)	0.0598
Total	100	76 (76)	74 (74)	13 (13) [17.1]	13 (13)	63 (63) [82.9]	11 (11)	0.0009*

dpi, days post infection; n, number of flies tested; S+, total number of flies harboring symbiont; T+, total number of trypanosomes infected flies; S+T+, flies with both *Sodalis* and trypanosome infection; S+T-, *Sodalis*-infected without parasite; S-T+, parasite infected flies lacking *Sodalis* ; S-T-, flies with neither symbiont nor parasite. % prevalence indicated in brackets calculated with reference to total flies at the specific time period after infection. Figures in square brackets represent parasite prevalence calculated with reference to the corresponding number of *Sodalis*-infected flies (s+). *p-value* Fisher's exact test for association between *Sodalis* and trypanosome infection. *statistically significant p<0.01

In the *T. b. rhodesiense*[ts] group, 76% of flies were infected with the symbiont and out of these, 82.9% had established the trypanosome infection by 28 dpi. In contrast, only 45.8% of those flies lacking the symbiont (S⁻T⁺) were able to establish trypanosomes by this time point (TABLE 4). Overall, in this group there was a highly significant association between infection with *S. glossinidius* and *T. b. rhodesiense* infection (p = 0.0009, Fisher's exact test). Analysis of temporal infection reveals that at 7, 14, 21 and 28 dpi, the proportion of *S. glossinidius*-positive flies that were infected by trypanosome parasites (S⁺T⁺) was constantly higher (76.5 ± 8.9%)

than those in which the parasite did not establish (S$^+$T$^-$) (17.9± 6.3%). Infection prevalence in the TBR^{35} group was 38.9% for *S. glossinidius* and 50% for trypanosomes. In this group as well, this association was statistically significant (*p* = 0.0023).

TABLE 4: *Sodalis* and parasite co-infection in experimentally-infected *G. pallidipes*

	TBR^{ts} (n=100)		TBR^{35} (n=18)		TBB (n=77)		TC (n=98)	
	T+	T-	T+	T-	T+	T-	T+	T-
S+	63 (63%) **[82.9]**	13 (13%)	0 (0%) **[0]**	7 (38.9%)	8 (10.4) **[61.5]**	5 (6.5)	26 (26.5%) **[81.3]**	6 (6.1%)
S-	11 (11%)	13 (13%)	9 (50%)	2(11.1%)	32 (41.6)	32 (41.6)	34 (34.7%)	32 (32.7%)
p – value	*p* = 0.0009		*p* = 0.0023		*p* = 0.5491 (NS)		*p* = 0.0074	

TBR^{ts}- TBR infection time series experiment; TBR^{35} - TBR infection with dissection at 35dpi; TBB- *T. b. brucei*; TC- *T. congolense*; S+/- *Sodalis* positive /negative; T+/- trypanosome positive/negative; NS - not significant Fisher's exact test. Bold values in square brackets indicates % of *Sodalis*- positive flies that were also parasite-positive.

Discussion and Conclusion

This study provides a significant insight into the contribution of endosymbiont *S. glossinidius glossinidius* to the outcome of exposing tsetse flies to trypanosome - infected mice. Tsetse flies reproduce by adenotrophic viviparity whereby the developing larva is nourished *in utero* by secretions from the milk gland. It is through these secretions that *S. glossinidius* is transferred from the mother to offspring (Balmand et al., 2013). *S. glossinidius* is therefore present at eclosion of the teneral fly, whereas trypanosomes are ingested by the fly at its first and/or subsequent infective feeds (Welburn and Maudlin, 1992). Previous workers have fed flies with various antibiotics to eliminate gut endosymbionts before performing similar comparative experiments (Weiss et al., 2013). However, antibiotic treatment has been shown to have negative effects on fly fecundity and longevity (Alam et al., 2011) and may ultimately have some effect on development of trypanosome infection. In this study, we had access to a laboratory population of *G. pallidipes* that was naturally heterogeneous with respect to *S. glossinidius* infection, thereby eliminating the need for antibiotic treatment.

The *S. glossinidius* prevalence of the time series group *(*TBR^{ts}*)* was 76% while for the TBR[35], TBB and TC groups, it was about 35%. This variation could be due to that fact that while flies in the last three groups were assayed when they were the same age, the time-series group was actually a composite group composed of four distinct groups that were assayed sequentially at different ages. In this study, we noted a negative correlation between *S. glossinidius* prevalence and the age of the specific group. This may have contributed to the much higher prevalence detected in the relatively younger composite group. It has been shown that although the relative density of *S. glossinidius* in individual flies may vary with age, the infection is permanent and is not lost in the tsetse's lifetime (Maudlin, 1991; Rio et al., 2006). We therefore surmise that this apparent temporal decrease in prevalence is not due to reduced density or total symbiont loss, but is a complete absence of the bacteria. This result introduces the hypothesis that *S. glossinidius* infection may affect tsetse longevity. This phenomenon has been reported in other arthropods such as the pea aphid whereby the secondary symbionts *Hamiltonella*, *Regiella* and *Spiroplasma* have caused negative effects on host longevity and fecundity (Maudlin, 1991). A similar scenario in tsetse flies would add a new angle to the proposed use of *S. glossinidius*-based paratransgenic approaches in tsetse fly control. We intend to conduct further research to verify the effects, if any, of *S. glossinidius* infection on longevity of *G. pallidipes*.

In our study, a significant association between the presence of *S. glossinidius* and concurrent trypanosome infection was noted in *T. b. rhodesiense* and *T. congolense* but not in *T. b. brucei*. The findings related to the last parasite may be considered to be anomalous, given that the synergistic effect of *S. glossinidius* on trypanosome establishment and maturation is hypothesized to apply to trypanosome species that pass through a midgut stage in the fly including *T. congolense, T. brucei* and *T. simiae* but excluding *T. vivax* (Welburn et al., 1993). The results we obtained agree with previously reported findings which postulate that *S. glossinidius* infection decreases the susceptibility of wild tsetse to infection with various trypanosomes (Farikou et al., 2010). However, the findings deviate from other studies which found no correlation between *S. glossinidius* infection and the ability of *G. p. gambiensis* to acquire *T. congolense* (Geiger et al., 2005). Studies using natural populations often reach contradictory conclusions, mainly due to the highly variable levels of *S. glossinidius* infection in wild flies. This prevalence varies depending on species and even populations, from apparently absent in *G. fuscipes fuscipes* (Lindh and Lehane, 2011; Alam et al., 2012) to more than 50% in *G. palpalis palpalis* (Farikou et al., 2010). In the latter, *S. glossinidius* was detected in about 55% of flies analyzed, and 59% of these were co-infected with various trypanosomes, primarily *T. congolense* and *T. brucei* sub-species (Farikou et al., 2010). While a strong co-relation was shown in *G. p. palpalis*, in contrast no co-relation was noted between infection with *S. glossinidius* and trypanosome establishment in Kenyan *G. austeni* and *G. pallidipes* with less than 2% of about 600 samples analyzed harboring both *S. glossinidius* and trypanosomes (Wamwiri et al., 2013). It is however evident that flies without *S. glossinidius* infection are also capable of developing trypanosome infections (Alam et al., 2012) as well. These divergent

conclusions highlight the considerable influence of vector - trypanosome species pairings on the success of infection establishment (Moloo et al., 1992).

In conclusion, this study reinforces the current opinion that concurrent *S. glossinidius* infection may increase susceptibility to trypanosome infection; however the extent of this effect is depends on the fly species and parasite involved. We also postulate that *S. glossinidius* infection may have a negative effect on longevity in *G. pallidipes*, which could have important implications for the application of *S. glossinidius*-based tsetse control interventions. However, a greater understanding of the interplay between the effects of *S. glossinidius* infection on fly survival and trypanosome-susceptibility is required.

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Detection and characterization of bacterial communities in tsetse flies

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Introduction

Tsetse flies (*Glossina* spp.) are found throughout tropical sub-Saharan Africa and are the sole vectors of African *Trypanosoma* spp., which cause African animal trypanosomosis (AAT – known also as nagana) in livestock or sleeping sickness (HAT) in humans (Van den Bossche et al., 2010). It has been estimated by the World Health Organization (WHO) that 60 million people in Africa are at risk of contracting sleeping sickness (about 40% of the continent's population) while the loss of local livestock from nagana amounts to 4.5 billion U.S. dollars annually (Aksoy, 2011; Barrett, 2006; Simarro et al., 2008). Knowing that the disease affects remote areas, it is likely that many HAT cases may remain unreported. In addition, in many parts of sub-Saharan Africa, the presence of tsetse and AAT is considered the major obstacle to the development of more efficient and sustainable livestock production systems and one of the most important causes of hunger and poverty (Cattand, 1995; Dyck et al., 2005; Feldmann et al., 2005; Kioy et al., 2004).

Controlling the vector, the tsetse fly, remains theoretically the most efficient and sustainable way of managing AAT (Jordan, 1986; Leak, 1998). There are currently several accepted environment-friendly methods of controlling tsetse: (i) the SAT (sequential aerosol technique) an aerial application of ultra-low volume, non-residual insecticides (Jordan, 1974), (ii) stationary bait techniques, i.e. use of insecticide-impregnated targets and traps that can be odour baited (Green, 1994), (iii) the live bait technique, i.e., application of residual insecticides on livestock (Thompson et al., 1991), and (iv) the release of sterile male insects, called the sterile insect technique (SIT) (Oladunmade et al., 1990; Politzar and Cuisance, 1984; Vreysen et al., 2000).

The tsetse flies heavily depend on the microbial flora to provide nutrients that are not present in their food (vertebrate blood). They harbor multiple symbiotic microbes, which display different levels of integration with their host. The main tsetse symbionts are: (a) *Wigglesworthia glossinidia* is present in all tsetse species. Sequencing of the genome confirmed that the symbiotic role is mainly associated with the nutrition of the insect since the bacterial symbiont carries many biosynthetic pathways of vitamins. Removal of the bacterium leads to reduced fertility and viability of the host. (b) *Sodalis glossinidius* has been identified in most tsetse flies

tested so far. Although the genome of the bacterium has been decoded, the role of *Sodalis glossinidius* is not fully elucidated. A working hypothesis is that it might be associated with the transmission of trypanosomes and (c) *Wolbachia pipientis* (*Wolbachia*) is regarded as the most widespread symbiotic microorganism since it has been estimated that is present in more than 65% of all insect species. The bacterium can affect insect hosts in many ways but it is mostly known due to its ability to control reproduction by inducing phenomena such as parthenogenesis, feminization, male killing and mainly Cytoplasmic Incompatibility, a form of embryonic mortality, which occurs in almost all classes of insects (Werren et al. 2008, Serbus et al. 2008, Saridaki and Bourtzis 2010). *Wolbachia*-based control strategies have been suggested as a tool to control pests and diseases and proof of concept has been provided in several cases (Apostolaki et al., 2011; Bourtzis, 2008; Bourtzis and Robinson, 2006; Brelsfoard and Dobson, 2009; Brelsfoard and Dobson, 2011; Xi et al., 2005; Zabalou et al., 2009; Zabalou et al., 2004; Walker et al. 2011; Hoffmann et al. 2011). During the last few years, studies have shown that *Wolbachia* blocks the transmission of major human pathogens including Dengue virus and the *Plasmodium* causing malaria (Moreira et al., 2009; Hancock et al., 2011; Walker et al. 2011; Hoffmann et al. 2011; Bian et al. 2013). To the best of our knowledge there are no studies on the possible interaction between *Wolbachia* and trypanosomes.

The aim of this study is to use the most advanced molecular tools and state-of-the-art technologies in order to characterize the microbial communities associated with the reproductive tissues and the gastrointestinal tract of *Glossina morsitans morsitans*, *Glossina pallidipes*, *Glossina fuscipes fuscipes* and *Glossina palpalis gambiensis*. More specifically we aim to: *(a)* detect and genotype reproductive parasites in laboratory and natural populations of *Glossina morsitans morsitans*, *Glossina pallidipes*, *Glossina fuscipes fuscipes* and *Glossina palpalis gambiensis* which are known to influence the reproduction of their insect hosts, *(b)* To characterize the prokaryotic communities associated with the gastrointestinal tract of laboratory and natural populations of *Glossina morsitans morsitans*, *Glossina pallidipes*, *Glossina fuscipes fuscipes* and *Glossina palpalis gambiensis*, and *(c)* to characterize the impact of irradiation treatments on the prokaryotic communties (both gut-associated bacteria and reproductive parasites) of laboratory populations of *Glossina morsitans*

morsitans, *Glossina pallidipes*, *Glossina fuscipes fuscipes* and *Glossina palpalis gambiensis*.

Materials and Methods

Sample collection and DNA isolation

Laboratory strains of *Glossina morsitans morsitans*, *Glossina pallidipes*, *Glossina fuscipes fuscipes* and *Glossina palpalis gambiensis* from FAO/Seibersdof and *G. f. fuscipes* natural populations were used. The dissected tissues, guts and reproductive organs, were used immediately for DNA extraction and DNA samples were stored at -20ºC until their use. DNA was isolated using the Qiagen DNeasy kit (Qiagen, Valencia, CA), following the manufacturers' instructions.

PCR Detection of reproductive parasites and sequencing

A total of 119 specimens from the four *Glossina* species were screened for the presence of *Wolbachia*, *Spiroplasma*, *Arsenophonus*, *Riskettsia* and *Cardinium* strains. Table 1 presents the PCR conditions and the primer list used for each reproductive parasite. For *G. fuscipes fuscipes* a total of 174 specimens were screened for the presence of *Spiroplasma*.

PCR amplifications were performed in 20 μl reactions containing 1 μl of DNA, 4 μl 5x reaction buffer (Promega), 1.6 μl $MgCl_2$ (25 mM), 0.1 μl deoxynucleotide triphosphate mixture (25 mM each), 0.5 μl of each primer (25 μM), 0.1 μl of *Taq* polymerase (Promega, 1 U/μl) and 12.2 μl water. Amplification was performed in a PTC-200 Thermal Cycler (MJ Research), using the following cycling conditions: 95ºC for 5min, followed by 34 cycles of 30 s at 94ºC, 30 s at the annealing temperature as designated in Table 1, 1min at 72ºC and a final extension of 10 min at 72ºC. PCR reactions were electrophoresed on a 1.5% agarose gel.

Spiroplasma multi locus genotyping

Spiroplasma genotyping was based on a multi-locus sequence approach using five marker genes (*rpoB, parE, dnaA, ftsZ* and *fruR*) as well as the region 16S rRNA-23S rRNA-5S rRNA with a total length of 4702 bp. All the primers and PCR conditions

used in the present study are presented in Table 2. Briefly, 5 min of denaturation at 95 °C preceded by thirty-five PCR cycles of 30 sec at 95°C, 30 sec at the appropriate temperature for each pair of primers and the appropriate extension time for each amplicon at 72 °C, with a final extension step at 72 °C for 10min. PCR reactions were carried out in 25 μl volume, included 12.5 μl High Fidelity Ready Mix Reaction Buffer (Promega), 0.3 μl of each primer (25 μM), 10.7 μl water and 1.2 μl of template DNA. The PCR products were purified following a PEG (Polyethylene glycol) - NaCl protocol (Sambrook et al. 1989). Positive samples were sequenced using a dye terminator-labelled cycle sequencing reaction conducted with the BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems). Reaction products were analysed using an ABI 3730 Genetic Analyzer (PE Applied Biosystems). All sequences generated in this study were assembled and manually edited with Geneious7.

Spiroplasma density in *G. fuscipes fuscipes*

Spiroplasma density was estimated by qPCR using the *dnaA* specific primers and normalized to the host *β-tubulin* gene amplified with the GmmtubqF/GmmtubqR primers (Table 2). Reactions were performed using the KAPA SYBR FAST qPCR Kit in a final volume of 10 ml (2 replicates for each reaction) containing: 5 μl 2x KAPA SYBR FAST qPCR Master Mix Universal, 0.08 μl from each primer (25 μM), 4.42 μl water and 0.5 μl total DNA (~ 20ng). Real-time PCR runs were conducted in an MJ Research Opticon 2. The amplifications were carried out in a 96-well plate and each biological sample had a minimum of three replicates. Statistical tests involved a one-way analysis of variance (ANOVA). *Spiroplasma* density is defined as the copy number of the *dnaA Spiroplasma* fragment relative to the copy number of the *β-tubulin* host fragment (Jingwen et al. 2012; Alam et al. 2012). Internal standard curves were generated for each primer set by cloning the amplified fragment into pGEM-Teasy vector (Promega). All assays were carried out in duplicate, and replicates were averaged for each sample. Negative controls were included in all amplification reactions.

Table 1 PCR primers and conditions used for the detection of reproductive parasites

Symbiont	Primer Sequence		Annealing	Expected size	Reference
Wolbachia	wspecF 5'-YATACCTATTCGAAGGGATAG-3'	wspecR 5'-AGCTTCGAGTGAAACCAATTC-3'	54°C	438	Werren & Windsor 2000
Ricketsia	16SA1 5'-AGAGTTTGATCTGGCTCAG-3'	Rick 16SR 5'-CATCCATCAGCGATAAATCTTTC-3'	55 °C	~200	Fukatsu and Nikoh 1998, Fukatsu et al. 2001
Cardinium	CLOF 5'-GCGGTGTAAAATGAGCGTG-3'	CLOR 5'-ACCTMTTCTTAACTCAAGCCT-3'	55 °C	450	Weeks et al. 2003
	CLO-f1 5'-GGAACCTTACCTGGGCTAGAATGTATT-3'	CLO-r1 5'-GCCACTGTCTTCAAGCTCTACCAAC-3'	56 °C	468	Gotoh et al. 2007
	CLO-f2 5'-GGTGCGTGGGCGGCTTATT-3'	CLO-r2 5'-AAAGGGTTTCGCTCGTTATAG-3'	56 °C	539	Gotoh et al. 2007
Spiroplasma	63F 5'-GCCTAATACATGCAAGTCGAAC-3'	TKSSpR 5'-TAGCCGTGGCTTTCTGGTAA-3'	58 °C	457	Mateos et al. 2006, Fukatsu and Nikoh 2000
Arsenophonus	ArsF 5'-GGGTTGTAAAGTACTTTCAGTCGT-3'	ArsR2 5'-GTAGCCCTRCTCGTAAGGGCC-3'	60 °C	800	Duron et al. 2008
		ArsR3 5'-CCTYTATCTCTAAAGGMTTCGCTGGATG-3'	60 °C	600	Duron et al. 2008

Table 2 Primers used for PCR, qPCR and sequencing (SR) reactions

Name	Primer Sequence 5'-3'	Gene region	Annealing temperature / extension time	Fragment size (bp)	Primer type	References
GmmtubqF	CCATTCCCACGTCTTCACTT	host *β-tubulin*	56 ℃ / 30sec	151	qPCR	Guz et al. 2007
GmmtubqR	GACCATGACGTGGATCACAG					Guz et al. 2007
12SCFR	GAG AGT GAC GGG CGA TAT GT	*mt 12S rRNA*	54 ℃ / 1min	377	PCR	Hanner&Fugate 1997
12SCRR	AAA CCA GGA TTA GAT ACC CTA TTA T					Hanner&Fugate 1997
63F	GCCTAATACATGCAAGTCGAAC	*16S rRNA*	67 ℃/ 1:30min	1334	PCR,	Fukatsu & Nikoh 2000
16STR1_Hasel	GGTGTGTACAAGACCCGAGAA				SR	Haselkorn et al.2009
16F2_Bi	GGTGCATGGTTGTCGTCAG	region 16S-23S	67 ℃ / 1min	1024	PCR,	Bi et al. 2008
23R1_Bi	TTCGCTCGCCGCTACTAAG				SR	Bi et al. 2008
23F1_Bi	GAATGGGGGAAACCCGGTGAG	region 23S-5S	67 ℃ / 3min	2914	PCR,	Bi et al. 2008
5R_Bi	TCGGGATGGGAACGGGTG				SR	Bi et al. 2008
23F2_BiDoud	ATAGACCCGAAACCAGGTGA	region 23S-5S	-	-	SR	this study
23F3_Bi	CCGTGAGGACTGCTGGACTG	region 23S-5S	-	-	SR	Bi et al. 2008
23F3_Heres	CCTAAGGCAAGCGAGATAAC	region 23S-5S	-	-	SR	Heres et al. 2010
23R2_Bi	GGATCACTAAGCCCAGCTTT	region 23S-5S	-	-	SR	Bi et al. 2008
fru-f	GTCATAATTGCAATTGCTGG	*fruR*	56 ℃ / 30sec	398	PCR,	Montenegro et al. 2005
fru-r	CAATGATTAAAGCGGAGGT				SR	Montenegro et al. 2005
SRdnaAF1	GGAGAYTCTGGAYTAGGAAA	*dnaA*	50 ℃ / 30sec	515	PCR,	Anbutsu & Fukatsu 2003
SRdnaAR1	CCYTCTAWYTTTCTRACATCA				SR	Anbutsu & Fukatsu 2003
FtsZF2	TGAACAAGTCGCGTCAATAAA	*ftsZ*	57 ℃ / 1min	774	PCR,	Haselkorn et al.2009
FtsZR3	CCACCAGTAACATTAATAATAGCATCA				SR	Haselkorn et al.2009
ParEF2	GGAAAATTTGGTGGTGATGG	*parE*	57 ℃ / 1min	1126	PCR,	Haselkorn et al.2009
ParER2	TGGCATTAATCATTACATTAATTTCT				SR	Haselkorn et al.2009
RpoBF1	ATGGATCAAACAAATCCATTAGCAGA	*rpoB*	60 ℃ / 1:30min	1703	PCR,	Haselkorn et al.2009
RpoBR2	GCATGTAATTTATCATCAACCATGTGTG				SR	Haselkorn et al.2009
RpoBR4	CTTTGTTTCCATGGCGTCCAGCC	*rpoB*	-	-	SR	Haselkorn et al.2009
FqPCR_dnaA	TGAAAAAAACAAACAAATTGTTATTACTTC	*dnaA*			qPCR	Harumoto et al. 2014
RqPCR_dnaA	TTAAGAGCAGTTTCAAAATC<u>A</u>GG	*dnaA*			qPCR	Harumoto et al. 2014

Bacterial Tag-Encoded FLX Amplicon Pyrosequencing Analysis

Bacterial tag-encoded FLX amplicon pyrosequencing was used to access the bacterial diversity of the dissected tsetse samples. In brief, all DNA samples were adjusted to 50 ng/μl. A 100 ng (2 μl) aliquot of each samples DNA was used for a 50 μl PCR reaction. The 16S rRNA universal Eubacterial primers 926F (5'- CTY AAA KGA ATT GRC GG -3') and 1392R (5'- ACG GGC GGT GTG TRC -3') were used for amplifying the ~470 bp region of 16S rRNA gene. PCR was performed under the following conditions: 94°C for 3 minutes followed by 32 cycles of 94°C for 30 seconds; 55°C for 40 seconds and 72°C for 1 minute; and a final elongation step at

72°C for 5 minutes. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents and following manufacturer's guidelines.

