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"*Spiroplasma* prevalence in *Glossina fuscipes fuscipes* colonies and its impact on mass rearing efficiency for sterile insect technique application"

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I dedicate this work to my parents, Esther and my late father, Rafael Eduard Shimonov.

List of Abbreviations

AAT	African Animal Trypanosomosis
AW-IMP	Area Wide Integrated Pest Management
FAO	Food and Agriculture Organization of the United Nations
Gff	*Glossena fuscipes fuscipes*
HAT	Human African Trypanosomosis
IAEA	International Atomic Energy Agency
IPCL	Insect Pest Control Laboratory
Joint FAO/IAEA	Division of Nuclear Techniques in Food and Agriculture
PAAT	Program against African Trypanosomosis
PATTEC	Pan African Tsetse and Trypanosomosis Eradication Campaign
PCR	Polymerase Chain Reaction
qPCR	Real-time Quantitative PCR
SIT	Sterile Insect Technique
WHO	World Health Organization
Kb	Kilo base

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ABSTRACT

Extensive hunger and poverty outline rural populations in sub-Saharan Africa countries. These populations sustainable upon agriculture, farming and livestock grow. The presence of the tsetse fly and trypanosomosis in 36 African countries, in addition to the irregular rainfalls reflected in drought and flood, which compromise crop production, compose a major limitation of livestock rearing. Substantial losses of cattle and other farm animals as a result of African Animal Trypanosomosis (AAT), costing an estimated $ 1.2 billion per year and Human African Trypanosomosis (HAT), which affects approximately half a million people per year. However, thanks to international efforts, various of control methods of trypanosomosis in Africa were developed, one of which is the Sterile Insect Technique (SIT), leading to a significant reduction of tsetse wild populations and in some cases even an eradication. This technique involves mass rearing of a specific species (colony development) and release of their irradiated sterilized males into the wild populations.

The presence of *Spiroplasma* infection in the laboratory colony of *Glossina fuscipes fuscipes* (Diptera: Glossinidae) (*Gff*) in the Kality Tsetse Rearing and Irradiation Centre, Addis Ababa, Ethiopia, might threaten colony performance by reducing female fertility, leading to insufficient males being available for sterilization and release for the control of tsetse and trypanosomosis by the SIT. High *Spiroplasma* prevalence might lead eventually to the collapse of the colony. This research was conducted in the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Insect Pest Control Laboratory (IPCL), Agriculture and Biotechnology Laboratories, Seibersdorf, Austria with the objective of monitoring the *Spiroplasma* prevalence in laboratory colonies of *Gff* (IPCL and Kality). To better understand the impact of *Spiroplasma* infection in laboratory colonies of *Gff* in IPCL and Kality and to follow the transmission to the offspring of infected and non-infected flies, two additional *Spiroplasma* positive and negative colonies of *Gff* were established. Presence or absence of *Spiroplasma* was determined by a non-destructive PCR method with one pair of primers to detect specifically the 16S rRNA gene and generate an amplicon of ~450 bp. The results showed that among all the colonized tsetse species in IPCL, *Spiroplasma* infection is only detected with these specific

primers in the *Gff* colony. Further screenings of *Spiroplasma* positive and negative newly *Gff* colonies were performed to ensure their purity and to provide insights on vertical bacterial transmission in tsetse flies. *Spiroplasma* negative colony of *Gff*, F1, showed only one infected individual out of 58. Later screening of 71 females and males of *Spiroplasma* negative *Gff* colony, F2, showed no 16S rRNA amplification on the agarose gel. This might suggest that the sample of the infected individual in F1 was as a result of cross contamination and that the *Spiroplasma* negative *Gff* colony is essentially infection free. *Spiroplasma* positive colony of *Gff* showed an increase of infection prevalence in latter generations. These results imply a vertical transmission of *Spiroplasma* from parent to offspring, however due to the infection increase in F2 of the positive colony, horizontal transmission can't be excluded. To determine whether high *Spiroplasma* prevalence impacts colony development, mortality and fecundity rates of F2 were analysed between the two colonies. Results showed that there was no significant difference found in mortality rate and fecundity between the newly established *Spiroplasma* positive and negative *Gff* colonies, although F2 *Spiroplasma* positive colony has reached similar *Spiroplasma* infection prevalence and loads in individual flies as the colony in Kality. Moreover, it exhibited stable colony development and a normal productivity rate. Sexual behaviour and mating compatibility was evaluated between the new lines in a field cage test. The results showed no tendency towards assortative mating, and that the flies select their mate randomly, regardless of the *Spiroplasma* infection status.

To summarise, it is obvious that high prevalence of *Spiroplasma* infection is not the core factor threatening colony collapse in the Kality *Gff* colony. Rather, it seems likely that poor colony management is leading to decreased immune protection and opportunistic pathogen infection. The results of this study aim to raise the importance of effective colony management protocols and strategies for tsetse rearing factories to minimize the risk of horizontal transmission and to enable further colony stable development.

1. INTRODUCTION

1.1. Tsetse and trypanosomosis in sub-Sahara Africa

Tsetse flies (Diptera: Glossinidae) are insects feeding on the blood of mammals, female as well as male, in tropical sub-Saharan Africa. Several members are known to be vectors of protozoan parasites of the genus *Trypanosoma* (Trypanosomatida: Trypanosomatidae) and therefore responsible for the transmission of two major parasitic diseases Human African Trypanosomosis (HAT) or sleeping sickness and African Animal Trypanosomosis (AAT) also known as nagana. HAT in humans caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense*, whereas AAT in livestock caused by various of species, such as *T. congolense*, *T. vivax* and *T. b. brucei* (Mulligan, 1981). In most cases the parasite is transmitted through a tsetse bite, however in humans mother-to-child infections and sexual contact have been observed as well. After a haemo-lymphatic stage, sleeping sickness causes fever, headaches and joint pain, the parasite develops rapidly and invades the nervous system, leads to changes of behaviour, confusion, poor coordination and disturbance in sleeping cycles and could be fatal if not treated on time (WHO 2016). African animal trypanosomiasis causes severe anaemia and farm animals become weak and unproductive, which leads to lost of fertility, low milk yields as well as reduced growth and work