Sequence analysis

Sequences were analyzed and processed using the QIIME package (Caporaso et al., 2010). Briefly, the QIIME pipeline takes all sequences from a single pyrosequencing run and assigns sample IDs using a mapping file and the barcode assigned to each sample. Sequences were removed from the analysis if they were <200 bp in length, had a quality score of <25, contained ambiguous characters, an uncorrectable barcode, or did not contain the primer sequence. The 16S rRNA gene sequences were clustered with uclust (Edgar, 2010) and assigned to operational taxonomic units (OTUs) with 97% similarity. Representative sequences from each OTU were aligned with Pynast (Caporaso et al., 2010) using the SILVA108 core set. Taxonomy was assigned using the SILVA108 16S rRNA gene database.

Results and Future Work

Detection of reproductive parasites

The presence of *Wolbachia*, *Spiroplasma*, *Cardinium*, *Rickettsia* and *Arsenophonus* was investigated in four species of *Glossina*. In total 119 adult flies were tested using a PCR-based method deploying specific primers for each reproductive parasite (Table 3). *G. f. fuscipes* (*Gff*) laboratory and natural populations were found to be infected with *Spiroplasma* and prevalence was estimated in natural and lab populations (Table 4). Prevalence of *Spiroplasma* in *Gff* populations was between 6.67 and 80%. In laboratory *Gff* populations, the Seibersdorf colony was found to be at least 33.3% infected while the Bratislava colony 80% of the adults tested were found to be infected to *Spiroplasma*. In natural populations from Uganda, the prevalence of *Spiroplasma* ranged from 35.3 to 60%.

Genotyping of the Spiroplasma strain present in G. fuscipes fuscipes

MLST characterization of the *Spiroplasma* strain present in *Gff* was carried out on two laboratory stocks from Seibersdorf (1 female and 1 male) and Bratislava (1 female and 1 male) as well as on one natural population from Uganda (Lukoma-Buvuma islands, 1 female). It was based on six gene markers (*16S rRNA, rpoB, parE, dnaA, ftsZ* and *fruR*) and the *16S rRNA-23S rRNA-5S rRNA* region. The results clearly indicated that both laboratory and field samples harbour identical *Spiroplasma* species (Table 4, 5 & Figure 1).

Spiroplasma **density in** *G. f. fuscipes*

The *Spiroplasma* density from adult teneral and 16day post eclosion old male and female flies was measured from the gut and the reproductive tissues. *Spiroplasma* density was significantly higher in testes than in ovaries (Figure 2A) with the teneral flies containing more *Spiroplasma* than the 16 day old flies (Figure 2B and 2C). Interestingly, the Spiroplasma density in testes from teneral adult flies was no statistically different from that of 16day old flies (Figure 2D).

Based on the current results, future work will focus on:

1. Natural populations from other Glossina species in order to investigate the prevalence of *Spiroplasma*
2. Natural populations of *Gff*, and *G. tachinoides* and the prevalence of *Spiroplasma* will be examined in more detail
3. *FISH* experiments to identify the location of *Spiroplasma* in the adult fly
4. The isolation of the *Spiroplasma* strain from *Gff* using specific media
5. Establishment of a *Spiroplasma* free strain in order to characterize the phenotype of the *Spiroplasma* strain

Table 3 Prevalence of reproductive parasites in different *Glossina* species

	Spiroplasma	*Cardinium*	*Rickettsia*	*Arsenophonus*	*Wolbachia*
G. m. morsitans – Seibersdorf	0/10	0/10	0/10	0/10	10/10
G. pallidipes – Seibersdorf	0/10	0/10	0/10	0/10	0/10
G. p. gambiensis - Seibersdorf	0/10	0/10	0/10	0/10	0/10
G. f. fuscipes – Seibersdorf	19/36 (52.8%)	0/10	0/10	0/10	4/10
G. f. fuscipes - Buvuma Island Uganda Field – GFKF2	12/26 (46.2%)	0/10	0/10	0/10	0/10
G. f. fuscipes - Buvuma Island Uganda Field – GFFBUV2	6/10 (60%)	0/10	0/10	0/10	0/10
G. f. fuscipes - Buvuma Island UgandaField – GFFTOR2	6/17 (35.3%)	0/10	0/10	0/10	0/10

Table 4 Prevalence of *Spiroplasma* in natural and lab populations of *Glossina fuscipes fuscipes*

Origin (Area, Collection Date, Population, Sex)	No. of infected insects/no. examined	Infection rate (%)
Bratislava lab-colony (2013, F)	16/20	80,00
Bratislava lab-colony (2013, M)	16/20	80,00
Seibersdorf lab-colony (1995, F)*	6/18	33,33
Seibersdorf lab-colony (1995, M)*	6/18	33,33
Uganda (Buvuma island, 1994, GFTF2, NA)	0/17	0,00
Uganda (Buvuma island, 1994, GFKF2, NA)	0/5	0,00
Uganda (Buvuma island, 1994, GFFBUV2, NA)	0/9	0,00
Uganda (Buvuma island, 1994, GFFTOR2, NA)	1/15	6,67
Uganda (Lukoma-Buvuma islands, 2014, F)	3/20	15,00
Uganda (Lukoma-Buvuma islands, 2014, M)	0/32	0,00
TOTAL	**48/174**	**27,59**

Table 5 Spiroplasma MLST genotyping of *Glossina fuscipes fuscipes*

| Origin (Area, Collection Date, Population, sex, tissue)	*Spiroplasma* MLST						
	16S rRNA	*16S rRNA-23S rRNA-5S rRNA*	*rpoB*	*dnaA*	*parE*	*fruR*	*ftsZ*
Bratislava lab-colony (2013, F, whole fly)	ID	ID	ID	ID	ID	ID	ID
Bratislava lab-colony (2013, M, whole fly)	ID	ID	ID	ID	ID	ID	ID
Uganda (Lukoma-Buvuma islands, 2014, 350, F, whole fly)	ID	ID	ID	ID	ID	ID	ID
Seibersdorf lab-colony (2013, F, gut)	ID	ID	ID	ID	ID	ID	ID
Seibersdorf lab-colony (1995, M, gut)	ID	ID	ID	ID	ID	ID	ID

ID: identical sequence
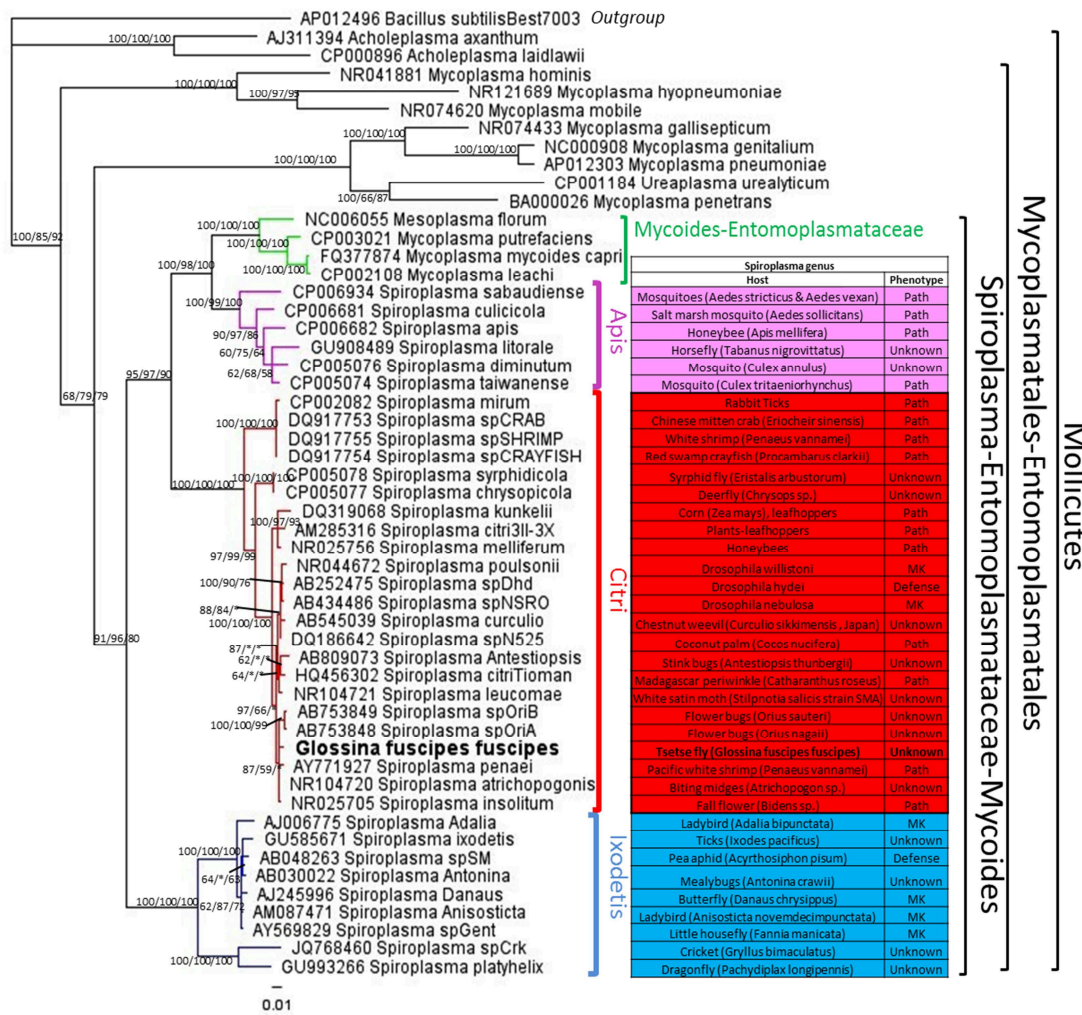
Figure 1. Bayesian inference phylogeny based on the *16S rRNA* sequence: The topologies resulting from the Maximum Likelihood (ML) and Neighbor Joining (NJ) methods were similar. Bayesian posterior probabilities, ML and NJ bootstrap values based on 1000 replicates are given at each node (only values >50% are indicated), respectively. Asterisks indicate support values lower than 50%. The *Spiroplasma* strain present in *Glossina* is indicated in bold letters. For each *Spiroplasma* species the GenBank accession number is given to the left of the name. The right table shows the host and phenotype of each *Spiroplasma* strain, respectively (Path: Pathogenic, MK: Male Killing).
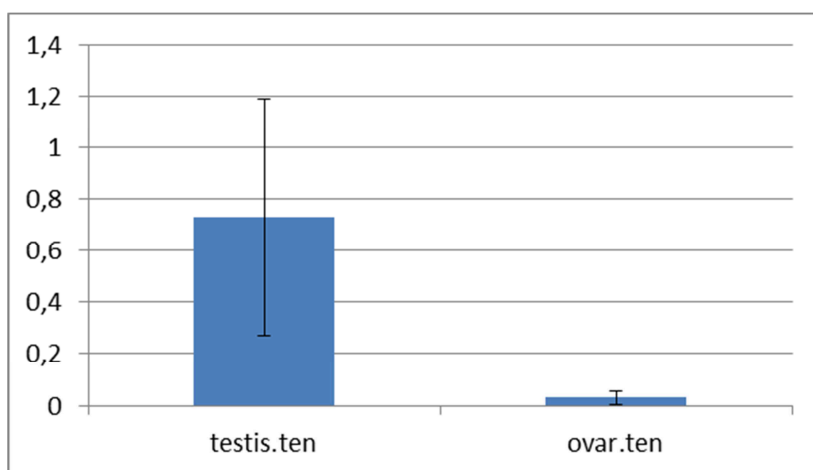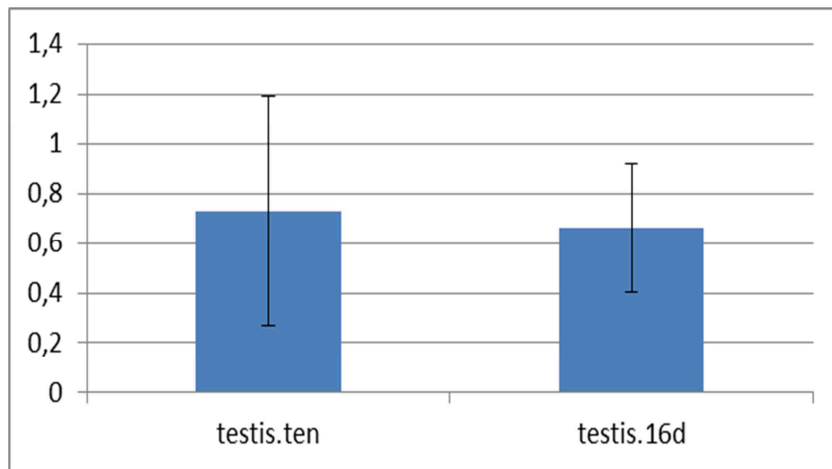
A.
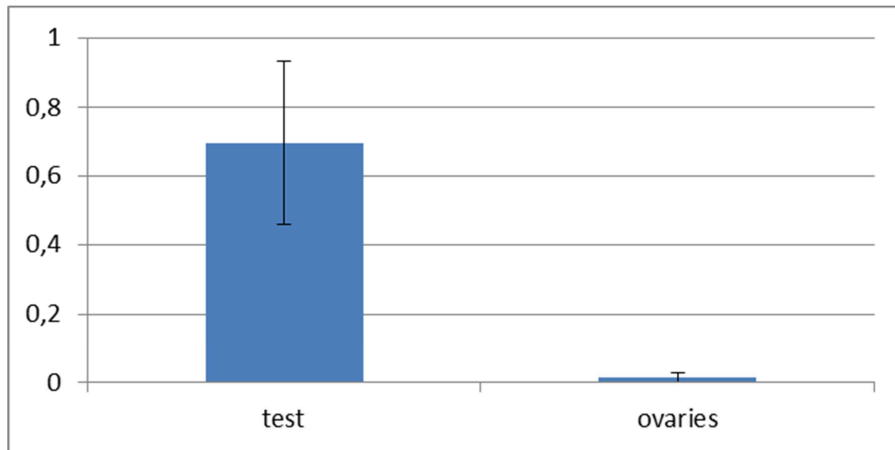
B.
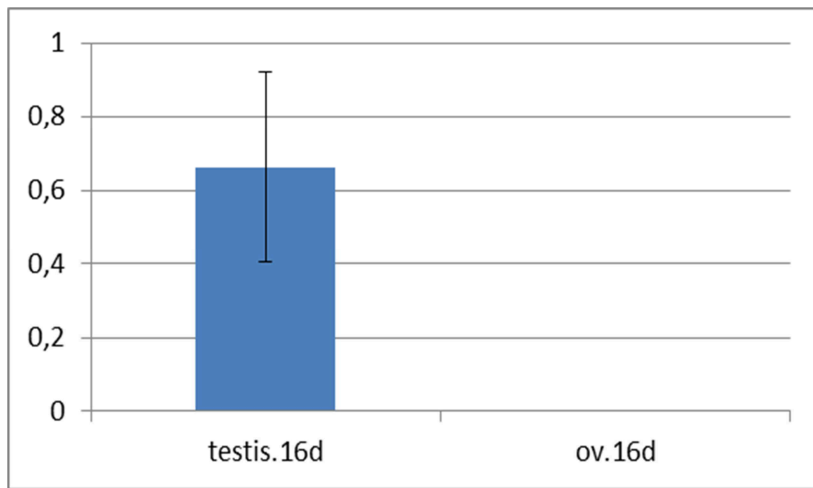
C.

D.

Figure 2 *Spiroplasma* density in *G. f. fuscipes* in testes and ovaries (**A**), testes and ovaries from 16 day old *Gff* flies (**B**), testes and ovaries from teneral *Gff* flies (**C**), testes from teneral and 16 day old flies (**D**).

Microbiota characterization of the gastrointestinal tract and gonadal tissues

A total of 550,443 high quality 16S *rRNA* gene sequences were produced through 454 FLX sequencing of gut and reproductive organs (testes and ovaries) extracts of *G. m. morsitans*, *G. palidipes*, *G. f. fuscipes* and *G. p. gambiensis* (32,379 reads per sample). These seqeunces were classified into OTUs based on 97% sequence identity using QIIME (Caporaso et al., 2010).

Analysis of the gastrointestinal track indicated that the bacterial diversity is limited with the Proteobacteria to have a dominant role. More than 95% of the bacterial diversity is dominated by Proteobacteria and in most of the cases by Gammaproteobacteria. Only in the male gut of *G. p. gambiensis* and the female gut of *G. f. fuscipes*, members of Alphaproteobacteria were detected. In the gut of teneral male of *G. f. fuscipes*, members of the Betaproteobacteria were dominant followed by Alpha and Gammaproteobacteria. Our analysis indicated also that *Wigglesworthia* and *Sodalis* are the dominat symbionts in the gastrointestinal tract of the tsetse species examined.
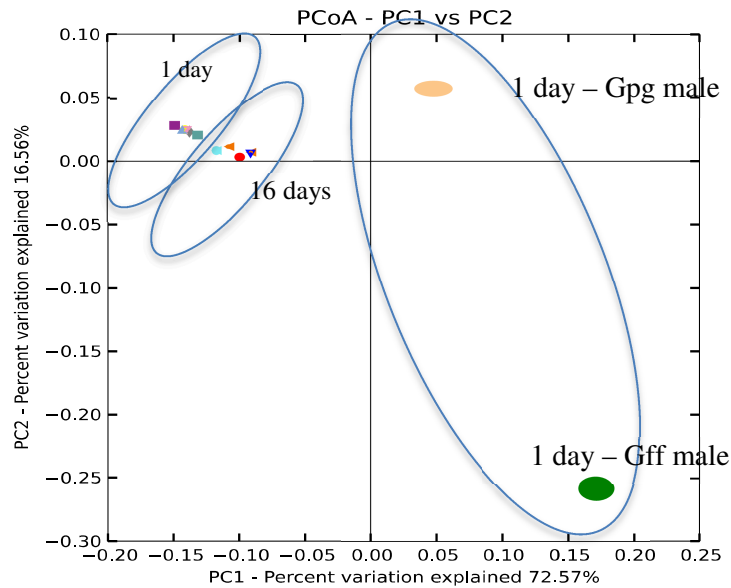
Figure 3 PCoA analysis performed on bacterial tag-encoded FLX amplicon pyrosequencing data from the *Glossina* species examined

However, prevalence varies depending on the species, the sex and the age. Our data indicate a negative interaction between *Wigglesworthia* and *Sodalis* which was evident in almost all samples. Finally, a PCoA analysis indicated that the Glossina species examined could be clustered based on age (Figure 3). The exceptions observed were due to the presence of *Wolbachia* and *Rhizobium*.

Microbiota characterization from testes and ovaries analysis has been completed for *G. f. fuscipes* (*Gff*) and *G. p. gambiensis* (*Gpg*). Our analysis indicated that the Proteobacteria is the dominant group in *Gpg* and *Gff*. 454 sequence data indicated that *Wolbachia* is present in teneral flies of *Gpg* while in 16 days old flies *Wolbachia* was not detectable by our assay. In *Gff*, *Wolbachia* was detected in males but not in females. Our preliminary data suggest the presence of competitive exclusion phenomena between Wolbachia and *Sodalis/Acinetobacter* in gonadal tissue. Finally, a PCoA analysis indicated that the *Gff* and *Gpg* samples can be clustered based on age (Figure 4).
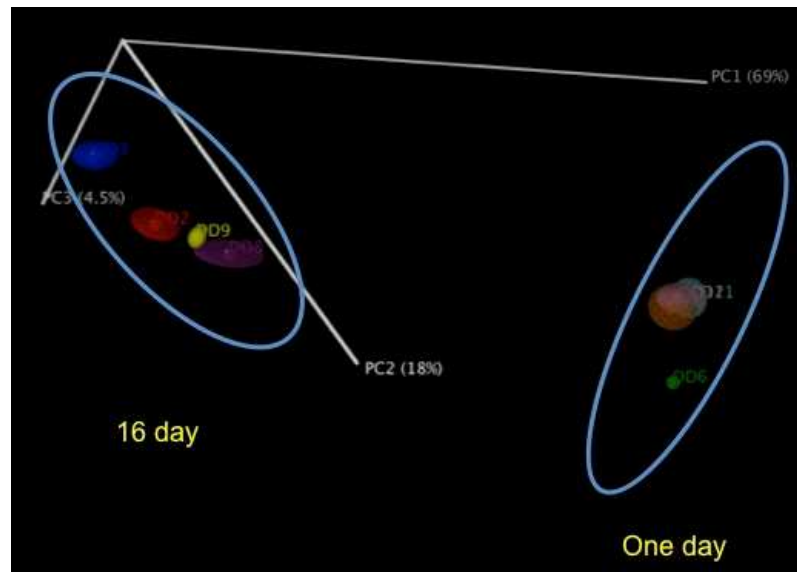
Figure 4 PCoA analysis performed on bacterial tag-encoded FLX amplicon pyrosequencing data from the gonadal tissues of *G. p. gambiensis* and *G. f. fuscipes*

Based on our results, future work will focus on:

1. collecting data from three biological replicates (our data are based on the analysis of one replicate) from lab populations.
2. performing qPCRs to confirm 16S rRNA pyrotagging data.
3. performing comparative analysis between larval and adult gut microbiota. Sex and age effects will also be considered.

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Infectomics of Glossina hytrosavirus

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Concept: The identification of expressed genes of viral entities is the main issue in recent genomic era. Expressomics, an integrated approach applying transcriptome and proteome outputs for data interpretation for *Glossina* hytrosavirus (GpSGHV) pathobiology was applied. Determination of the siRNA targeting profile and the 3-prime untranslated regions (UTRs) have been performed and following to these progresses exploring the conserved mechanisms related to viral pathogenesis will be undertaken. In addition, an integrated approach using transcriptome and proteome of infected cells performed to further knowledge on interactions between different tsetse isolates and the GpSGHV isolates are comparatively analysing currently by generation of genome and proteome maps. Knockdown of host protein factors related to viral infection will be investigated. As there is no available genetic recombination system for host as well as for virus (e.g. bacmid), *in vitro* dsRNA production will be used for generating knockdowns of target genes for clarifying the transcriptional regulation of this new group of virus (Hytrosavirus). Production of genetic recombination systems as bacmids, will greatly enhance progress of this research proposal. The ultimate aim is to unravel the key virus-host factors responsible for viral infection and latency. This research will serve for the establishment of the effective mitigation strategies for tsetse rearing factories.

Introduction

This research aims to unravel fundamental molecular mechanisms underlying interactions between the *Glossina hytrosavirus* and the tsetse fly to understand the viral infection process. It is also equally important to understand temporal regulation and UTRs information of the viral transcripts to classify them in to certain functional group e.g. related to host response, viral latency etc. After the mapping genome and the transcriptome of the different SGHV isolates followed by correlation of the transcriptome with total proteome of the virus, the realization of this objective will be facilitated by the availability of comparative genomic information for both the host and the virus that has different pathogenesis on host. This approach will avail new opportunities to understand various aspects related to the infection, assembly and dissemination of the virus in tsetse rearing facilities.

Material and Method

After performing successful infection models for *Glossina hytrosavirus* in tsetse colonies in order to unravel the difference in pathogenesis of the two GpSGHV isolates. The deep-RNA sequencing of total infected cell RNA to map viral transcriptome as well as infected cell RNA transcripts have successfully been performed. The main anticipated outcome was the confirmation of the presence of the transcript of the 160 proposed ORF in GpSGHV published sequence. This approach also opened us now an opportunity for modelling 5- and 3-prime UTR regions of RNA transcripts which will

propose the understanding the mechanisms for transcriptional regulation of viral genes of this new group of virus (Hytrosavirus). The investigation of infected cell proteins for unravelling crucial host factor which responsible for successful viral infection or keep host with virus in an asymptomatic manner. Further testing knock outs of host protein factors related to viral infection will be informative. However, there is no available genetic recombination system for host as well as not for virus e.g. bacmid. Production of possible genetic recombination systems as bacmid will be great progress for the research proposal. The aftermath this approach will contribute establishment of the effective mitigation strategies for tsetse rearing factories. After following the deep sequencing of total RNA and small RNA with Illumina HiSeq2000 platform, the transcript reads should be sorted into those originating from the positive and negative strands in order to generate bidirectional transcription profiles. This will be further confirmed by RT-PCR. Genome and transcriptome sequences of Ugandan (Seibersdorf) and Ethiopian strain of GpSGHV are available. Ugandan strain has a size of 190.032 bp genome while Ethiopian one has 189975 which encode 160 putative ORFs (Abd-Alla *et al.*, 2008). The Ethiopian isolate has recently sequenced revealing the sequence similarity of 98.7% (Table 1, Manuscript in preparation, 2015).

Table1. Comparative genomic information of two GpSGHV isolate.

	GpSGHV-Ug	GpSGHV-Eth
Genome size (bp)	190,032	189,975
ORF number	160	156
GC (%)	28	27.9

The observed differences in genome opens up the research questions below;
1. How we can determine the clear relationship between these two viruses?
2. Are THEY different viruses? different strains? or different isolates?
3. How the differences in genome is linked with the virus pathology?

To answer above questions, the expressomics approach will be used. Next generation sequencing and mass spectrometry is the main methods. The data from transcriptomic and proteomic analyses facilitated to finalize the ORF selection for Ethiopian isolate annotation (156 ORFs). The outcome of this analysis validated by comparative transcriptome and proteome assay to map cellular factors in virus-host interaction.

Next generation sequencing of the total and small RNA samples provided information on;

1. Complete transcriptome map
2. Mapping promotors, 5 and 3 prime UTRs
3. small RNA mapping
4. Convergent transcription phenomenon of viral transcripts

We used the proteomic data to understand how expressomics data can contribute to the quality of the viral genome annotation. Mass spectrometry data provided the main quality check step for

understanding truly expressed transcripts. We considered the detected proteins are the final product of the gene expression so the approach provide us a real time identification of gene products thus the confirmation of the truly expressed ORFs.

Protein samples for were prepared for LC-MS/MS. Briefly, proteins were extracted from hypertrophied salivary glands dissected from G. pallidipes, analysed by 12% SDS-PAGE, followed by in-gel trypsin digestion. The resultant tryptic peptides were analysed by liquid chromatography coupled to electrospray and tandem mass spectrometry (LC-MS/MS). The MS/MS spectra were searched against a home-made GpSGHV-Eth database, a contaminant database containing sequences of common contaminants, and a decoy database (derived from reversing the target database). The MS searches were performed using MaxQuant v 1.3.0.5, with the Andromeda search engine using default settings including maximum FDR of ≤ 0.01 using the decoy database search. The resulting MaxQuant protein list was filtered to show only those proteins with minimally two peptides matching the same protein, of which at least one peptide was unique, and at least one peptide was unmodified. The cut off length for the peptides was at least seven amino acid residues. Any hits to the decoy sequences and hits with modified peptides only were deleted (Ince et al., 2015).

The proteomic part of the proposal contributed for quantitative identification of the proteins in the various phases of viral infection. Recent advances in proteomics allow us to measure expression levels for thousands of genes simultaneously, across different conditions and over time. Analysis of the label free quantitation of the infected cell proteomic data unravels two main important phenomena. First of all, an opportunity to perform comparative global measurement of the relative protein expression levels in a related sample series (e.g. time course) offering a universal method so called expression proteomics. Secondly, this approach is technically similar with a microarray study in which transcriptome measurement is taken as a basis. However, expression proteomics obviously give a better insight as it looks at the end product of a gene expression cascade. This is better than message levels to unravel the biological functions of the protein(s) of interest. Combining these two important outcomes of the approach provides us a powerful tool to identify and quantify the different proteome states with greater details on the primary structure of proteins, protein-protein interactions and provides further insight in cellular or organismal level proteomes.

Total RNA was isolated from hypertrophied salivary gland collected from and Ethiopia tsetse production facilities. Purified RNA sample prepared for sequencing using TruSeq™ RNA Sample Preparation protocol (Illumina Inc., San Diego, CA), and sequenced using Illumina HiSeq2000 platform (Illumina). RNA libraries were prepared for sequencing using standard Illumina protocols. The data were pair ended and strand specific, data set of unaligned 178-base reads in fastq format with associated phred quality scores was generated by using Fastq Quality Encoding: Sanger Quality ASCII Character Code = Phred Quality Value + 33, and Illumina Pipeline (CASAVA) v1.8.2. The read data set was assembled against the non-annotated six frame translation of GpSGHV-Eth genomic sequence. To annotate the viral transcriptome, computational pre-processing of the sequences was performed using EA-Utils. Mapping of the paired reads to the viral genomic sequence was done using Bowtie. The viral gene expression was estimated using EDGE-pro, while gene modelling was done using EVidenceModeler. Read pairs were quality filtered using the program EA-Utils (Aronesty, 2011) to remove reads with average Phred quality scores less than 30, to trim low quality bases from the read ends, to remove residual Illumina adapter sequence, and to remove reads that were less than 75 nt in length after quality filtering and adapter removal. Filtered read pairs were mapped against GpSGHV-Eth genomic sequence using the program gsnap (Wu et al, 2010). Mapped reads were subsequently assembled using Trinity (Grabherr et al, 2011) in strand specific (RF) mode. The Jaccard clip was applied to split chimeric contigs resulting from overlapping transcripts to aid in the assembly of transcripts with overlapping UTRs, which are common in gene-

dense viral genomes. Assembled contigs that exceeded 200 nt in length were retained and screened to remove polyA tails and potential host contaminants using the program seqclean. Filtered transcripts were mapped back to the genome using the aligners gmap and blat. Contigs with overlapping regions were assembled into putative transcripts and gene models were predicted using the program PASA (Haas et al, 2003). Transcripts that mapped to the same genomic interval (with >=97% similarity at the nucleotide level) were considered redundant and were collapsed to a single transcript/gene model. PASA assembled transcripts were screened for putative open reading frames using the program Transdecoder; ORFs containing contiguous coding regions of >=50 AA were retained for comparison to GpSGHV-Eth annotations. Differential expression analysis; Expression level of each gene was calculated using Edge-PRO (Magoc *et al.*, 2013). Annotation of transcripts performed by Blast+ Blast2Go (Götz et el., 2008, 2011).

Future Remarks and Milestones

In the following research activities, we will aim to find fundamental expressed transcripts of the an hytrosavirus including determination of the 5- and 3-prime untranslated regions (UTRs), and the conserved motif for extrapolation of possible mechanisms related to viral gene transcription. In addition, an integrated approach of transcriptome and infected cell proteome will be performed to further knowledge on the interactions between the tsetse fly and the *Glossina hytrosavirus*. Viral Pathogens and Development of new control tolls are the potential direct interaction points in this case. This proposal will deliver fundamental scientific basis for understanding virus-host interaction and tsetse biology.

Milestones of the project;

- To unravel the evidence that *Glossina hytrosavirus* (SGHV) involves RNAi response, the total RNA and small RNA sequencing virus-infected and uninfected in tsetse flies will be analysed in *Glossina pallipides* as well as *Glossina morsitans*.
- Preliminary mapping of small RNA is achieved. This contributes to determine the targeting hot spots of host response to viral infection. We are aiming to find out functional hotspots for elimination of viral infection.
- Mapping of 3' untranslated region of *Glossina hytrosavirus* transcript has been performed to contribute fine mapping of viral transcriptome and understand the termination motifs of viral transcripts. Termination motifs are important for development antivirals.
- The DNA sequence of GpSGHV-Ethiopia and the re-sequencing of the GpSGHV Uganda isolate is completed, annotated and comparative genome, transcriptome and proteome were performed among two isolates with GpSGHV. Related to this achievement, the difference in pathogenesis between GpSGHV Ugandan and Ethiopian strains will be investigated.
- Comparison of the proteomic profiles of symptomatic (*Glossina pallidipes*) and asymptomatic (*Glossina morsitans*) infection state of Tsetse species to establish the fundamental mechanism behind the formation of salivary gland hypertrophy.

The ultimate aim is to unravel the key virus-host factors responsible for viral infection and latency.

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ENHANCING TSETSE FLY REFRACTORINESS TO TRYPANOSOME INFECTION

Progress report on project: Microfauna host interactions for trypanosome infection rates in wild tsetse species in tanzania and the effects of tsetse irradiations on trypanosomes and gut microfauna establishment

CONTRACT No. 17679

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1. Introduction and rationale

Tsetse midgut contain microfauna that tend to play a role in the vectorial capacity of tsetse. Research indicate that there is a close interaction of tsetse with symbionts and pathogens such as Sodalis, Wolbachia and trypanosomes; such that flies habouring Sodalis are 3 times more likely to be infested with trypanosomes than without Sodalis and Wolbachia (Geiger et al., 2007). Tsetse tend to carry a number of symbionts (Sodalis, Wigglesworthia, Wolbachia) whose effects on the physiology of the insect have been studied in depth. Other epidemiological studies on tsetse fly populations from different geographic sites revealed the presence of a variety of bacteria in the midgut and the common bacterias are genera Entrobacter (most common), Enterococcus, and Acinetobacter.

The role of resident midgut bacterial flora on the physiology of the fly and vector competence is still unknown. Lindlh and Lehane (2011) reported that *G. f. fuscipes* from Kenya harbored a number of microfauna in the gut is thought to be responsible for arresting trypanosome infection. Recent detailed study on *G. f. fuscipes* in Uganda have shown that *G. f. fuscipes* there is a close association of host genotype and pathogens (*Wolbachia* and SGHV) where as host groups were inversely correlated for *Wolbachia* and SGHV. Trypanosomes infections were significantly influenced by the presence of *Wolbachia* and SGHV. The prevalence of *Wolbachia* and SGHV were negatively correlated whereas the prevalence of SGHV and trypanosomes were positively correlated. *Wolbachia* tended to have a negative impact on SGHV and trypanosomes (Alam *et al.*, 2012). Observations from the last CRP showed that *G. f. fuscipes* had a SGHV microscopic infection rate of about 2/97 (2.1%) and 51/236 (21.6%) by PCR (Malele *et al.,* 2012). However, the trypanosome infection rate by PCR was only 1/71 (1.4%) (Unpublished). The mechanism that hinders the establishment of trypanosomes in a highly infected species with SGHV remains unknown. The data from the palpalis tsetse necessitated a need to carry out detailed investigations on the role of resident bacteria on the physiology of the fly and vector competence of tsetse *G. pallidipes and G. morsitans morsitans* (Savannah tsetse group) in Tanzania. The two species (*G. pallidipes and G. morsitans morsitans)* are among species of economic importance due to its vectorial capacity and are widely distributed and found in many areas with habitats and climatic conditions that support the breeding and survival of the two species. A thorough research on the microfauna on the species named above will increase our understanding on the role and the existing interactions between Tsetse, trypanosomes and SGHV in the gut of tsetse flies. The generated knowledge will be instrumental towards generation of refractory tsetse strains that once released in the field will not accelerate the transmission of African Trypanosomiasis in released areas.

This report covers the progress report on activities conducted at TTRI for the past 16 months with regards to "**Enhancing tsetse fly refractoriness to trypanosome infection**" through a research on "*Microfauna (SGHV and Sodalis) host interactions for trypanosomes infection rates in tsetse species in Tanzania*"

Specific objectives
1. To determine if there is association between trypanosome infections with SGHV in tsetse from HAT and non HAT endemic areas
2. To determine the diversity and association with other midgut content (Sodalis) in tsetse in HAT endemic and non-HAT endemic areas.
3. To determine the effect of midgut microfauna on the establishment of trypanosomes in the lab flies

ACTIVITIES COVERED FOR THE PAST 16 MONTHS

1. METHODS
1.1. Field collection of tsetse flies
Flies were collected from the HAT endemic area near the Serengeti Ecosystem the northern part of Tanzania. The traps were generally set up just after sunrise and live flies were removed in the collection vessels after 24 hrs since trap deployment. Trap used were Biconical traps (Challier & Laveissiere, 1973) baited with acetone.

1.2. Dissection of tsetse to examine for trypanosome infection and DNA extraction
Flies were dissected in PBS using instruments that were cleaned between each individual dissection and examined for the presence of trypanosomes and the carcasses of individual fly were placed in clean tubes containing 100% EtOH. DNA from the carcasses was extracted using Qiagen Kit. DNA was suspended in 100 µl of elution buffer and stored at -20°C (Ferreira et *al.,* 2008). For microbiota (bacterial screening), trapped tsetse were dissected to remove gut content and cultured by culture– dependent and by culture independent method as in Geiger et al., 2009). DNA extracted by Dneasy Qiagen kit for amplification for trypanosomes ITS, Sodalis, SGHV.

1.3. Trypanosomes, GpSGHV-PCR and Sodalis PCR analyses
1.3.1. Trypanosome
In the lab, DNA Amplification using Phusion Taq (FinnEnzyme) for Internal transcribed spacer (ITS) (Adams *et al* 2008) was carried out in a DNA thermal cycler (Applied Biosystem) in a final volume of 20µl reaction mixture containing 11.4µl distilled water, 4µl of 5x Phusion HF buffer (FinnEnzyme), 0.4µl 10mM dNTPs, 1µl for each Primer, 0.2ul Phusion DNA polymerase and 2µl DNA template. The polymerase chain reaction (PCR) condition involved an initial denaturation step at 98°C for 30 seconds, followed by 30 cycles of 98°C for 30s, 60°C for 40s, 72°C for 30s with a final elongation step at 72°C for 5min. 5µl of PCR product was mixed with standard loading dye (Hyperladder) and electrophoresed in 1.5% agarose stained with ethidium bromide (5µg/ml) and the product visualized and photographed under ultraviolet illumination. A positive control (with reference genomic DNA) and negative control (without DNA, only with distilled water) were included in each set of reactions.

1.3.2. *GpSGHV-PCR*

25µl PCR reaction, 12.5 µl PCR master mix (Applied Biosystem), 1 µl DNA template, 10.5 µl sterile dist water, 0.5 µl P1 and 0.5 µl P2. Cycling temperatures were as indicated by (Abd-Alla *et al*., 2007). Primers used were GpSGHV1F and GpSGHV1R and GpSGHV2F and GpSGHV2R. The PCR's were analyzed by 1.5% agarose gel electrophoreses.

1.3.2. S*odalis PCR*

DNA obtained from section 1.2 above reaction was also used for analysis of *Sodalis.* The presence of Sodalis was determined using the primers GPO1F/GPO1R (Dale & maudlin, 1999). PCR assays were performed using 1 µl of template DNA and standard conditions (Alam *et al* 2012). For each assay, a negative control (no DNA) as well as a positive control using DNA isolated from *Sodalis morsitans* was included. The quality of sample DNA was verified using insect-specific 12S mitochondrial markers (Simon et al., 1994). After completion of the PCR run, 10 µl of the amplification product was analyzed by electrophoresis in TBE buffer on a 1.5% agarose gel electrophoreses together with a 100 bp DNA ladder size standard and visualized using ethidium bromide.

2. **RESULTS**

2.1 Microscopic investigation of wild flies for trypanosomes, SGHV and Sodalis

Only 5 flies were microscopically positive in the gut. Upon PCR analysis, all 80 *G. pallidipes* samples were positive for different trypanosomes and other symbionts as tabulated below.

Table 1: Diversity of trypanosome species, Sodalis and SGHV from G. pallidipes midguts

Gut fauna	Occurrences
SGHV only	0
Sodalis only	0
Trypanosome /Sodalis/SGHV	19/80
Trypanosome /Sodalis	43/80
Trypanosome/SGHV	33/80
Trypasome only (single infection)	19/80
T. congolense genotypes/*T. brucei*	15/80
T. congolense genotypes	55/80
T. brucei	30/80
T.godfreyi and *T. simiae* each	4/80
T. vivax	7/80
T.congolense/Sodalis	15/43
T. congolense/SGHV	3/33

The proportional of trypanosome infection amounted to 58%, Sodalis infection was 24% and that of SGHV was 18%. The proportional of occurrence of all gut residents was 35% for T. Congolense genotypes; 16% for *T. brucei; T.godfreyi* and *T. Simiae* each 2%; T. vivax 5%; Sodalis 24% and the SGHV 18%. The pattern of occurrence were 10% for triple infection of Tryps/Sodalis/SGHV; double infection of Tryps/ Sodalis were 22%; Tryps/SGHV 17%; T. congolense amounted to 28% and *T. brucei* 15% as indicated in figure 1.

2.2 Interaction of SGHV, Sodalis and Trypanosome infection
The trypanosomes appeared common as single, double, triple mixed infections. The common double trypanosome infections in the triple infection of TRYPS+ SGHV+ SODALIS were *T. congolense* genotypes and *T. brucei* (6). The least were *T.vivax* (2) and *T. simiae* (2).
The common single trypanosome infection in the triple TRYPS+ SGHV+SODALIS infection were *T.congolense* (6) and or *T. brucei* (4)
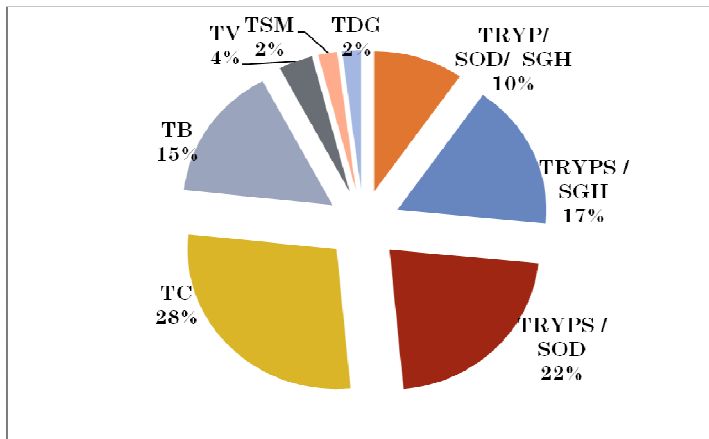

Figure 1: Pattern of occurrence of trypanosomes and symbionts

Table 2: **Interaction of *T. vivax* with other symbionts and trypanosomes in tsetse guts**

Microscopic	ITS Trypanosomes	SGHV	Sodalis	Trypanosome species
+	+	+	+	Tb/Tgod/T.vivax
	+	+	+	T.vivax
+	+	+	+	Tc/Tb/T.vivax
	+	+	+	T.vivax
	+	+	+	Tb/T.vivax
+	+	+	+	Ts/Tgod/T.vivax
	+	+	+	Ts/T.vivax
+	+	+	+	T.vivax
+	+	+	+	Tc/T.vivax

T.vivax interacted well with other trypanosomes and SGHV and *Sodalis* as indicated in the Table 2; however in order to get mature infection only of *T. vivax*; in the future study we will analyse both the mouthpart as well as the gut of tsetse

Entomopathogenic fungi vs tsetse
Preparations were completed through initial data collection, in terms of tsetse species, density and blood meal collection. Few flies were also dissected. The data were meant to have the baseline data before the implementation of the study. Analysis of collected samples is ongoing. However implementation of fungi (*Metarhizium anisopliae* isolate ICIPE 30) against tsetse was postponed till next year after the rain season. This work will be implemented jointly with ICIPE at Mwiba Hunting block outside Serengeti ecosystem. The dominant species found were *G. pallidipes* and *G.swynnertoni*

SUMMARY

Tsetse flies are the vectors of trypanosomes, the causative organisms of trypanosomiasis: nagana, in animals and sleeping sickness in human or commonly known as Human African Trypanosomiasis (HAT). Control of the disease is most effective through the control of the vector, tsetse flies. One of the efficient ways of controlling the vector is through the release of sterile insect technique. Before release, sterilized flies are introduced to a diet treated with antitrypanocidal drugs in order to minimize the risks of the released flies transmitting the diseases. However it has been published that the use of trypanocidal treated diet prior release doesn't entirely prevent released males from transmitting the disease, and this can be fatal in areas which are HAT endemic. This necessitates finding ways of minimizing the risk of released flies magnifying the epidemiology of African trypanosomiasis. Tsetse midgut contains micro fauna that tend to influence the vectorial capacity of tsetse through interaction with symbionts and pathogens such as *Sodalis, Wolbachia* and trypanosomes.

In this study we present results from the analysis of 80 tsetse samples (*G. pallidipes*) from the HAT endemic area of north western Tanzania. Of 80 flies, all tsetse flies were found infected by trypanosomes by PCR analysis and these were infected by different types of trypanosomes. Analysis for SGHV, 33 were positive, where as 43 were positive for *Sodalis*. Infection by trypanosomes, *Sodalis* and salivary gland hyperplasia virus were 19, double infections of trypanosomes with salivary gland were 11, and trypanosomes with *Sodalis* were 24. Single infections by trypanosomes only were 23. Common pattern of triple infection were *T. brucei, T. congolense* savannah (6) followed by *T. c. savannah* (4) and *T. brucei* (4). So far, results shows that the presence of *Sodalis*, have no influence on the susceptibility of tsetse to trypanosomes. Samples from this area were all infected by trypanosomes. Metagenomic analysis is ongoing to investigate on the diversity of gut micro-fauna found in tsetse from the two areas one which is endemic for HAT and a non HAT area. Results will be presented in the next RCM. The generated knowledge will be instrumental towards management of released tsetse in order to minimize the risk of released tsetse taking an active role in the epidemiology of trypanosomiasis especially in HAT endemic areas

CONCLUSION

So far, from 80 analysed, there is no clear picture of the assocation of trypanosomes, Sodalis, salivary gland hyperplasia virus. However the plan is to sequence the gut from the tsetse collected samples and this will give us the detailed and accurate information on the microbiota diversity in the midguts of tsetse flies from the HAT and non HAT sites in Tanzania. Results from sequencing will give us a true picture on the diversity and association of microfauna and their influence on the establishment of trypanosomes. This is important in order to have the epidemiological implication of the microfauna found in the gut of a tsetse fly.

FUTURE PLANS

Symbionts and trypanosome infected tsetse species from the wild populations will be identified and characterized followed by

- Sequencing gut microbiota for the samples from the HAT and non HAT endemic areas and results (diversity compared between the two sites)
- Investigate on the SGHV and microbiota interaction (pathology) from wild tsetse population

○ Investigate on the use of entomopathogenic fungi (*Metarhizium anisopliae* isolate ICIPE 30) as a tool for tsetse control.

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Working paper

Update on the *Sodalis* expression of functional anti-trypanosome nanobodies in different tsetse fly tissues.

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Introduction.

The contemporary response to vector-borne infectious diseases still mainly relies on low-technology interventions, with a major emphasis on vector control through the use of insecticides. As vector-borne diseases continue to present significant threats to human, animal and plant health, there is an urgent need to develop control efforts that remain effective over time. Genetically modified disease vectors that are rendered resistant (refractory) to pathogen transmission can provide unique tools for developing new or complementing existing control strategies (reviewed by [1]). Paratransgenesis is one such approach that aims to reduce vector competence by genetically modifying symbionts of disease vectors and has been demonstrated for *Rhodnius prolixus*, the triatomine vector of Chagas disease [2] and *Anopheles gambiae* [3-4], the principal malaria vector in Africa.

A paratransgenic approach in tsetse flies, the sole vector of major African trypanosome parasites (*T. brucei* sp. and *T. congolense*), is of high interest since tsetse flies are not amenable to germ-line transformation due to their viviparous reproductive biology (intrauterine development and parturition of live offspring [5]). The tsetse fly harbors a natural commensal bacterium i.e. *Sodalis glossinidius*, which is ideally suited as a paratransgenic platform organism since it i) resides in different tsetse tissues that are in close proximity to pathogenic trypanosomes (e.g. midgut) [6]; ii) can be cultured and genetically modified in vitro [7-8]; iii) is maternally transmitted to the offspring [9] and iv) is restricted to the tsetse host niche ensuring that this symbiont is a safe candidate for use in the paratransgenic strategy [10].

The characteristics of the selected effector molecule will largely determine the efficacy and specificity of this paratransgenesis approach. Low specificity will generally result in bystander effects that could have a direct or indirect fitness cost for the arthropod. The use of highly specific compounds from the adaptive immune system of vertebrates such as antibody derived fragments is likely to enable highly specific effects without conferring a selective disadvantage to the (para)transgenic arthropods [11]. Nanobodies® (Nbs), representing the smallest known intact antigen-binding fragments derived from camelid heavy-chain only antibodies (HCAbs) [12], are therefore considered as excellent candidates to increase the immune competence of tsetse. Nanobodies targeting distinct epitopes of the variant-specific surface glycoprotein (VSG), abundantly present on the surface of bloodstream trypanosomes have already been identified, some of which were shown to exert direct *in vitro* and *in vivo* trypanolytic activity by interfering with the complex

endocytotic machinery that is organized in the flagellar pocket of this parasite [13]. Recently, we developed recombinant *Sodalis* (rec*Sodalis*) strains expressing functional anti-trypanosome nanobodies. These strains were shown to release considerable amounts of functional anti-trypanosome Nbs to the extracellular culture environment and to have no negative effects on the bacterium in an *in vitro* context [14].

Another prerequisite in developing paratransgenic tsetse flies is the development of a methodology that allows the stable repopulation of tsetse flies with rec*Sodalis* strains expressing trypanosome-interfering proteins in insect tissues where trypanosome parasites reside. Previously, rec*Sodalis* expressing green fluorescent protein (GFP) has been successfully introduced into tsetse through thoracic microinjection [6]. Here, GFP-expressing *Sodalis* was found to be present in the haemolymph and gut tissues of injected females and their progeny. However, to date no studies have focused on the densities whereby genetically modified *Sodalis*, expressing heterologous genes, are maintained within the fly and the efficiency of their transmission to the offspring.

In this study, a rec*Sodalis* strain was tested for its ability to deliver functional anti-trypanosome Nbs in the tsetse fly *Glossina morsitans morsitans*. The *in vivo* long-term stability of the recombinant strain and transmission to the progeny was measured using a quantitative PCR (qPCR) analysis. We show that rec*Sodali*s expressing a potent trypanolytic nanobody, i.e. Nb_An46, was stably maintained in vivo only when the WT *Sodalis* population was significantly reduced (> 95% of the normal population) prior to rec*Sodalis* introduction. Furthermore, we demonstrated that significant levels of functional Nb_An46 accumulated in different fly tissues, including the midgut where an important developmental stage of the trypanosome parasite occurs.

Results

In vitro culture characteristics of recombinant Sodalis expressing Nb_An46

Prior to the introduction of rec*Sodalis* into experimental flies, the Nb expression profile, in vitro growth rate and plasmid stability of rec*Sodalis* expressing a FliCpelBNb_An46 fusion protein (Sod:FliCpelBNb46fliC) was established. Extracellular Nb_An46 expression was confirmed by Western blot analysis of supernatant from cultures grown to the beginning of stationary phase (OD600 0.5-0.6). Nb_An46 expression and release was quantified at different time points during bacterial growth over a 10-day period by measuring the concentration of active Nb in the whole cell lysate and culture medium using a VSG-binding ELISA assay. Functional Nb_An46 was expressed from day 2 onwards and accumulated in the culture medium to a concentration of 88 ng/ml by day 10. Rec*Sodalis* showed normal growth kinetics with cell population doubling times comparable to a WT *Sodalis* strain i.e., 15.03 hrs and 14.82 hrs respectively. The number of plasmid copies per cell was estimated to be approximately 20 during the lag and exponential phases of Sod:FliCpelBNb46fliC grown in the presence of antibiotic selection. The stability of the FliCpelBNb46fliC plasmid in rec*Sodalis* was measured by maintaining the recombinant bacteria in log phase growth for 27 generations in liquid MM medium in the absence of antibiotic selection. Colony counts on antibiotic-selective plates showed that 94% of the *Sodalis* population remained antibiotic resistant after 27 generations (corresponding to a 2-month test period) (Table 1).

Prior reduction of WT Sodalis is a prerequisite for an efficient host colonization with recSodalis

We explored the capability of rec*Sodalis* to colonize the tsetse fly after introduction through intrathoracic injection. We first evaluated the necessity of the prior reduction of the WT *Sodalis* population in tsetse for rec*Sodalis* to establish and colonize its host. In female flies that received 3 streptozotocin supplemented blood meals, the WT *Sodalis* population was reduced by 95% and 88% in abdomen and thorax tissues respectively, compared to flies fed on normal blood. This treatment did not affect the obligatory *Wigglesworthia* symbiont population that mainly resides in the tsetse fly abdomen. Next, the in vivo persistence and growth of the recombinant bacteria in streptozotocin-treated and non-treated flies injected intrathoracically with $5x10^4$ CFU Sod_FliCpelBNb46fliC was evaluated using qPCR. In streptozotocin-treated flies, rec*Sodalis* was able to proliferate inside its host, reaching densities of 10- and 20-fold the initial injected dose in respectively abdomen and thorax, whereas in non-treated flies the injected rec*Sodalis* population was not able to expand and remained present at its initial density.

The effect of differential doses of recSodalis inoculum on tsetse fly colonization and viability
We determined the optimal dose for recSodalis injection in terms of host colonization and its viability. For this, streptozotocin-treated male flies were microinjected with either $5×10^4$, $5×10^5$, 10^7 and $5×10^7$ CFU Sod_FliCpelBNb46fliC and rec*Sodalis* densities in abdomen and thorax tissues were determined 7 and 14 days post-injection (dpi) using qPCR. Injection of $5×10^7$ CFU resulted in an increased fly mortality (up to 59% mortality 14 dpi), whereas limited mortality (≤ 25% 14 dpi) was observed within the other injection groups. In these groups, rec*Sodalis* was able to repopulate the abdomen and thorax to densities comparable to natural *Sodalis* levels present in WT flies (on average respectively $2.5×10^6$ and $1.5×10^6$ CFU), demonstrating that a wide range of rec*Sodalis* doses (i.e. $5×10^4$ to $1×10^7$) are suitable to initiate colonization without affecting fly viability.

recSodalis persists in the tsetse fly and is vertical transmitted to the offspring but at a low extent.
The *in vivo* persistence of Sod_FliCpelBNb46fliC was evaluated by qPCR based estimation of the amount of rec*Sodalis* CFU in abdomen and thorax tissues of streptozotocin-treated male flies injected with $1×10^7$ recombinant CFU over a 28 day period. Sod_FliCpelBNb46fliC was able to remain present at high densities in abdomen and thorax tissues of experimental flies throughout the course of the 28-day observation period. In flies injected with Sod_FliCpelBNb46fliC, the entire *Sodalis* population in abdomen and thorax remained recombinant. Next, we evaluated rec*Sodalis* densities in the haemolymph and midgut tissues of flies injected with $1×10^7$ recombinant CFU. Sod_FliCpelBNb46fliC was able to reach the fly midgut where it persisted at densities between $5×10^4$ and $1×10^5$ CFU (DNA equivalent) throughout the 21-day observation period. In these flies the obligatory *Wigglesworthia* symbiont population was not affected by the presence of Sod_FliCpelBNb46fliC nor did we observe any effect upon the fecundity of female rec*Sodalis* colonized flies. Transmission dynamics of the recombinant bacteria to the F1 progeny was evaluated by qPCR. Sod_FliCpelBNb46fliC was transmitted to the F1 generation, although non-plasmid containing *Sodalis* were dominant in these flies. Indeed, Sod_FliCpelBNb46fliC constituted only 7 and 5 % of the entire *Sodalis* population in respectively abdomen and thorax.

Functional Nb_An46 is expressed in different tsetse fly tissues.

Nb_An46 expression in flies injected with 1×107 Sod_FliCpelBNb46fliC CFU was quantified using a VSG-binding ELISA. Nanobody concentrations were determined at different time points post-injection in whole abdomen and thorax extracts, haemolymph and midgut. Functional Nb_An46 accumulated in haemolymph and thorax samples of injected flies over time, indicating a continuous transgene expression by recSodalis in these tissues. In the thorax the Nb_An46 concentration increased from 22 ng on day 14 post-injection to 35 ng on day 21. Although significant quantities of active Nb_An46 were detected in abdomen and midgut using a VSG-binding ELISA, the accuracy of this quantitation could have been hampered by the abundant presence of proteolytic enzymes in the tsetse fly gut, probably resulting in fast degradation of the nanobodies in the tissue homogenates and possibly resulting in the underestimation of the active Nb content of the non-digestive part of the tsetse fly gut.

Discussion

The aim of this study was to develop a functional *Sodalis*-based shuttle system that is able to deliver and express anti-trypanosome nanobodies within the tsetse fly. In this study a recombinant *Sodalis* strain was used expressing a potent trypanolytic nanobody Nb_An46 through a plasmid-based expression system. The functionality of the system was assessed by monitoring if rec*Sodalis* bacteria can (1) establish a stable population in the tsetse fly over time, (2) express significant levels of active anti-trypanosome nanobodies in the tsetse fly and (3) be efficiently transferred to the offspring.

Although the growth characteristics of Nb-expressing rec*Sodalis* were shown to be similar with those of the WT *Sodalis* in *in vitro* culture conditions it is plausible that in the tsetse fly in vivo environment the endogenous WT bacteria could have a competitive advantage over the introduced recombinant bacteria. Our results clearly suggest that rec*Sodalis* is competitive with WT *Sodalis* in *in vivo* conditions, however the maximal total *Sodalis* density in the inner tsetse fly environment seems to be limited to approximately 5x106 CFU. Indeed, only when the existing WT *Sodalis* population is priorly reduced (> 95%) by a selective antibiotic treatment, the introduced rec*Sodalis* population is able to maintain and proliferate to comparable density levels observed for wildtype *Sodalis* in normal tsetse flies. This rec*Sodalis* population is then able to outgrow the WT population which remains

present at low density. In contrast, in non-treated flies rec*Sodalis* is not able to displace the WT *Sodalis* population but remains present at low density, confirming the importance of creating a WT *Sodalis* deprived niche that allows rec*Sodalis* to proliferate inside its host.

An important factor when using episomally located plasmids in a paratransgenic system is the persistence of these recombinant strains in the tsetse fly in the absence of antibiotic selection as premature loss of expression due to plasmid instability would not be desirable. Therefore, we assessed the stability of the recombinant strains during in vitro culture maintenance and after re-introduction in the tsetse fly. Results from the bacterial plate assay showed that in the absence of selection pressure in the culture medium the FliCpelBNb46fliC plasmid proved to be stably maintained with 94% of the *Sodalis* population retaining the plasmid after 27 generations. This strong long-term plasmid retention in rec*Sodalis* was not always observed as for another strain (containing the FliCpelBNb33fliC plasmid) a complete plasmid loss in the total *Sodalis* population after 20 generations in culture. We postulate that the strong persistence of the FliCpelBNb46fliC plasmid in the rec*Sodalis* culture in the absence of a selection pressure is related to the high plasmid copy number that is observed in this strain and which was 20 fold higher than in the unstable Sod:FliCpelBNb33fliC strain.

Sod_FliCpelBNb46fliC remained present at high densities in the fly throughout the 28 day observation period. Furthermore, rec*Sodalis* was able to disseminate into the digestive tract reaching densities comparable to those in flies harboring WT symbionts. Furthermore, the majority of the total *Sodalis* population remained recombinant for the duration of the experiment indicating that the high plasmid stability that was observed in the in vitro culture is also present in the *in vivo* tsetse fly environment.

Although transmission to the F1 generation was observed, this seemed to be highly inefficient as only a small percentage of the total *Sodalis* population was found to be recombinant. This could be due to plasmid loss by rec*Sodalis* during the 30-day pupal stage or the inability of rec*Sodalis* to efficiently colonize the milk glands in the adult female fly which is a prerequisite for rec*Sodalis* transmission to the intra-uterine larvae through the milk secretion. Indeed analysis of transcripts encoding *Sodalis* motility genes, fliC and motA, and cell invasion genes, invA1 and invA2, are up regulated in the larval and early pupal stages, and not in adult tsetse flies [15]. These results indicate that the Type-III secretion system and flagellum may be important for the transmission and establishment of symbiont infections in the intra-uterine progeny. Our results clearly indicate that for a successful use of *Sodalis* as paratransgenic vehicle in the tsetse fly several methodologies should still be improved allowing i) the generation of more stable rec*Sodalis* i.e. through an efficient methodology to insert exogenous DNA directly into the bacterial genome and ii) a more efficient transfer of the rec*Sodalis* to the next generations.

Rec*Sodalis* was found to continuously express functional nanobody in the tsetse fly as indicated by the Nb_An46 accumulation in the haemolymph over time. Abdomen and midgut extracts from the majority of insects that carried the recombinant nanobody-producing bacteria were positive in the ELISA, indicating the presence of functional Nb in these tissues. However the abundant presence of proteolytic enzymes in the tsetse fly midgut probably interfered with detection, prohibiting accurate quantification of the Nb expression in this tissue. These results suggest the benefit of lowering the susceptibility of potential effector proteins to proteolytic degradation especially when they have to be

functional in a digestive tissue such as the tsetse fly midgut. Nbs have the advantage that they can be mutagenized and selected for increased proteolytic stability [16].

From our experimental data, the expected levels of *in vivo* Nb expression are in the lower ng range. However, these levels of Nb expression should be sufficient to interfere with trypanosome development given that an infective blood meal of a tsetse fly in nature is estimated to contain around 103 parasites as the average parasitaemia in *T. brucei* infected cattle fluctuates from 1.5×105 parasites per ml during the acute phase of infection to 5×104 parasites per ml during the chronic phase [17] and in vitro studies have shown that nanomolar concentrations of trypanolytic Nbs are sufficient to saturate >95% of the surface VSG molecules and to provoke efficient trypanosome lysis [13, 18]. Indeed, since a VSG:Nb molar ratio of 1:20 is sufficient to cause efficient lysis, 5 ng of Nb_An46 should be adequate to efficiently lyse 103 parasites.

Conclusion

These data are the first to show the potential of *Sodalis* as a delivery system for anti-trypanosome effector molecules in tsetse fly tissues relevant for trypanosome development. Given the ability of recombinant *S. glossinidius* to efficiently establish in different tsetse fly tissues at high densities and their capacity to release significant levels of functional anti-trypanosome Nbs in tissues were trypanosomes reside, the foundation has been laid for further exploration of the inhibitory effect on trypanosome development in the tsetse fly. Moreover, a paratransgenic approach using *Sodalis* to deliver Nbs that target the trypanosome-tsetse fly crosstalk could open a new avenue to unravel the molecular determinants of this specific parasite-vector association.

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Research Report / working document CRP-2, July 2013 – December 2014

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The major objectives for the next period of sixteen months' of the Coordinated Research Project (CRP) were: (i) to understand the status of *Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV) in symptomatic and asymptomatic tsetse flies, and (ii) to make further investigations into the genetic diversity of GpSGHV in wild populations within or among *Glossina spp.* in Africa.

(i)   Status of GpSGHV in (a)symptomatic flies
One of the outcomes of the first CRP has been the hypothesis that the covert asymptomatic infection of GpSGHV in the tsetse flies (Abd-Alla *et al.*, 2010; Kariithi *et al.*, 2013) may represent a case of viral latency. GpSGHV has been detected in many *Glossina* spp. by PCR, but generally without hypertrophy or hyperplasia of the salivary glands (SGH). *G. pallidipes* is an exception, where SGH can reach 100% in laboratory cultures. It is likely that GpSGHV is present in 100% of the *G. pallidipes* flies (Abd-Alla *et al.*, 2007). As such SGHV may be part of the microbiome of the tsetse fly, together with the bacterial endosymbionts (*Wolbachia* spp., *Sodalis* spp., *Wigglesworthia* spp.), gut flora and other bioagents (viruses, Rickettsiae, spiroplasms, etc.) (Boucias *et al.*, 2013).

In *G. pallidipes* in particular, but also in other *Glossina* species, SGHV can proceed into SGH. Among the viral pathological effects are reduced fecundity, reproductive dysfunction and ultimately collapse of the laboratory colonies. These effects not only endangers the application of Sterile Insect technique (SIT) to control tsetse flies and hence the debilitating zoonotic African trypanosomoses, but also jeopardizes research into tsetse biology/genetics because the tsetse colonies are primary source of research materials. The occurrence of SGH can be prevented by application of antiviral drugs such as valcyclovir and valacyclovir, which block viral replication by inhibit the viral DNA polymerase (Abd-Alla *et al.*, 2012; 2014) and by clean feeding protocols (to prevent horizontal viral transmission during the *in vitro* membrane feeding). The major difference between *G. pallidipes* and the other *Glossina* species is the absence of detectable *Wolbachia* in the former, suggesting a role of *Wolbachia* in controlling GpSGHV in other *Glossina* spp. *Wolbachia* has been implied in provoking an RNAi response (Merkling and Van Rij, 2013).

In the natural tsetse populations, GpSGHV is primarily transmitted vertically (mother-to-progeny), either trans-ovum or infected milk gland secretions (Jura *et al.*, 1989; Kokwaro *et al.*, 1990; Sang *et al.*, 1998; Sang *et al.*, 1999; Boucias *et al.,* 2013). In laboratory tsetse fly colonies, GpSGHV can also transmitted horizontally through the membrane feeding regime via salivary secretions of the fly into the blood (Abd-Alla *et al.*, 2010; Feldmann, 1994; Kariithi *et al.*, 2011). In the case of *G. pallidipes*, artificial infection of the first (parental) generation flies with GpSGHV via microinjections does not result to any SGH symptoms, rather, the SGH symptoms are noted from the F_1 onwards with later progenies exhibiting up to 100% SGH prevalence (Boucias *et al.*, 2013). Noteworthy, so far, oral infections of the flies via contaminations of blood meals with GpSGHV does not result to SGH either in the parental generation or their subsequent progenies. During asymptomatic infection state, GpSGHV is likely to be present in the milk glands, salivary glands and the reproductive organs. The question is how the virus is 'maintained' in the fly: (i) integrated in the chromosomes, (ii) as an episome in the nuclei or (iii) as a low-level, covert infection in these organs. In all cases the virus is 'under control' of the host and the question is what type of control is exerted. A specific RNAi response is one of the possibilities.

The model systems in this work package were *G. pallidipes*, where SGH frequently occurs, and *G. morsitans morsitans,* which is mostly asymptomatic. We assume that GpSGHV is not present in the host DNA or as an episome, since the amount of GpSGHV copies in the target organs are much lower than would be expected, when the virus was in the host genome or present as an episome in each cell. Currently, we hypothesize that GpSGHV is under RNAi control, as has been initially demonstrated for invertebrate iridoviruses in dipterans (Bronkhorst *et al.*, 2012; Kemp et al., 2013) and for baculoviruses in lepidopterans (Jayanchandran *et al.*, 2012). Since then, more publications appeared showing that RNAi is involved in the surveillance of invertebrate viruses, such as densovirus (Ma *et al.*, 2011), whispoviruses (Huang et al., 2013) and even plant DNA viruses (Blevins *et al.*, 2011). Thus it has become clear that both RNA and DNA viruses are under RNAi surveillance (Figure 1; Merkling and Van Rij, 2013) and it is very likely that SGHVs are also under the same kind of host surveillance. GpSGHV infections are frequently observed, but stay asymptomatic and seldom result in SGH in nature. Thus, the question is also how GpSGHV infection progresses from a covert asymptomatic infection to an overt symptomatic infection, and what the role of the host RNAi defense and possibly the microbiome may be.
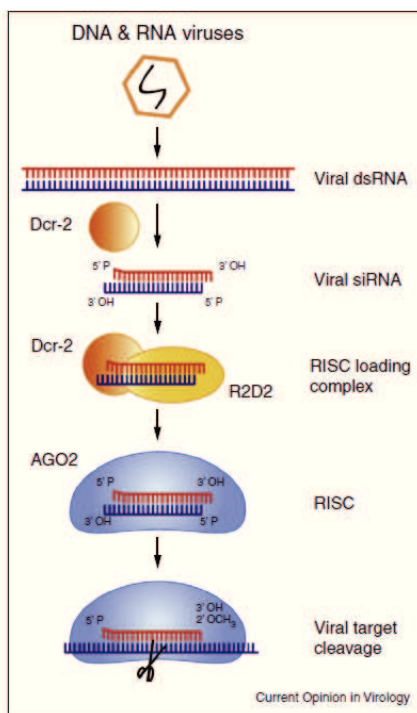

Figure 1: RNAi pathway in invertebrates

We attempted to answer these questions, by setting up two types of experiments whose objectives were aimed to: (1) Compare the mRNA levels of the key players in the RNAi defence, Dicer-2 and Ago-2, in asymptomatic and symptomatic *G. pallidipes* flies, and (2) eliminate Ago-2 and Dicer-2 transcripts in tsetse flies by using Ago-2 and Dicer-2-specific dsRNA. To achieve these objectives, the first task was to identify the Ago and Dicer genes in the genome of *G. m. morsitans* (IGGI-consortium, 2014) and to screen the *G. pallidipes* genome for similar genes using PCR with degenerated primers. In this way the Ago-2 and Dicer-2 genes of both *Glossina* species were identified.

Using Ago-2 and Dicer-2-specific primers mRNAs for these genes were detected by RTqPCR and quantitated. The result indicated that both Ago-2 and Dicer-2 were up-regulated four times and twelve times, respectively, in symptomatic F_1 flies, as compared to asymptomatic flies in the F_1. This clearly indicated that the RNAi pathway is up-regulated upon SGHV infection. Currently, the viral small RNAs (vsiRNAs; 21-24nt) are being analyzed and assigned to the SGHV genome to find their origin. This strongly suggests that the RNA pathway is involved in the surveillance of SGHV infection in tsetse flies. It would be interesting to see what happens (in terms of induction

of SGH symptoms), when the Ago-2 and Dicer-2 genes of asymptomatic *G. pallipides* flies are knocked down. In parallel report (Drion Boucias), there are also indications in the housefly, that infected flies Ago-2 and Dicer-2 genes are up-regulated, suggesting the hytrosaviruses could be under RNAi surveillance.
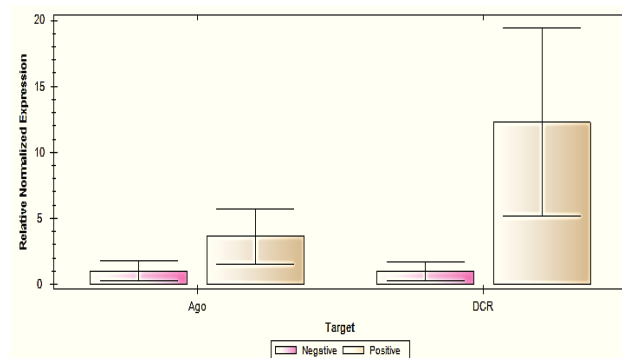

Figure 2. Ago-2 and Dicer-2 expression in *G. pallidipes* flies with hyperplasia

To find out whether RNAi is is also involved in the surveillance of SGHV in *G. m. morsitans*, Ago-2 and Dicer-2-specific RNA knock down was applied as a strategy to see whether the replication of SGHV is increased in this fly species and possibly induction of the SGH. SGH is rarely seen in this species in the field and in the laboratory.

In the laboratory of Jan Van Den Abbeele (ITM, Antwerp), *G. morsitans morsitans* flies were injected with either water (-ve control), 15 µg of long dsRNA (300bp) of GFP (+ve control) or *G. m. morsitans*-specific Ago-2 and Dicer-2. Long dsRNA is routinely used in this laboratory to effectively knock down host genes of tsetse flies. At 8 and 12 days post injection (d-pi) RNA was extracted from the abdomens of the flies, and used for cDNA synthesis. qPCR was carried out with Ago-2 and Dicer-2-specific primers, in locations on these genes other than the 300bp, and two housekeeping genes (tubulin and GAPDH) for normalization. The expression levels were corrected for the water control. Per condition five tsetse flies (n=5) were used.
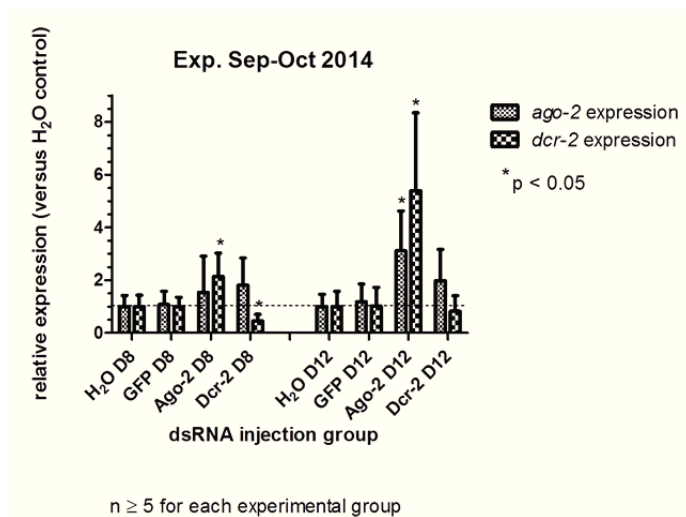

Figure 3. Knockdown of Ago-2 and Dicer-2 genes in *G. m. morsitans*.

After 8 d-pi only Dicer-2 mRNA was silenced (54%), Ago-2 mRNA level was as the wild type. At 12 d-pi, both Ago-2 and Dicer-2 mRNA were significantly up-regulated in the Ago-2 dsRNA injection group.. Surprisingly, no up-regulation of either Dicer-2 nor Ago-2 was observed upon treatment of the flies with the GFP-dsRNA (+ve control). (Figure 2). qPCR on SGHV DNA of all flies was about the same, suggesting no effect on SGHV replication.

The most likely explanation is that after a brief period of silencing the silencing machine was activated rather than down-regulated. A preliminary conclusion can be that dsRNA in this particular case did not work so well and that is may better to keep the Dicer and Ago machinery intact and use siRNAs for knock-down of the RNAi pathway. In addition to looking at mRNA of Dicer-2 and Ago-2 mRNA levels and virus replication, it may be worthwhile to look at the F1 for SGH or virus levels.

(ii) Comparative salivary gland proteomes of GpSGHV-infected *G. pallidipes* and *G. morsitans*

Infection of the tsetse fly by GpSGHV compromises maintenance of healthy colonies of some tsetse fly species. Tsetse fly colonies are a key component of African trypanosomiasis eradication campaigns, as well as for research on physiology/genetics of the disease vector. SGHV replicates in the salivary glands (SGs) and is both horizontally and vertically transmitted. During SGHV infection, the damage to the SGs is obvious, and is accompanied by a reduction in the host's general fitness and survival. Majority of the GpSGHV-infected flies are asymptomatic. It is only in a small proportion of *G. pallidipes* that the disease symptom - salivary gland hypertrophy (SGH) - is overt under some unknown conditions/triggers. Although it has been postulated that SGHV favors development of trypanosomes in the SGs, the interplay between SGHV/trypanosome/microbiome/tsetse host is not well understood. However, our efforts to elucidate this interactomics have revealed that SGHV modifies the SG epithelial cells, and reduces tsetse immunity, thus favoring colonization of the glands by trypanosomes, and increases the virus transmission opportunities. We also have indications that the microbiome greatly influences the mother-to-progeny transmission of the virus, and that the microbiome may play role in the maintenance of asymptomatic infection state of the virus. However, it is not known: (1) what factors are responsible for expression of overt SGH symptoms in a selection of *G. pallidipes*; (2) why some tsetse species such as *G. morsitans* do remain asymptomatic despite high SGHV titers; (3) What is the role of SGHV in vectorial ability of tsetse fly to transmit African trypanosomes.

In an attempt to answer these questions, we first infected both *G. pallidipes* and *G. morsitans* with GpSGHV via micro-injections. After the injections, seven intact pairs of salivary glands were dissected out from the F_1 progenies and the proteome determined by mass spectrometry. prior to the dissections, we verified whether GpSGHV is transmitted to the F_1 progenies in *G. morsitans* as is the case in *G. pallidipes* (Boucias *et al.*, 2013). To achieve this, viral titres were quantified by qPCR using a single intermediate leg excised from each of the seven live flies. The results presented in figure 4 below clearly indicates that indeed GpSGHV is transmitted to progenies in *G. morsitans* but the titres are significantly lower than in *G. pallidipes*.
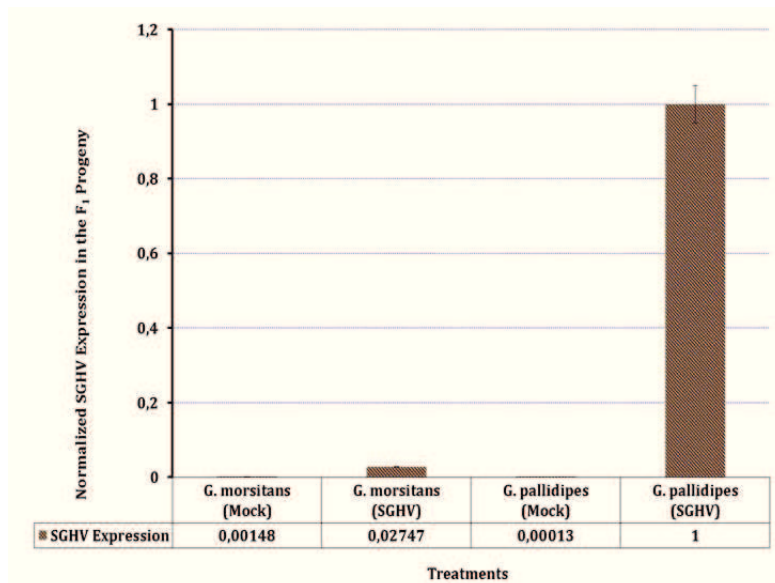
Figure 4: Quantification of GpSGHV titres in the F1 generations of *G morsitans* and *G. pallidipes*. The viral titres were quantified by qPCR using a single intermediate leg of live flies, whose mothers had been micro-injected with 2 μl of virus suspension. The mocks were injected with equal volumes of filter-sterilized PBS.

Next, we compared the proteomes of SGHV-infected salivary glands of the two model tsetse species i.e. *G. pallidipes* and *G. morsitans.* By this approach, we identified 218 host (*Glossina*) proteins, 33 endosymbiont proteins, and 73 viral proteins. The identified proteins are depicted in the log-log plot in figure 5 below.
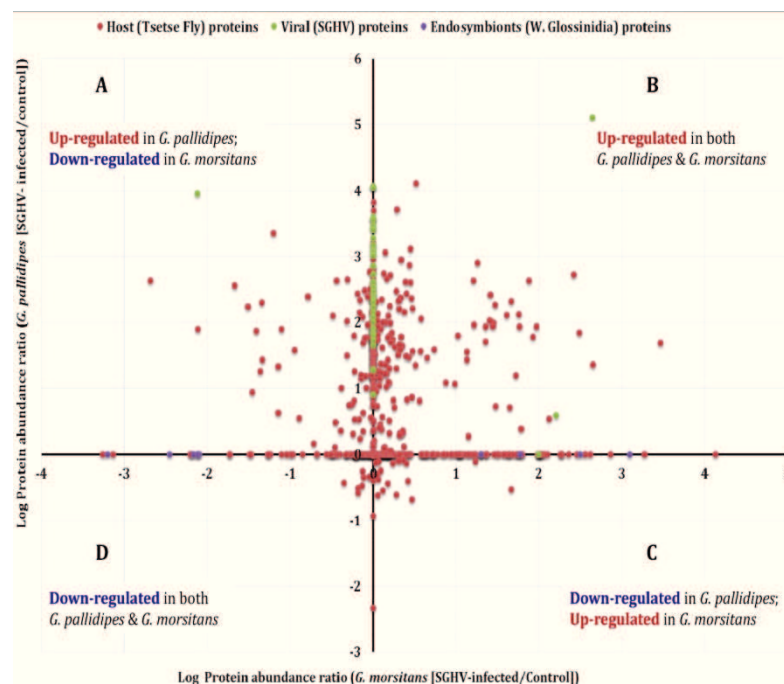

Figure 5: Log-log plot of the abundance distribution ratios of *G. morsitans* and *G. pallidipes* salivary gland proteins: The figure shows six protein groups. (A) Proteins that were down-regulated in the proteome of *G. morsitans* but up-regulated in that of *G. pallidipes* upon SGHV-infection. (B) Proteins that were up-regulated in the proteomes of both *G. morsitans* and *G. pallidipes* upon SGHV-infection. (C) Proteins that were down-regulated in the proteome of *G. pallidipes* but up-regulated in that of *G. morsitans* upon SGHV-infection. (D) Proteins that were down-regulated in the proteomes of both *G. morsitans* and *G. pallidipes* upon SGHV-infection. The other two groups are proteins that were detectable in the proteome of *G. pallidipes* but were not measurably detectable in that of *G. morsitans* (aligned on the Y-axis), and proteins detectable in the proteome of *G. morsitans* but not detectable in that of *G. pallidipes* (aligned on the X-axis).

To identify proteins that may be responsible for the SGHV pathobiology in *G. pallidipes* compared to *G. morsitans*, we focused on the proteins that are differentially expressed in the proteomes of these two tsetse spp. Of the 218 host proteins that were identified, 57 were up-regulated in the proteome of SGHV-infected *G. pallidipes* but were down-regulated in the proteome of virus-infected *G. morsitans.* Of the 57 proteins, 23 were ≥ 100-fold up-regulated. Notably, our analyses revealed that, the 23 proteins identified in this study have homologs in various hosts, and that viruses infecting these hosts do hijack these proteins to facilitate their replication and transmission. Table 1 below provides a summary of the analysis of the 23 host proteins. Further, this virus-hijack of host proteins involve host's signaling pathways that more often than not result in cellular proliferation. Preliminary analyses of this cohort of 24 proteins indicate that upon infection, SGHV modulates at least 10 different host pathways, some of which may explain expression of overt SGH symptoms in *G. pallidipes* as opposed to *G. morsitans*. In addition, our pathway analyses and interactome reconstructions give indicate that the cohort of viral and endosymbiont proteins that we identified in this study interact at various levels to influence the outcomes of SGHV infection and transmission, with the potential of influencing trypanosome-tsetse interface. Taken together the data from this study offers various potential targets for development of rationally designed strategies to control SGHV in tsetse fly colonies, as well as control of trypanosomes in wild tsetse populations

Table 1: Twenty-three proteins that were ≥ 100-fold up-regulated in the SGHV-infected *G. pallidipes* but were down-regulated in the proteome of virus-infected *G. morsitans*: - .

No.	UniProt IDs	Protein names	Length [aa]	Mol. weight [kDa]	Functional characteristics/Annotation (Roles during virus infection)
1	D3TP07	Proteasome subunit alpha-4 type	248	27.918	ATP/ubiquitin-dependent non-lysosomal proteolytic pathway; (interacts with viruses; crucial for virus replication by blocking protease activity and stimulates transcription trans-activation by viruses)
2	D3TS03	ER glucose-regulated protein; (Hsp90)	716	81.953	Molecular chaperone that promote maturation, structural maintenance and regulation of proper folding of proteins involved in signal transduction; (virus-controlled transcriptional/translational switches)
3	D3TSB7	Ubiquitin/SUMO (small ubiquitin-related modifier) activating enzyme uBA1	567	64.293	Coiled coils; Alters the function, location or trafficking of a protein, or targets it to the 26S proteasome for degradation; (SUMO is a partner protein to viral replication centres/virus assembly; the proteins sumoylate and therefore prompt viral gene expression, benefit viral replication)
4	D3TMU2	Integrin-linked kinase (ILK)	448	50.871	ILK is involved in processes that diverse signaling pathways; ILKs are up-regulated in unregulated cell proliferation, migration, and inhibition of apoptotic arrest; (receptor-mediated viral entry and egress)
5	D3TM51	Mitochondrial oxoglutarate/malate carrier protein (OGC)	318	35.072	Mitochondria carrier (MC) protein family; (OGC is up-regulated during virus infection as adaptive response to prevent mitochondrial injury);
6	D3TS86	40S ribosomal protein S16	141	15.952	Protein synthesis; Constituent proteins of stress granules (SGs) and processing bodies (P-bodies) that are involved in mRNA turnover in; (viruses modulate SGs and P-bodies to promote synthesis of viral proteins)
10	D3TQA9	Ribosomal protein L19	204	24.083	
13	D3TQT3	Ribosomal protein L5	297	33.983	
14	D3TLP1	60s ribosomal protein L7	255	29.731	
7	D3TM09	Transketolase protein (TKTL) 2-like	627	68.103	TKTL provide a link between the glycolytic, pentose-phosphate, and nucleotide synthesis pathways; (during virus infections when rapid DNA synthesis is required, glucose carbon molecules are channelled towards nucleotides synthesis through TKTL pathway)
8	D3TSC0	Hemomucin	549	60.823	(Salivary gland mucins are up-regulated during virus infection; represent insect host defence response to arbovirus infection)
9	D3TRZ3	E3 - ubiquitin ligase	150	16.537	Zinc ion-binding protein with specialized functions; (during virus infection, the protein targets specific cellular proteins for destruction by the ubiquitin proteasome system (UPS); viruses hijack UPS to promote favourable cellular environment or to block activation of host's defence mechanisms)

No.	UniProt IDs	Protein names	Length [aa]	Mol. weight [kDa]	Functional characteristics/Annotation (Roles during virus infection)
11	D3TRS6	Annexin	324	35.941	(Annexin II complex family proteins are implicated in virus assembly on lipid rafts, and directing virions to the cellular exocytotic machinery, thus aiding in non-lytic virus egress from infected cells)
15	D3TME1	Annexin	319	35.299	
12	D3TMI0	Protein ZASP (z band alternatively-spliced PDZ-motif protein)	302	33.498	PDZ domains are found in cytoplasmic and adapter proteins involved in diverse cellular processes of significance to virus infection including maintenance of cell-cell junctions, establishment of cellular polarity, apoptosis, and signalling pathways in insects; viruses modulate PDZ proteins to enhance their replication, dissemination in the host, and transmission to new hosts.
16	D3TMN6	Eukaryotic translation initiation factor 3 subunit M (eIF3m)	387	44.081	Protein synthesis; eIF3m plays critical roles in promoting the initial translation of viral immediate early proteins; inhibition of eIF3m blocks virus infection
17	D3TNJ0	26S proteasome regulatory complex subunit RPN2/PSMD1	1005	111.24	Protein synthesis; enzyme regulatory activity; (the ubiquitin/26S proteasome system (UPS) is part of the unfolded protein response (UPR) machinery, an early event essential for persistent virus infection that benefits virus replication)
18	D3TMA0	GTPase Rab2	213	23.568	Ras-like small GTPases functioning as 'molecular switches, and key regulators of (vesicular) membrane traffic by ensuring proper protein fusion in the cytoplasm; (Rab GTPases regulate anterograde traffic between the ER and the Golgi complex, and the cellular membranes)
19	D3TLN8	Protein phosphatase 2A (PP2A)-29B	591	65.501	Hippo signaling pathway; Viruses target specific PP2A enzymes to deregulate cellular pathways to specifically counteract the host's antiviral defences and promote production of viral progenies
20	D3TPG7	G protein β-subunit-like protein	318	35.485	WD-40 repeat containing protein implicated in signal transduction and transcription regulation to cell cycle control, cellular proliferation, and apoptosis; (viruses highjack G-protein mediated signaling to drastically facilitate their infection and transmission)
21	D3TN12	Serine-arginine rich protein 55 (SRp55)	351	40.1	SRp55 are a conserved family of pre-mRNA splicing regulators that are hijacked by viruses to increase production of virus progeny.
22	D3TP27	Hypothetical conserved (TcP-1-like) protein	174	20.525	Potentially involved in skeletal muscle myosin thick filament assembly
23	D3TMK9	Tailless-complex polypeptide-1 (TcP-1) zeta subunit	531	58.183	A chaperonin involved in the assembly of viral capsid

(iii)   GpSGHV diversity and evolution

It is important to determine the extent of genetic variation of GpSGHV: (i) for accurate detection of GpSGHV (to prevent underreporting in field samples), (ii) for specific antiviral strategies (siRNA or antibody-related interventions) and (iii) to understand the evolution of GpSGHV in *G. pallidipes* and other *Glossina* spp. Since *G. pallidipes* is a solitary insect and since the transmission is most likely only vertical, the GpSGHV is very likely of single lineages. Therefore, it would be of interest to see how variable GpSGHV is in (i) *G. pallidipes* from as many regions as possible, including West Africa, and (ii) as many *Glossina* species other than *G. pallidipes*. The hypothesis is that in the case of (ii) GpSGHV has a greater genetic variation than in (i), and that for GpSGHV within *G. pallidipes*, there are separate clades of GpSGHV in East and West Africa, respectively. Recently, a paper has been published (Kariithi *et al.*, 2013) on the genetic diversity of GpSGHV in *Glossina pallidipes* flies from different geographic regions of Africa. From this limited study it was concluded that (i) there seems to be little variation among GpSGHV isolates in *G. pallidipes* throughout Africa, (ii) the isolates from East-Africa are somewhat more related, with various dominant haplotypes, and (iii) that the genes used for the analysis were too conserved for an extended detailed study.

There has been little progress in this objective, primarily due to the fact that the sequence of the Ethiopian strain of SGHV was not complete until very recently. Now the genetic variation among the GpSGHV lineages from *G. pallidipes* could be better investigated and exploited. The previously used genes (*p74*, *pif-1*, *pif-2*, *pif-3*, *dnapol*) appeared to be too conserved (only few nucleotide differences) to allow robust statistical relatedness analysis (Kariithi et al. 2013). As outlined in the next work plan, an effort will be made to use this comparative genetic information to test the hypothesis that SGHV has distinct lineages between *Glossina* species and that the diversity between species is larger than among species.

Major conclusions

- First evidence that Salivary Gland Hypertrophy Virus (SGHV) provokes an RNAi defense response (up-regulation of Ago-2 and Dicer-2 genes) in tsetse flies (*Glossina pallipides*).
- The DNA sequence of GpSGHV-Ethiopia is completed, annotated and compared with GpSGHV-Uganda (manuscript in progress).
- The transcriptome and proteome of GpSGHV-Eth is available allowing the annotation of functional genes. (manuscript in progress)
- Comparison of the proteome of salivary glands of symptomatic and asymptomatic tsetse (*Glossina pallidipes*) flies revealed the up-regulation of virus-modulating pathways.

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Genetic bottlenecks in natural populations of the tsetse fly *Glossina pallidipes*: impact of vector control

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Introduction

Vector control programs have had limited impact on *Glossina pallidipes* populations in East Africa. After the end of control programs responsible for 95-99% reductions in population density, this vector of Human and Animal African trypanosomiasis invariably re-establishes, possibly by reinvasion from neighbouring populations or local population recovery. This raises the question: which of these processes are causing the absence of long-term effects of control on *G. pallidipes* populations?

Previous studies have found no detectable impact of vector control on the population genetic diversity in this species, thus suggesting that reinvasion from neighbouring populations explains the re-establishment of *G. pallidipes* after the end of control programs. We used Approximate Bayesian Computation to infer the demographic history of two *G. pallidipes* populations exposed to vector control in the 1980's and 1990's. Two populations not exposed to control have been included for comparison.

Materials and methods

Sample collection and DNA extraction

G. pallidipes were obtained from the 4 populations (Table 1). Flies sampled in Busia were dried after collection and stored in 95 % ethanol. Flies from Nguruman and Shimba Hills were collected in the field using NG2G traps (BRIGHTWELL *et al.* 1991) baited with cow urine and acetone and stored in 95% ethanol. For Busia, DNA extractions were carried out from the abdomen of each fly using the Qiagen DNeasy blood and tissue kit following the manufacturer's indications and using a final elution of 100µl. For Nguruman and Shimba Hills, DNA extractions were carried out from the head of each fly using the same procedure but with a 60µl elution. DNA extractions from the Rukomeshi sample were carried out as indicated in Kariithi et al (2013).

Table 1: *G. pallidipes* samples used in this study

Area/country	Sites	Collection date	Latitude(°N), Longitude(°E)	Sample size
North Zimbabwe	Rukomeshi	2006	-16.13, 29.40	34
Uganda/Kenya border	Busia	April 2000	0.61, 34.30	31
Rift Valley/Kenya	Nguruman	April 2011	-1.84, 36.08	26
Coast/Kenya	Shimba Hills	May 2011	-4.24, 39.48	20

Sample size: number of individuals genotyped per sample.

Microsatellite genotyping

Flies were genotyped at 9 microsatellite loci using two multiplex PCRs (PCR multiplex α: GmmK06, GmmC17, GpC10b, GpC101, GpB115, GpCAG133; PCR multiplex β: GmmA06, GpA19a and GpC26) as indicated in Ciosi et al (2014).

Bottleneck test

For each population, tests for a recent reduction in population size in the last 2Ne-4Ne generations were performed using the program BOTTLENECK 1.2 (CORNUET and LUIKART 1996; PIRY *et al.* 1999). These analyses were carried out assuming a generalised stepwise mutation (GSM) with a variance of 0.36 (ESTOUP *et al.* 2001; EXCOFFIER *et al.* 2005; MILLER *et al.* 2005). One-tailed Wilcoxon sign-rank tests were used to determine whether observed heterozygosity deviates from expectations at mutation-drift equilibrium. Estimations were based on 10000 replications. Reductions in population size were also tested using the "modeshift" indicator of the distortion of allele frequency classes' distributions (LUIKART *et al.* 1998).

ABC analyses

We used an Approximate Bayesian Computation (ABC) approach to infer past variation of the effective population size in the field populations under study. Microsatellite data were combined with prior information on the history and demography of those populations. Analyses were performed with DIYABC v 1.0.4.46 (CORNUET *et al.* 2008; CORNUET *et al.* 2010).

Analyses were performed in the same way as analyses 1 and 2 in Ciosi et al (2014). Briefly, the following scenarios were compared for each population (Figure 1): (A) Strong population bottleneck with recovery - the timing of reduction of population size and recovery match the timing of the control program in each location. (B) Lighter reduction in population size that happened earlier than the control programs performed in the 1980's and 1990's. (C) The effective population size remains constant.

For all analyses, the posterior probabilities of competing scenarios were estimated as indicated in Ciosi et al (2014).

Results

Bottleneck test

The tests for mode shift of the distribution of allele frequency classes and for heterozygosity excess (Figure 2) indicated that the Busia population recently experienced a genetic bottleneck while those tests revealed no evidence of such bottlenecks in Rukomeshi, Shimba Hills or Nguruman populations. An L-shaped distribution was really obvious for Shimba Hills and Rukomeshi. However, although the bottleneck test were not significant for Nguruman, the L-shape of the distribution was definitely not obvious.
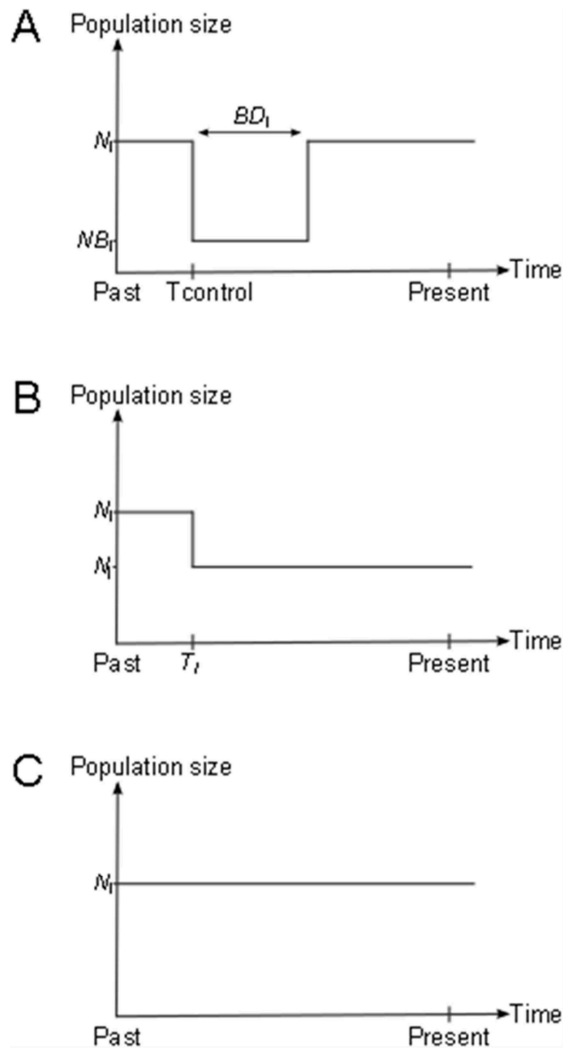
Figure 1: Demographic scenario considered to infer the demographic history of the *Glossina pallidipes* field populations considered. A: Demographic diagrams of scenario of strong population bottleneck with recovery. B: Demographic diagrams of scenario of lighter reduction in population size. C: Demographic diagrams of scenario of constant population size. N_i: stable effective size of the population. NB_i: effective size of the population during the population bottleneck. BD_i: duration of the population bottleneck. *Tcontrol*: date at which the control program started. For Rukomeshi and Shimba Hills that date was allowed to vary between 1950 and the year of sample collection. N_j: effective size of the population after the reduction of population size in scenario B. Ti: date at which the reduction of population size starts in scenario B.

ABC analyses

The scenario with a strong bottleneck (scenario A) obtained a posterior probability of 0.99 and 0.88 for Busia and Nguruman respectively. For the other two populations the selected scenario was the scenario with no variation of population size (scenario C) with posterior probabilities of 0.75 and 0.74 for Shimba Hills and Rukomeshi respectively. To conclude, the results of the ABC analyses show that a genetic bottleneck occurred recently in Busia and Nguruman but provide no support for such a bottleneck in Shimba Hills and Rukomeshi.

Discussion

Illustration of the usefulness of model based inferences in population genetics

The data set analysed in the present study is a good illustration of the usefulness of model based inferences, such as the ABC, over more classical population genetics analyses. The current study clearly demonstrates that model based inferences, such as ABC, are more powerful at detecting genetic bottlenecks compared to moment based methods such as the ones implemented in the program BOTTLENECK (PIRY *et al.* 1999) that was unable to detect any bottleneck in Nguruman from contemporary samples. This observation accords with previous findings both empirical (e.g. HOFFMAN *et al.* 2011) and by simulation (GIROD *et al.* 2011).

Moreover, Hoban et al. (2013) recently showed by simulation that a "recovery after a moderate amount of time" is associated with an important reduction of power in bottleneck detection when using moment based methods. This finding would be consistent with a rapid recovery of the *G. pallidipes* population in Nguruman after the bottleneck inflicted by the tsetse control program.
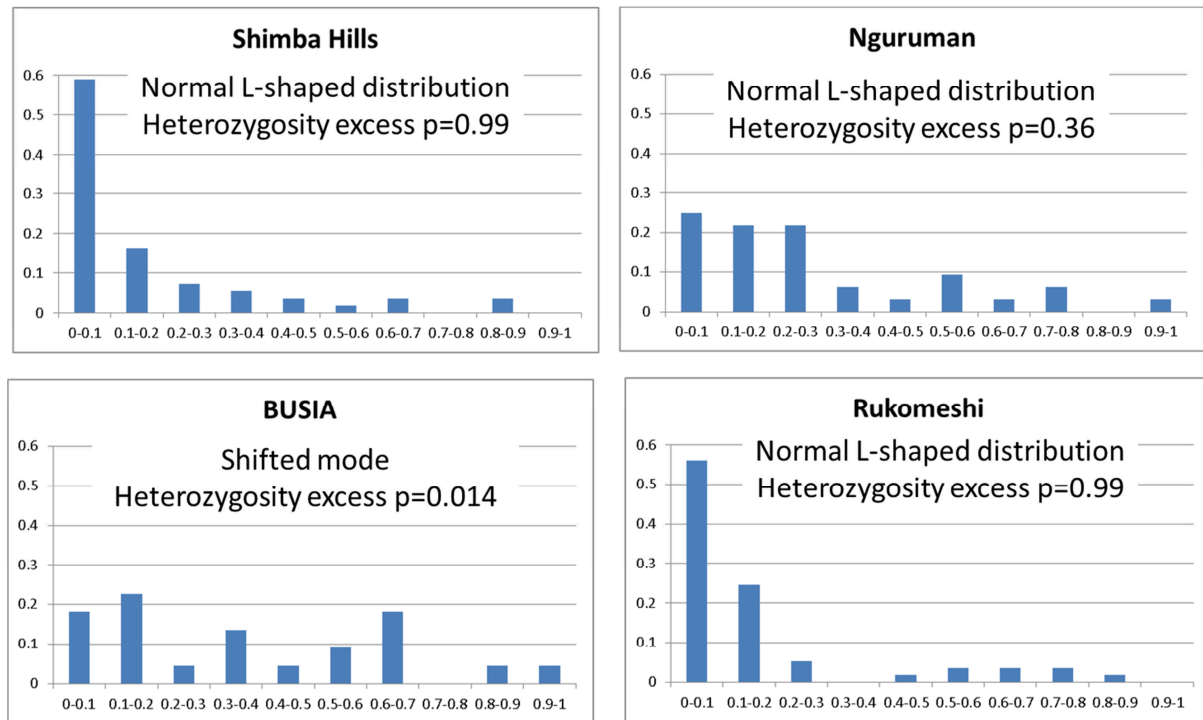

Figure 2: **Distribution of the allele frequency classes**. The result of the bottleneck tests (Wilcoxon' tests on heterozygosity excess, and the mode shift tests) are indicated for each panel. Heterozygosity excess and/or a shifted mode in the distribution of allele frequency classes indicate a recent reduction in population size.

Genetic bottleneck associated to control

The bottlenecks detected in Busia and Nguruman are have most likely been caused by tsetse control measures carried out in these areas in the 1980's and 1990's (BRIGHTWELL *et al.* 1997; MAGONA *et al.* 1998) while no variation of population size was inferred for the populations not exposed to control. These results thus suggest that the lack of long term demographic effects of vector control programs on *G. pallidipes* populations is better explained by a local population recovery rather than reinvasion from a neighbouring population.

Conclusion

Identification of a genetic impact of vector control in tsetse is encouraging. Indeed, reduced genetic diversity in populations exposed to control may decrease their adaptive potential and thus make them more vulnerable to future control or future modifications of their environment. Future work should focus on the impact of the genetic bottlenecks detected here on the genetic polymorphisms associated with tsetse refractoriness to trypanosomes.

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Influence of symbiotic bacteria on cuticular hydrocarbon profiles in tsetse flies (*Glossina m. morsitans*)

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Abstract

Microbial symbionts play important roles for the ecology and evolution of many insects, with their most well-known effects being nutritional supplementation, reproductive manipulation, and defense against antagonists. However, recent studies indicated that symbionts can also alter the chemical profile of the host and thereby affect mate choice decisions, which can ultimately result in reproductive isolation and speciation. Here, we investigated the effect of bacterial symbionts on the host's cuticular hydrocarbon profiles in the multipartite symbiosis between tsetse flies (*Glossina morsitans morsitans*) and their associated microbial symbionts, i.e. *Wigglesworthia, Sodalis,* and *Wolbachia*. We manipulated symbiont infection status by treatment with ampicillin (reducing *Wigglesworthia* and to a lesser extent *Sodalis* titers) and tetracycline (reducing titers of all symbionts, i.e. *Wigglesworthia, Sodalis* and *Wolbachia*), respectively, and subsequently analyzed CHC profiles of male and female offspring five and ten days after eclosion. The results revealed that both ampicillin and tetracycline treatment affected CHC composition in female and male offspring. Interestingly, the relative amount of the females' contact sex pheromone 15,19,23-trimethyl-heptatriacontane decreased upon tetracycline treatment in 10-day old flies. However, inter-individual exchange of CHCs in the rearing cages might have confounded the present results, and replications of the experiments with individually reared flies are underway to exclude the risk of pseudoreplication. If substantiated, the results provide evidence for an influence of *Wolbachia*, *Sodalis*, and/or *Wigglesworthia* on the amount and composition of CHC profiles in *G. m. morsitans*, which can have important implications for reproductive success and mate choice in tsetse flies.

Introduction

Cuticular hydrocarbons (CHCs) are ubiquitous and both structurally and functionally diverse in insects. Although the primary function of CHCs is the protection of the insect cuticle from water loss,

they have secondarily adopted a multitude of function in intra- and interspecific communication in a solitary as well as social context (Blomquist and Bagnéres 2010). In several tsetse fly species, long-chain methyl-branched CHCs have been implicated in eliciting sexual behavior of males after contact with the females (Carlson *et al.* 1978; Carlson *et al.* 2005; Carlson *et al.* 1984; Carlson *et al.* 1998; Langley *et al.* 1975). It remains elusive, however, whether CHCs also play a role for female mate choice decisions in tsetse.

Facultative bacterial symbionts can have a range of effects on their insect hosts, the most well-known of which are nutritional supplementation, reproductive manipulation, and defense against antagonists (Douglas 2009; Feldhaar 2011; Kaltenpoth 2009). Interestingly, however, recent studies on fruit flies have shown that symbionts can also alter the chemical profile of the host and thereby affect mate choice decisions (Sharon *et al.* 2010). These pheromonal changes may constitute the first steps towards premating isolation and hence initiate speciation processes (Brucker& Bordenstein 2012; Miller *et al.* 2010).

The tsetse fly symbiosis system presents an exceptional multi-level association between at least three bacterial endosymbionts, the gut microbiome, and viral as well as protozoan pathogens. *Wigglesworthia glossinidia* is an intracellular mutualist that serves an important nutitional function by providing B-complex vitamins, and it is essential for the development of the host immune system, thereby enhancing resistance to the protozoan parasites of the genus *Trypanosoma*. The other two microbial symbionts, *Sodalis* and *Wolbachia*, are less prevalent in tsetse populations, and their function is not yet well established, but *Wolbachia* is known to affect host reproduction across a wide range of insect hosts.

In the proposed project, we aim to investigate the impact of the bacterial symbionts *Wigglesworthia, Sodalis*, and *Wolbachia* as well as the trypanosome parasites on CHC profiles of *Glossina morsitans morsitans* and their possible influence on sexual selection and mate choice. We will use gas chromatography coupled to mass spectrometry (GC-MS) as a rapid tool to analyze CHC profiles (see Kroiss *et al.* 2011) of tsetse flies after artificial perturbations of the host-symbiont-parasite equilibrium. Our collaborators at Yale University, Serap Aksoy and Veronika Michalkova have established procedures to cure flies of *Wigglesworthia* only (by ampicillin treatment) or of the complete symbiotic bacterial community (by tetracycline treatment) and subsequently rear the flies successfully on artificial diets. Jan van den Abbeele (University of Antwerp) can experimentally manipulate *Sodalis* and *Trypanosoma* infection in *G. m. morsitans*, and Wolfgang Miller and Daniela Schneider (Medical University of Vienna) have established procedures to manipulate *Wolbachia* titers via depletion by antibiotics or overreplication by hybrid introgression.

We first plan to characterize CHC profiles of male and female (mated and unmted) *G. m. morsitans* of defined age groups in order to establish age- and mating-related changes in CHC profiles. Subsequently, we will analyse profiles of flies with different symbiont/parasite infection status (with and without all three symbionts, without one of the symbionts, after infection with *Trypanosoma*, and after irradiation for SIT) to assess whether the microbial community affects chemical profiles in *G. m. morsitans*. Symbiont and parasite infection status will be monitored for all flies by quantitative PCRs. These experiments will allow us to assess whether symbionts and/or parasites via changes in chemical profiles may influence (i) male mating behaviour in *G. m. morsitans*, (ii) female mate choice decisions based on male CHCs, and they will thereby provide a baseline for behavioural analyses of the mating behaviour and sexual selection in this species.

The comparative analyses of the temporal and spatial symbiont dynamics upon defined host-symbiont conditions as well as their impact on host chemical profiles will deepen our understanding of the functional complexity and the biological interplay of tsetse flies and their symbionts. 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.

Materials and methods

Sampling and treatments

Glossina morsitans morsitans individuals were obtained from Serap Aksoy's laboratory at Yale University. Three different treatment groups were established: wildtype (WT), ampicillin-treated (Amp), and tetracycline-treated (Tet). Antibiotics were delivered (50mg/ml, 25mg/ml for Tet) via bovine blood meals, and the Tet-diet was supplemented with 10mg/ml of yeast extract. The offspring of these established lines was reared on antibiotic-free bovine blood and sampled for chemical analyses. For each treatment group, 10 unmated male and 10 virgin female flies were sampled at day 5 and day 10 after adult emergence, respectively.

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 (Nelson& Carlson 1986). 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.

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 (Carlson *et al.* 1978), 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 groups by ANOVA with Tukey post-hoc comparisons.

For all other analyses, relative amounts were calculated from the peak areas and then log-ratio-transformed according to Aitchison (1986). 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. All statistical analyses were done with SPSS 17.0.

Results

As described earlier, 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 (Table 1). 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 65% of the complete CHCs in WT flies. In males, 2-methyl-triacontane and 11,15-dimethyl-tritriacontane dominated, amounting to about 70% of the total CHC profile in WT flies.

Table 1: CHC profile of 5- and 10-day-old virgin male and female *Glossina m. morsitans*. Compounds are sorted by class (mono-, di-, and trimethyl-alkanes).Values indicate average relative amounts of individual substances within groups, as well as the average absolute total amount of hydrocarbons per fly (in µg).

| | Females | | | | | | Males | | | | | |
| | 10-day old flies | | | 5-day old flies | | | 10-day old flies | | | 5-day old flies | | |
Compound	WT	Amp	Tet	WT	Amp	Tet	WT	Amp	Tet	WT	Amp	Tet
2Me-C28	0.28	0.79	0.74	0.41	0.58	0.83	0.45	0.84	2.15	0.42	1.36	1.20
2Me-C29	1.26	1.76	1.88	0.77	1.81	1.32	8.83	8.10	7.59	4.36	6.21	5.74
2Me-C30	20.65	20.47	25.98	13.16	21.57	13.89	20.42	22.96	29.22	19.52	21.89	21.81
2Me-C31+9,13-diMe-C31?	0.62	0.79	0.97	0.47	0.80	0.57	3.79	5.11	5.03	4.39	5.59	4.62
2Me-C32+diMe-C32?	0.89	0.89	1.11	0.89	1.55	1.13	3.32	3.78	4.40	4.47	5.79	5.48
2Me-C34	0.06	0.12	0.11	0.08	0.14	0.10						
11,15-diMe-C33	0.42	0.45	0.98	0.46	1.04	0.96	51.62	46.12	36.18	49.51	37.92	39.70
diMe-C34	0.20	0.22	0.24	0.36	0.44	0.45	3.30	2.96	3.77	5.59	5.85	5.20
15,19-diMe-C35	5.52	5.35	5.58	9.13	8.51	8.06	5.36	5.09	8.17	9.28	11.70	12.64
15,19+16,20-diMe-C36	4.33	3.69	4.40	6.85	6.35	7.03						
15,19+17,21-diMe-C37	13.68	13.12	15.02	21.80	17.61	17.60						
diMe-C38	0.90	0.65	1.06	1.57	1.47	1.83						
7,11,15-triMe-C33							0.91	2.00	1.27	1.06	1.45	1.48
13,17,21-triMe-C35	0.57	1.17	0.42	0.64	0.51	0.69						
triMe-C36?	1.80	1.83	1.38	1.86	1.65	2.17						
15,19,23-triMe-C37	36.46	37.17	28.70	27.35	23.05	27.85						
15,19,23-triMe-C38	7.85	6.67	6.96	8.40	7.90	9.93						
15,19,23-triMe-C39?	3.77	2.85	3.41	5.41	4.46	5.24						
unknown1	0.32	1.34	0.64									
unknown2	0.41	0.69	0.44	0.37	0.53	0.36						
unknown3							0.75	0.10	0.23	0.11	0.18	0.13
unknown4							0.37	1.17	0.69	0.39	0.61	0.62
unknown5							0.20	0.52	0.30	0.28	0.38	0.38
unknown6							0.68	1.26	0.99	0.62	1.07	0.99
Total CHC amount (µg)	33.4	24.2	18.5	35.5	30.1	43.9	25.0	19.6	4.3	17.2	21.0	15.9

Female CHC amounts and profiles

There were no differences in total amounts of CHCs across groups in either 5-day-old or 10-day-old female flies (ANOVA, p>0.3 for both age groups).

In 5-day-old flies, a DA based on the four PCs including all three treatment groups yielded a significant difference in CHC profiles across groups (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 WT and Amp flies (Wilks' Lambda = 0.232, X^2=23.37, df=4, p<0.001), between WT and Tet flies (Wilks' Lambda = 0.367, X^2=16.0, df=4, p=0.003) and also between Amp and Tet flies (Wilks' Lambda = 0.405, X^2=14.5, df=4, p=0.006), respectively.

In 10-day-old flies, the DA based on four PCs including all three treatment groups yielded a significant difference in CHC profiles across groups (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 WT and Amp flies (Wilks' Lambda = 0.595, X^2=8.30, df=4, p=0.081), but significant differences between WT and Tet flies Wilks' Lambda = 0.498, X^2=11.1, df=4, p=0.025) and between Amp and Tet flies (Wilks' Lambda = 0.402, X^2=14.6, df=4, p=0.006), respectively.

Female sex pheromone

In 5-day-old flies, Amp females showed a non-significant tendency towards lower absolute amounts of 15,19,23-trimethyl-heptatriacontane (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 Amp females as compared to wiltype and Tet flies (ANOVA, $F_{2,27}$=6.981, p=0.004; Tukey HSD p=0.014 for WT-Amp and p=0.001 for Amp-Tet). In 10-day-old flies, Tet females showed a non-significant tendency towards lower absolute amounts of 15,19,23-trimethyl-heptatriacontane (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 Tet females as compared to wiltype and Amp flies (ANOVA, $F_{2,27}$=6.291, p=0.006).

Male CHC amounts

In 5-day-old males, there were no differences in total amounts of CHCs across groups (ANOVA, $F_{2,27}$=1.565, p=0.227). In 10-day-old flies, WT and Amp flies showed on average 5-6 times higher total amounts of CHCs than did Tet flies (ANOVA, $F_{2,25}$=10.03, p=0.001). Posthoc comparisons (Tukey HSD) revealed these differences to be significant (p=0.001 and p=0.009 for WT-Tet and Amp-Tet, respectively), while there was no difference between WT and Amp flies (p=0.457).

Male CHC profiles

In 5-day-old males, the DA based on the four PCs including all three treatment groups yielded a significant difference in CHC profiles across groups (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 WT vs. Amp males (Wilks' Lambda = 0.253, X^2=21.3, df=5, p<0.001); WT vs. Tet males (Wilks' Lambda = 0.146, X^2=29.8, df=5, p<0.001), but not between Amp and Tet males (Wilks' Lambda = 0.727, X^2=4.9, df=5, p<0.424.

In 10-day-old males, a DA based on the four PCs including all three treatment groups yielded a significant difference in CHC profiles across groups (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: WT vs. Amp: Wilks' Lambda = 0.233, X^2=23.3, df=4, p<0.001; WT vs. Tet: Wilks' Lambda = 0.076, X^2=36.0, df=4, p<0.001; Amp vs. Tet: Wilks' Lambda = 0.177, X^2=24.2, df=4, p<0.001.

Future perspectives

Our results provide first insights into changes in CHC profiles upon symbiont manipulation by antibiotic treatment in *G. m. morsitans*. However, inter-individual exchange of CHCs in the rearing cages might have confounded the present results, and replications of the experiments with individually reared flies are underway to exclude the risk of pseudoreplication. If substantiated, the results provide evidence for an influence of *Wolbachia* and possibly *Sodalis*, as well as – to a lesser extent – *Wigglesworthia* on the amount and composition of CHC profiles in *G. m. morsitans*, which can have important implications for reproductive success and mate choice in tsetse flies. Furthermore, analyses on the influence of irradiation or infection by trypanosomes as well as salivary gland hypertrophy virus will be conducted to assess their influence on CHC profiles, and possibly, mate choice.

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Effect of fungal infection on *Trypanosoma congolense* load in *Glossina fuscipes fuscipes*

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SECOND RESEARCH CO-ORDINATION MEETING

on "Enhancing Vector Refractoriness to Trypanosome Infection"

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Abstract

Entomopathogenic fungi (EPF) infect their target host organism through the cuticle in four stages: adhesion, germination, penetration and intra-haemocoellian development, resulting to the host's death in 3-14 days depending on the concentration. In addition to killing the host, they can reduce blood feeding in haematophagous arthropods such as mosquitoes, tsetse and ticks, which may subsequently impact on the development and transmission of the parasite. We therefore investigated the effect of fungal infection by *Metarhizium anisopliae* on the development of *Trypanosoma congolense* in *Glossina fuscipes* flies and the acquisition of *T. congolense* by fungus-infected flies. In the control treatment, although the titer of parasite decreased from day 2 to day 7 post-infection, the parasite was still present in the flies. The titer of the parasite also decreased in both fungal treatments; however, no parasite was detected in fungus treatments after 3 days post-infection. More flies in the control acquired trypanosome parasite for more than 4 days while significantly less flies acquired trypanosome in fungus treatments. No parasite acquisition was observed in 4-day old fungus-infected flies, which may correspond to the peak of the disease.

Materials and methods

Insect
Glossina fuscipes used in this study were obtained from the *icipe*'s Animal Rearing and Containment Unit (ARCU) where they were maintained under controlled conditions of temperature (25 ± 2 °C) and 70% relative humidity (RH). Flies were fed regularly *in vivo* on rabbit blood.

Parasite cultivation

Trypanosoma congolense was used in the present study. It was obtained from Molecular Biology and Biochemistry Department (MBBD) of *icipe* and was stored in liquid nitrogen at -140 ^{o}C.

Mice were immuno-suppressed for 24 hours using cyclophosphamide in order to increase the rate of infection. Blood stream forms (BSF) of *T. congolense* in cryopreserved aliquots was thawed and used to infect the mice. Parasitemia levels were monitored through microscopic observation (X100 magnification) of a drop of blood placed on a glass slide which was obtained by prickling the mice tail. The parasitemia concentration of 4 x 10^6 trypanosome ml^{-1} was used. Once the optimal parasitemia was reached, mouse was exsanguinated and the blood used to infect pig blood which was then used to infect the flies.

Trypanosoma infection of fly

Parasite infection of flies was done through silicone membrane feeding. Sterile Pig blood was obtained from Farmer's Choice slaughter house, Zimmerman, Kasarani. Before being used, blood was tested and cleared of any bacteria present using standard procedures established at the *icipe's* ARCU. The blood was then mixed with L-glutathione (Sigma Aldrich, GmbH, Germany) at the concentration of 10 mM. Glutathione has been reported to make flies susceptible to trypanosome infection. Teneral flies (48 h-old) were allowed to feed on the trypanosome-infected blood for 15 min through a silicone membrane, after which were denied blood meal for 5 days after the initial feeding. They were maintained on the sterile pig blood thereafter.

Fungal Culture

Two strains of *Metarhizium anisopliae* were used in the present study: a wild type strain of ICIPE 30 (WT) and a strain of ICIPE 30 transformed to express the green fluorescing protein (GPF) (GZP). The latter was obtained from Professor Raymond St Leger, University of Maryland, USA. The cultures were maintained at 25 ±1 °C on Sabouraud Dextrose Agar (SDA). Conidia were obtained from two-week old culture by scrapping the surface plate using a sterile spatula.

Conidial viability was determined before any experiment by spread-plating 0.1ml of the suspension (3 x 10^6 conidia ml^{-1}) on SDA plates. Sterile microscope cover slips were placed on each plate. Plates were then incubated at 24-28 ˚C, 12:12 L: D photoperiod and examined after 16-20 h. Percentage germination was determined by counting the number of germ tubes formed among 100 random conidia for each plate at 400 x under a light microscope. Conidial germination above 85% was considered acceptable. Four replicate plates were used.

Fungus contamination technique

Test-insects were contaminated using velvet carpet material impregnated with conidia that covered the inside of a cylindrical plastic tube (95 x 48 mm) and had white nylon netting over one end (Dimbi et al., 2003). Dry conidia (0.1 g) were spread evenly onto

the velvet. Ten flies were transferred to the tube which was then closed. Flies were exposed to the fungus for 24hours after which they were removed and placed in clean cylindrical plastic tubes and maintained at 25 ± 2 $^\circ$C and 55% RH.

Effects of fungal infection on parasite development

Forty-eight (48) hour-old teneral flies were exposed to trypanosome-infected pig blood for 2 hr and then exposed to fungal conidia for 24 hr. Test-flies were dissected on day 2, 3, 5 and 7 for the presence of the parasite in the midgut. The alimentary tracts from proventriculus to the rectum were dissected onto a glass slide containing a drop of 1X PBS and viewed as wet mounts under phase contrast at X100 magnification, in search for trypanosomes. The midgut and proboscis were dissected then separated and used in fluorescent microscopy, mounted using cytological techniques and used in PCR analysis. In the control treatment, flies were not exposed to fungal infection. Treatments were randomized and consisted of 10 flies per replicate and the experiment was repeated three times.

Effects of fungal infection on parasite acquisition by flies

Two-day-old fungus-inoculated flies were offered trypanosome-infected blood meal at different day intervals. In the control treatment, flies were not exposed to fungal infection. Flies were then dissected to determine parasite acquisition following the technique described above. Treatments were also randomized and consisted of 10 flies per replicate and the experiment was repeated three times.

Results

Effects of fungal infection on parasite development

In the control treatment, the trypanosome load was 8.7 x 10^7, 3 4.6 x10^5, 5 1.7 x 10^5, and 7 8.3 x 10^4 ml^{-1} on day 2, 3, 5 and 7, respectively. The parasite load in flies treated with *M. anisopliae* WT was 8.7 x 10^7 and 8.3 x 10^4 ml^{-1} on day 2 and 3, respectively, while it was 8.7 x 10^7 and 1.3 x 10^5 ml^{-1} on day 2 and 3, respectively, in flies treated with *M. anisopliae* GZP. No parasites were observed on day 5 and 7 in both fungal treatments as compared to control treatment.

Effects of fungal infection on parasite acquisition by flies

In control treatment, 90, 60 and 50% of flies had acquired the parasite at day 1 and 2, day 3 and 4, respectively. In the *M. anisopliae* WT treatment, 50, 40 and 30% had acquired parasite at day 1, 2 and 3, respectively. In the *M. anisopliae* GZP treatment, parasite acquisition was 60% at day 1, 50% at day 2 and 50% at day 3. No presence of parasite was observed in flies on day 4 in both fungal treatments.

Microscopic examination results seem to indicate that fungal infection by *M. anisopliae* may induce refractoriness of tsetse to *Trypanosoma* parasite. This finding is supported by the qPCR Cycle threshold (Ct) results. Indeed, values for the flies exposed to fungal

infection stood at 28.68 while the ones in the control flies at 15.66. Since Ct values are inversely proportional to the amount of target nucleic acid in the sample, the higher the Ct value, the lower the expression of the target gene, in this case *T. congolense* gene. Samples will be processed for fluorescent microscopy in order to determine the type of interaction between fungal propagules and the parasite..

Vitamin B6 generated by symbionts is necessary for tsetse fly proline homeostasis and fecundity

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Tsetse flies are exclusively haematophagous and this highly restricted diet requires the presence of an obligate symbiont *Wigglesworthia glossinidae*. This bacterium is capable of synthesizing a range of B vitamins including pyridoxal phosphate (vitamin B6). Tsetse flies utilize proline instead of trehalose as a circulating nutrient source. Proline biosynthesis from alanine involves alanine-glyoxylate aminotransferase (agat), which requires pyridoxal phosphate as a co-factor (Fig. 1).

We examined the role of *Wigglesworthia*-produced vitamin B6 for maintenance of proline homeostasis, specifically during the energetically expensive lactation period of the tsetse's reproductive cycle. The expression of *agat*, as well as genes involved in vitamin B6 metabolism in both host and symbiont, increases in lactating flies (Fig. 2, 3). Removal of symbionts via antibiotic treatment of flies (=aposymbiotic flies) led to hypoprolinemia, reduced levels of vitamin B6 in lactating females, and decreased fecundity (Fig. 4, 6). Proline homeostasis and fecundity recovered partially when aposymbiotic tsetse were fed a diet supplemented with either yeast or *Wigglesworthia* extracts (Fig. 5, 6). RNA interference-mediated knockdown of *agat* in wild-type flies reduced hemolymph proline levels to that of aposymbiotic females (Fig. 5A). Aposymbiotic flies treated with *agat* short interfering RNA (siRNA) remained hypoprolinemic even upon dietary supplementation with microbial extracts or B vitamins. Flies infected with parasitic African trypanosomes display lower hemolymph proline levels, suggesting that the reduced fecundity observed in parasitized flies could result from parasite interference with proline homeostasis (Fig. 7). This interference could be manifested by competition between tsetse and trypanosomes for vitamins, proline, or other factors involved in their synthesis.

These results indicate that the presence of *Wigglesworthia* in tsetse is critical for the maintenance of proline homeostasis through vitamin B6 production. We show that *Wigglesworthia*-produced vitamin B6 is critical for the prevention of hypoprolinemia, specifically during lactation when proline is required both as a blood-borne nutrient resource and as a key amino acid for milk production. Interestingly, vitamin B6 alone does not recover fecundity completely, suggesting that the multiple B vitamins produced by *Wigglesworthia*, including folate and thiamine, likely also have distinct roles in relation to tsetse physiology and reproduction. Since vitamin B6 is a required factor for most transamination reactions, it may also be involved in other processes beyond acting as an AGAT cofactor that are critical to tsetse biology.

Fig. 1: Biosynthesis of vitamin B6 by *Wigglesworthia* with salvage of vitamin B6 and proline synthesis in tsetse flies.
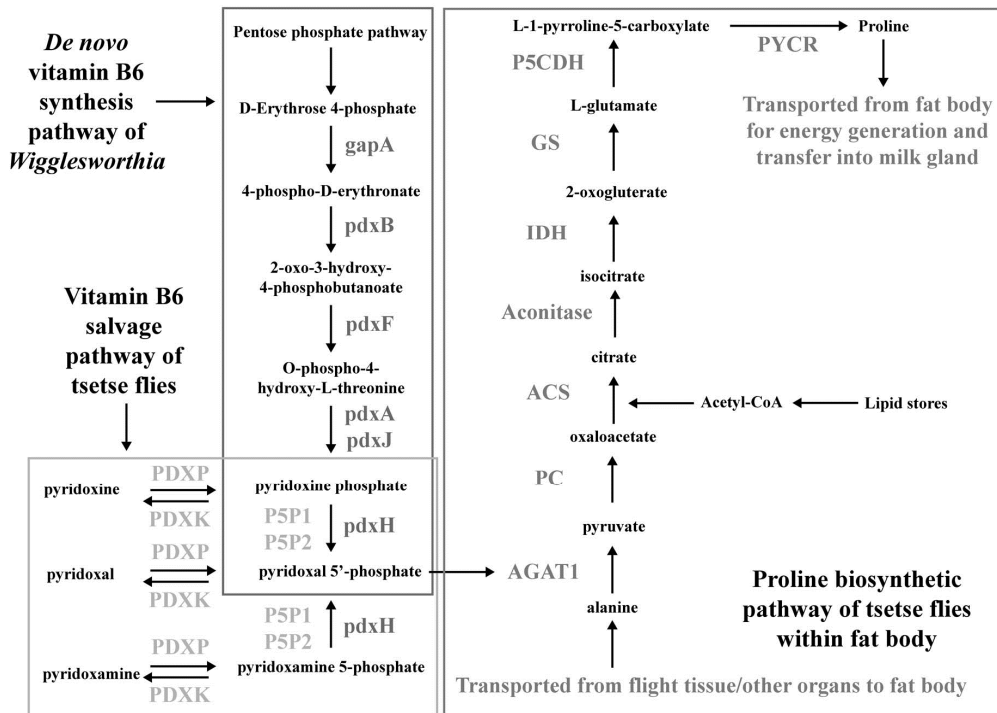

Fig. 2: Expression levels of selected genes from *Wigglesworthia* (A) and *Glossina* (B) associated with vitamin B6 metabolism.
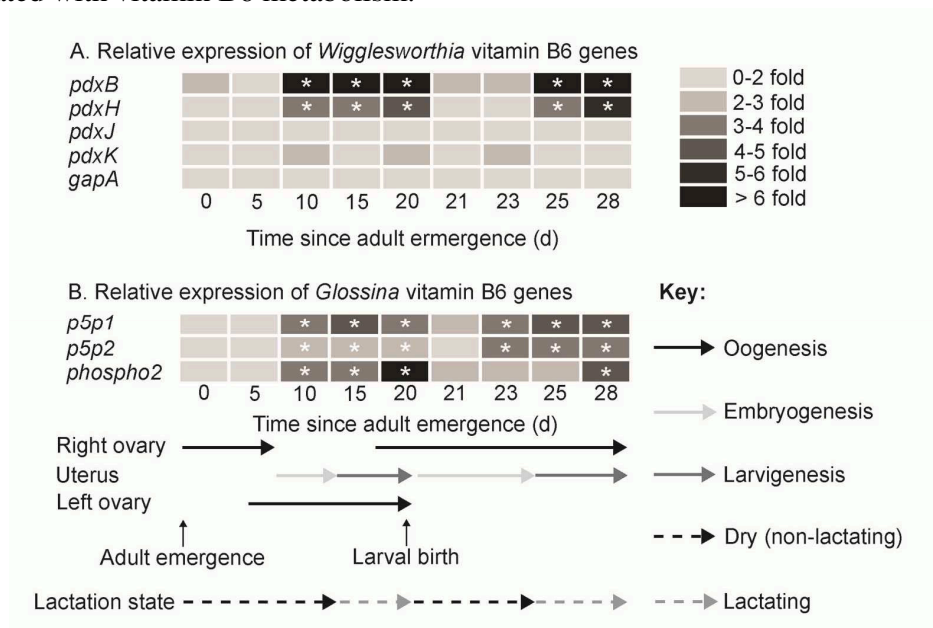
Fig.3: Expression of alanine-glyoxylate transaminase 1 gene (agat1) throughout tsetse lactation. Expression levels reaching the peak in lactation period.
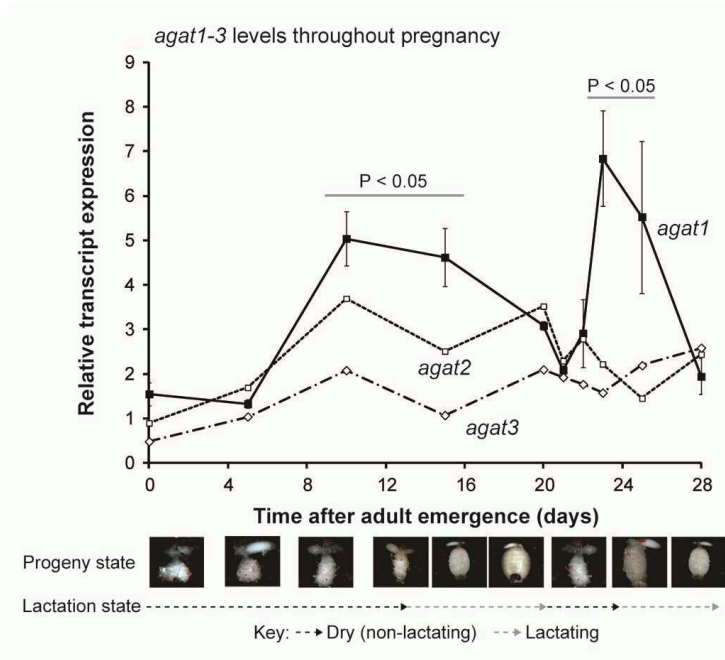

Fig. 4: Proline levels in hemolymph during lactation in wild-type and aposymbiotic flies. Proline levels failed to increase during lactation in aposymbiotic flies.
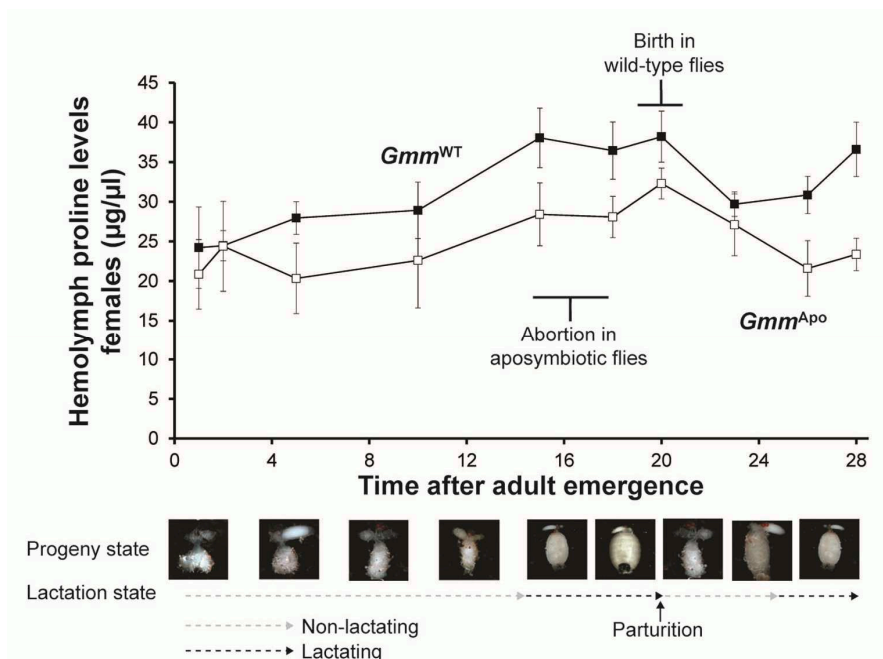
Fig. 5: Proline levels in flies after knockdown of agat and after supplementation with microbial extracts and B vitamin cocktails. (A) Knockdown of agat1 through utilization of injection of siRNA. (B) Supplementation of aposymbiotic flies with yeast extract, bacteriome *Wigglesworthia* extract, and B vitamins. (C) Knockdown of agat1 in aposymbiotic flies followed by treatment with yeast extract. (D) Knockdown of agat1 in aposymbiotic flies followed by treatment with B vitamins.

(A) Knockdown of agat leads to a reduction in proline levels in lactating flies (similar to aposymbiotic flies). (B) Yeast extracts and B vitamin coctails recover the proline levels in aposymbiotic flies. (C-D) Agat knockdown and symbiont removal inhibits proline recovery via yeast extract or B vitamins.
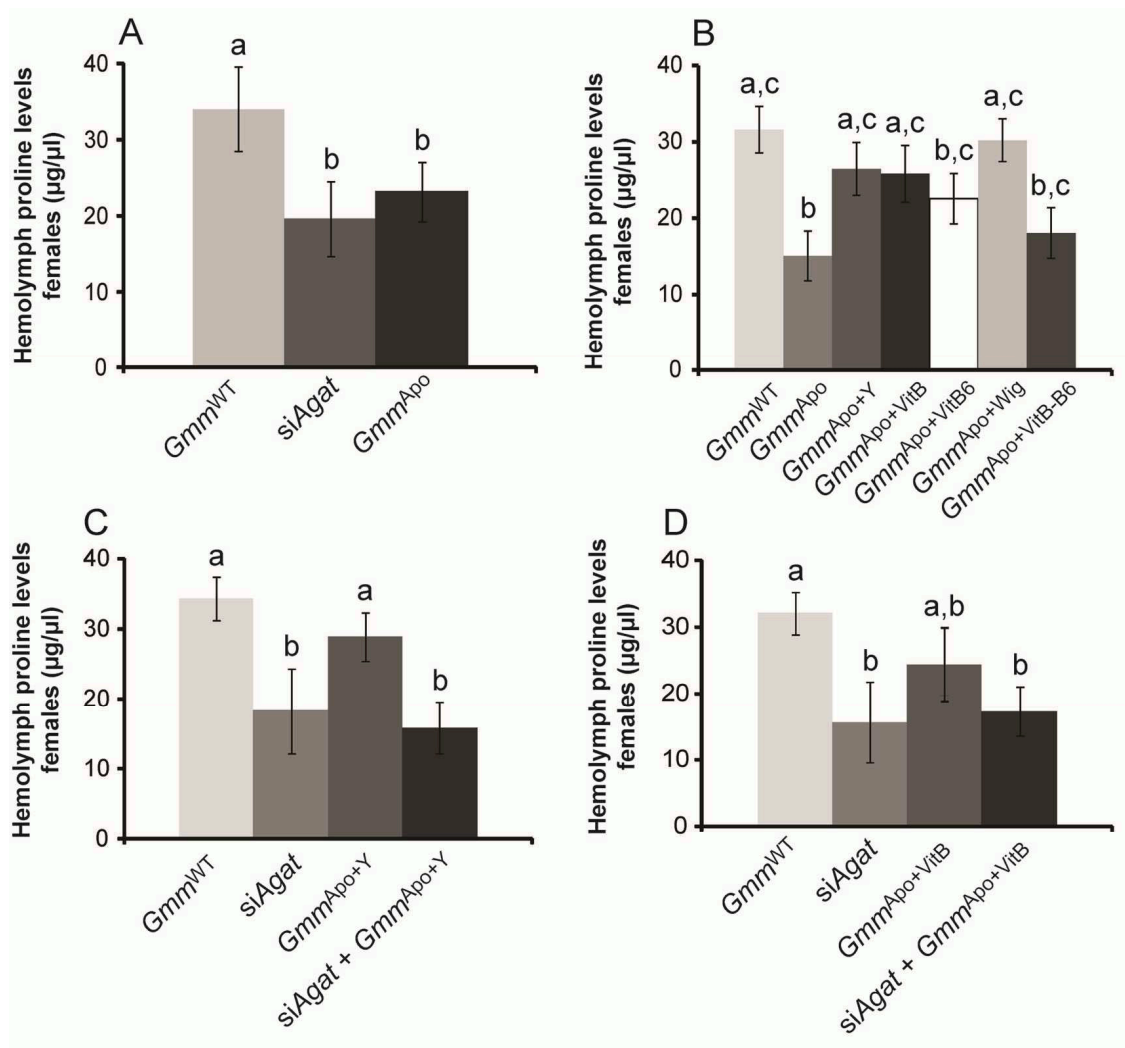
Fig. 6: Fecundity in wild type flies (WT) and aposymbiotic (Apo) flies following supplementation with yeast extracts (Y), microbial extracts (Wig), B vitamins cocktails (VitB) or with vitamin B6 only (B6) or without vitamin B6 (VitB-B6) and after knockdown of agat1.

Significant reductions in fecundity in agat knockdown flies and aposymbiotic flies. Yeast and VitB supplementation partially recovers fecundity.
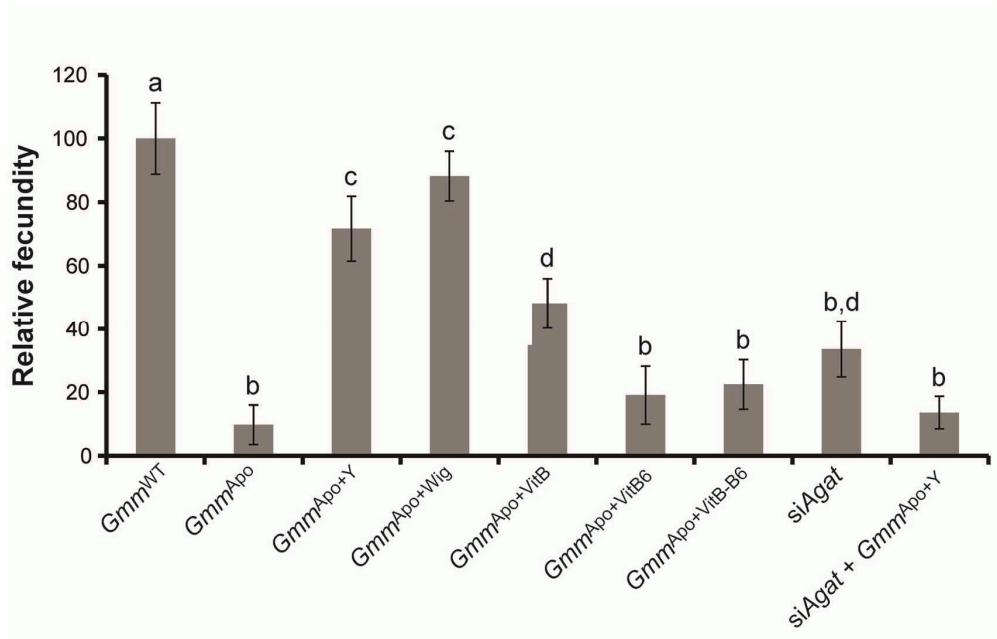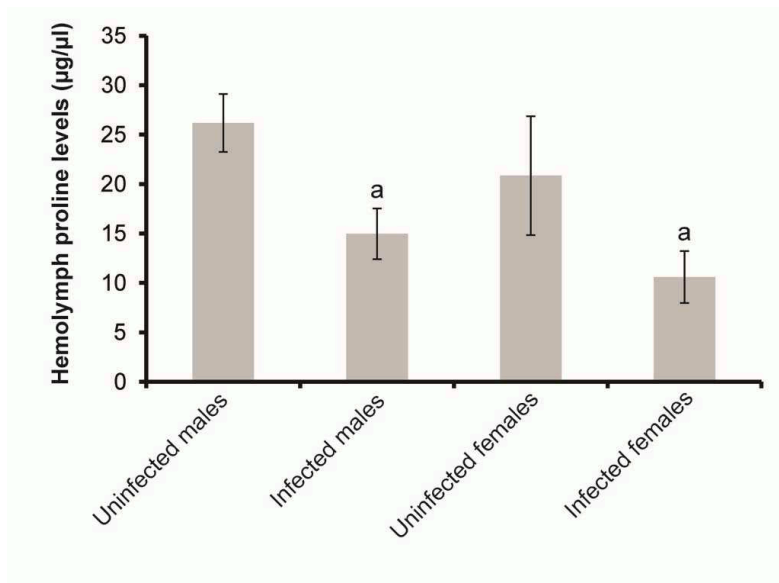

Fig. 7: Comparison of proline levels in wild type flies and flies infected with trypanosomes.

Symbiont-triggered Speciation – Lessons from Tsetse Fly Hybrids

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PROJECT SUMMARY

In this proposal, we aim to decipher the potential impact of tsetse fly symbionts on host speciation by monitoring their temporal and spatial dynamics of *Wigglesworthia, Sodalis* and *Wolbachia* in interspecies tsetse hybrids and, to its end, by breaking host species borders by means of symbiont knock-down of the male partner via antibiotic feeding before heterogamic crossings. This approach allows us to rescue hybrid male sterility and hence to generate stable hybrid lines, not existing in nature. In such, we aim to create artificial tsetse fly species (*species nova*) for improving the success of the Sterile Insect Technique (SIT). In detail, we propose to create novel *Glossina spp.* strains out of interspecies hybrid lines of *G. morsitans morsitans* and *G. morsitans centralis,* where *species nova* males are post-zygotically incompatible with both parental species due to their natural hybrid sterility. Such pseudo-sterile *species nova* tsetse males can then be released in nature just after moderate gamma-irradiation at lower dosages, which are still high enough to ensure complete sterilization of females that potentially escape rigorous sexing procedures. For their future applied success in pest control management, however, it will be pivotal that upon their release in nature such artificial *species nova* tsetse males are attractive to, but completely incompatible with, females of native species at the release site.

Although this idea is not new and was already proposed by Ron Gooding in the 1970s (reviewed in Gooding, 1990), we recently have demonstrated that the causative agent for inter-species incompatibilities between closely related tsetse flies are endosymbiotic bacteria belonging to the genus *Wolbachia* (Schneider et al. 2013). We have shown that similar to the neotropical *Drosophila paulistorum* speciation system (Miller et al. 2010; Miller & Schneider 2012), such naïve *Wolbachia* symbionts of tsetse are over-replicating in interspecies tsetse hybrids, thereby causing high embryonic lethality due to bidirectional cytoplasmic incompatibilities expressed by the symbiont itself (Schneider et al. 2013). Furthermore, we recently have demonstrated that partial depletion of *Wolbachia* titre by feeding antibiotics to the heterogamic male before interspecies crossing is sufficient to decrease F1 embryonic lethality and, most importantly, to partially rescue native hybrid sterility of F1 males (Miller & Schneider, unpublished). In such by knocking down the *Wolbachia*-expressed *mod* factor via mild antibiotic feeding during spermatogenesis in males (*i.e.*, the causative but still uncharacterized molecular factor causing *Wolbachia*-induced CI (reviewed in Serbus & Sullivan 2007), it is now possible to overcome symbiont-triggered species borders and to obtain fertile F1 males that further propagate in sibling mating with their hybrid sisters. We have recently generated stablized F8 hybrid tsetse colonies, originated from crosses of *G. morsitans morsitans* females and transiently antibiotic-fed *G. morsitans centralis* males (Parker et al. unpublished).

Hence the generation of artificial *species nova* tsetse colonies at the IAEA/FAO Laboratories in Seibersdorf, not existing in nature, is feasible and hence could serve as an important improvement to standard SIT, where conspecific males, *i.e.*, species members of the target population, are sterilized by high dosages that presumably affect male fitness severely. Side effects of standard gamma irradiation might affect cell organelles such as mitochondria, dividing cells of the gut epithelia, the gut microbiome, pheromone profiles as well as tsetse-specific microbial symbionts. Some if not all such native tsetse fly symbionts can serve mutualistic or commensalistic functions to their respective hosts by affecting important fitness traits such as pathogen protection, fertility, as well as their sexual attractiveness to females. To this end, the generation of artificial hybrid tsetse colonies that express complete natural sterility in mating with native females in the field would be an important step to minimize irradiation side effects in males, to enhance their sexual performance and finally to improve the success rate of SIT in nature.

1. BIOLOGICAL BACKGROUND AND SIGNIFICANCE

Tsetse flies are biting flies from the genus *Glossina* (family *Glossinidae*) inhabiting a region on the African continent stretching from the Sahara to the Kalahari Desert. This region forms a 'belt', in which sleeping sickness occurs due to the presence of the disease vector. Tsetse flies feed on host blood and through this route trypanosomes can be transmitted to humans and animals via the salivary glands. Tsetse flies have evolved an intimate relationship with three distinct bacterial endosymbionts, *i.e.*, *Wigglesworthia glossinida*, *Sodalis glossinidus*, and *Wolbachia* (**Table 1**; reviewed in Aksoy and Rio 2005). *Wigglesworthia* (γ-proteobacteria) represents the primary endosymbiont of tsetse, hence they are essential for host survival. *Sodalis* (γ-proteobacteria) and *Wolbachia* (α-proteobacteria) in contrast, are considered secondary symbionts. It was shown that this triple symbiont association in tsetse flies also affects many aspects of host physiology (**Table 1**). The primary symbiont *Wigglesworthia* is necessary for female fecundity and larval development and immune mounting Removal of *Wigglesworthia* renders the flies sterile and, most interesting, much more susceptible to trypanosome infection (Wang et al. 2009). The role of *Sodalis* for the tsetse host is not fully understood yet but an influence on host susceptibility to trypanosome infections has been reported (Geiger et al. 2006). In addition, *Sodalis* is considered as a candidate for para-transgenesis to introduce anti-trypanosidal genes of interest into a tsetse population (Van Den Abbeele et al. 2013). These bacteria can be cultured, genetically engineered and then be re-introduced into the fly host (Rio et al. 2004).

Symbiont	Host trait that is impacted
Wigglesworthia glossinida	female fecundity, development, resistance to trypanosomes
Sodalis glossinidus	longevity, refractoriness to trypanosome infection
Wolbachia	host reproduction (CI), parasite immunity

Table 1. Major bacterial symbionts of the tsetse fly and their interaction with host biology.
_____

As recently demonstrated, some *Wolbachia* strains of tsetse flies can trigger cytoplasmic incompatibility (CI) within (Alam et al. 2011) and between *Glossina* species (Schneider et al. 2013). *Wolbachia*-triggered CI leads to high levels of embryonic lethality among the offspring from mating between *Wolbachia*-carrying males and females devoid of the symbiont. Hence, population size of the host can be dramatically reduced. Also, CI acts as an efficient post-mating barrier to hybrid formation and thus is an important parameter in preserving species borders (Miller et al. 2010).
During the past decades, hybridization experiments have been conducted, under laboratory conditions, between various members of the genus *Glossina*, showing that they mate readily. However, the produced hybrid offspring is characterized by reduced female fecundity and complete male sterility (Gooding 1999). This shows that in tsetse flies, post-mating isolation is very strong, whereas pre-mating barriers are very weak or completely absent (reviewed in Gooding 1990). Hybrids have drawn attention already several years ago when they were considered for application strategies in order to control and suppress vector population (reviewed in Gooding et al. 1990). We have recently observed that quantities of *Wolbachia* are significantly alternated upon hybridization (Schneider et al. 2013). Hence, it is a key question how mixed hybrid nuclear backgrounds affect titre and tropism of all three symbionts of tsetse flies in general, and what the consequences are for interaction with the host plus parasites like trypanosomes.

2. PROJECT OUTLINE

In the course of this CRP project, we aim at addressing the impact of tsetse host-hybrid formation on (*i*) quantitative symbiont titre regulation, *(ii)* spatial symbiont distribution in host tissues, and (*iii*) the potential to generate stable colonies of artificial hybrid tsetse lines, a *species nova*, by rescuing *Wolbachia*-induced CI and hybrid male sterility.

Specific aims for the CRP year 2015:

***(i)* Coevolved symbionts escape host-directed titre regulations in hybrid backgrounds**
Detecting symbionts, particularly *Wolbachia,* in some *Glossina* subspecies is challenging as their prevalence and distribution is patchy (Doudoumis et al. 2012), and their natural symbiont titres can range at levels far below detection limit of standard molecular techniques (Schneider et al. 2013; 2014). Hence reliable estimation of symbiont infection frequency, however, especially with regard to interrelations between the symbionts and their potential impact on host biology, is of pivotal interest in order to control and eradiate African Trypanosomiasis in the near future (Van Den Abbeele et al. 2013).
It has been shown recently, that host hybrid backgrounds massively influence symbiont load in independent biological systems (reviewed in Schneider et al. 2011). In the neotropic species complex *D.rosophila paulistorum*, a drastic titre increase of the closely evolved, mutualistic *Wolbachia,* was reported recently (Miller et al. 2010). Most similar to this system, we have demonstrated significant *Wolbachia* titre increase upon artificial hybridization events in the genus *Glossina*. We now have demonstrated that in the *Glossina morsitans* species complex, interspecies hybrids also exhibit massively increased *Wolbachia* loads compared to their corresponding non-hybrid parents (Schneider et al. 2013). In addition, we have uncovered in a pilot screen accompanying titre alterations in *Sodalis* and *Wigglesworthia* upon hybrid formation (Schneider, Nagy & Miller, unpublished). These studies demonstrate that hybrid formation obviously has a severe impact on symbiont titre level control and that generating hybrids in the laboratory facilitates detection of even low titre infections that usually escape standard detection protocols. Application of this biological tool to subspecies of *Glossina*, which have yet not been studied in detail for their native symbiont status will allow to better estimate and decipher their natural symbiont loads and functional consequences. By doing so, we will be able to reliably determine whether species like *G. palpalis palpalis* and *G. fuscipes fuscipes* are really devoid of *Wolbachia* as it is currently stated (Doudoumis et al. 2012). Particularly, these subspecies with high relevance to the medical field due to their capacity to transmit trypanosomes are of great interest to be analysed for their native symbiont status and effects in mixed hybrid backgrounds.

Experimental Strategy
In order to test whether symbionts density changes in hybrid tsetse flies , we have already generated set of hybrids between members of different 'species groups' within the genus *Glossina*. We have shown recently the success of artificial hybridization in the lab for members of the *G. morsitans* group (Schneider et al. 2013).

***(ii) Spatial distribution of* symbionts is alternated in hybrid backgrounds**
Within their tsetse fly host *Wigglesworthia*, *Sodalis* and *Wolbachia* are located in defined tissues (**Figure 1**; Balmand et al. 2013). The primary symbiont *Wigglesworthia* is housed within a specialized organ, the bacteriome that is intimately attached to the intestine. It also infects the female milk gland, from which it is transmitted to the offspring via the secreted milk. *Wolbachia* reside primarily in the reproductive tract of the fly, and *Sodalis* seems in contrast to have a wider distribution in the host since it has been seen in several host tissues, including the uterus, the milk gland and even the fat body (Balmand et al., 2013). Here, we aim at analysing the consequences of hybrid formation on the strict maintenance of the symbionts to their spatial niches inside the host. We hypothesize that hybrid formation may trigger loss of host-directed spatial restriction of symbionts and thus, we expect mild to severe alterations in the symbiont-tissue tropism of yet unknown impact on tsetse biology. This part is currently performed in close collaboration with Abdelaziz Heddi´s research group in Lyon.

Experimental Strategy

To track down *Wigglesworthia*, *Sodalis* and *Wolbachia* in tsetse fly tissues (**Figure 1**), we will apply Fluorescent *in situ* Hybridization (FISH) as well as immunostainings on whole mount samples and histological sections of hybrids and their corresponding parents (wildtype). Fluorescent *in situ* hybridization (FISH) is a histo-molecular technique with a broad spectrum of application. Within the area of symbiosis research, FISH has proven a highly efficient tool for analysing in a specific way persistence and distribution of symbiotic residents in invertebrate host tissues by targeting either symbiont RNA or DNA. DNA-RNA FISH using fluorophore end-labelled short oligoprobes has been established and successfully employed in the lab of A. Heddi at the INSA Lyon, France (Heddi et al. 1999; Login et al. 2011; Balmand et al. 2013). Probes that selectively target one of the three bacterial symbionts will allow to perform triple stainings and consequently analyse tissue tropism of these three symbionts in parallel in one single individual. In addition to FISH, we will use antibodies against the symbionts to detect their location inside the tsetse host in wildtype and hybrid background via immunostaining procedure. Immunostaining of tsetse *tubulin* and *actin* proteins with allow determining symbiont distribution within host cells as well as extracellularily.
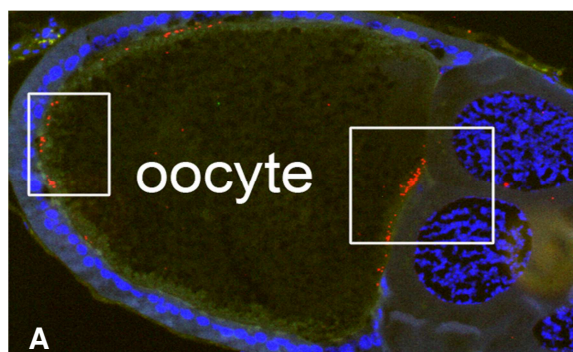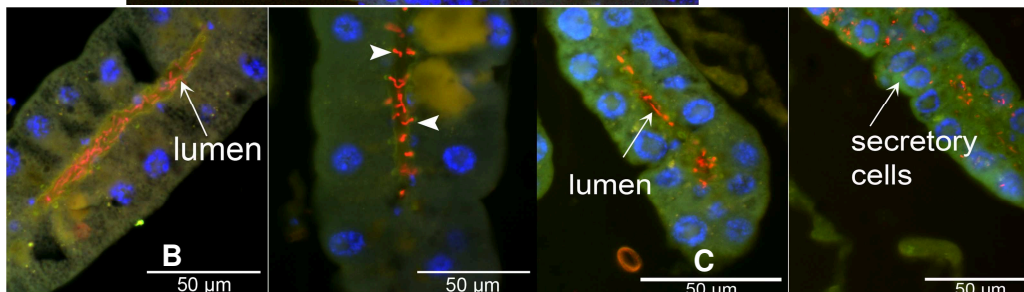

Figure 1. FISH on tsetse fly tissues. **(A)** *Wolbachia* in the oocyte, **(B)** *Sodalis* in the milk gland indicated by white arrows, and **(C)** *Wigglesworthia* in the milk gland indicated by white arrows. Symbionts are marked in red, host DNA in blue. Modified from Balmand et al. 2013.

(iii) Generation of artificial tsetse hybrid colonies by transient Wolbachia-knockdown

In full accordance with the definition of the biological species concept formulated by T. Dobzhansky, (1937) and E. Mayr (1942), F1 hybrids between different *Glossina* species suffer severely from high embryonic lethality, plus complete hybrid male sterility (Gooding 1999). Due to their natural capacity to abolish gene flow when mass-released in nature, the idea of the sterile hybrid male technique has drawn attention already several years ago when artificially produced hybrids were considered for application strategies in order to control and suppress vector population of tsetse flies (reviewed in Gooding et al. 1990).

Theoretically, mass-release of naturally sterile hybrids would also allow to significantly reduce dosages of gamma irradiation usually applied at high levels in SIT to ensure complete sterilization of colony-bred males. Recent studies, however, strongly suggest that such rigorous irradiation treatments of male insects not only damage host spermatogenesis, but also harm dividing somatic cells of the gut epithelia, cell organelles, the gut microbiota, as well as their native endosymbionts. As recently demonstrated many symbiotic microbes can significantly affect insect host fitness and fecundity, pathogen protection as well as their mating competence and success (reviewed in Schneider et al. 2011). Therefore, rigorous sterilization of tsetse

males at high dosages of gamma-rays could also destabilize and/or even demolish the complex symbiotic interactions between the tsetse host and its native symbionts *Wigglesworthia, Sodalis* and *Wolbachia* (summarized in **Table 1**). The tempting concept of applying hybrid-males that express innate sterility, however, was hindered by the fact that the potential large-scale generation of F1 hybrids for mass production and release was extremely laborious and time consuming and hence not feasible at all. Only the establishment to stable hybrid colonies, *i.e.*, the creation of a *species nova* tsetse fly, being incompatible *per se* with native *Glossina* species at their release site would circumvent this important biological and technical issue for future and successful pest control management.

In pilot experiments we have recently demonstrated that transient feeding of tsetse males with antibiotics before mating to untreated heterogamic females belonging to another but related species is sufficient to significantly reduce hybrid lethality as well as to partially rescue innate hybrid male sterility (Schneider & Miller, unpublished). By consecutive sibling mass mating we have succeeded to establish hybrid lines, of which we have currently reached generation F9 without hybrid breakdown, ready to build up a stable colony at the IAEA/FAO at Seibersdorf (Parker et al. unpublished).

Experimental Strategy

In the first part we aim to monitor the temporal and spatial dynamics of symbiont titre and tissue-tropism in parental (aim *i* and *ii*) and consecutive hybrid generations (F1 to F9) of both sexes by means of qPCR and FISH technique. We speculate that upon stabilization of the hybrid line in consecutive generations respective symbiont titre and tropism of *Wigglesworthia, Sodalis* and *Wolbachia* will immediately adapt to their mixed genetic host background to their normal levels and tissues due to natural selection acting on host fitness and fecundity. In the second part, we aim to perform incompatibility assays between stabilized isofemale hybrid lines and parental flies, as well as behavioural female mate choice assays between natural tsetse females and natural and hybrid males for testing male sexual attractiveness of artificial hybrids.

3. PERSPECTIVES:

We also aim to generate additional hybrids between members deriving from this as well as other species groups, particularly *Glossina palpalis palpalis* and *Glossina palpalis gambiensis*. These hybrids will be generated in close collaboration with the Insect Pest Control Laboratory, Joint FAO/IAEA Vienna (Seibersdorf). Upon successful hybridization, we will test symbiont loads in hybrids and compare these to their corresponding parents (wildtype). Classic non-quantitative Polymerase Chain Reaction (PCR) and quantitative real-time PCR (qRT-PCR) targeting symbiont 16S ribosomal RNA as well as other marker genes will be employed to track titre levels of *Wolbachia*, *Wigglesworthia*, and *Sodalis* symbionts. *Wolbachia*-specific 16S rRNA-PCR will follow methods described in Schneider et al. 2013; *Wigglesworthia* and *Sodalis* analyses are outlined in Boucias et al. 2013. Symbiont DNA levels will be normalized with host RNA through qRT-PCR quantification of the tsetse *tubulin* gene.

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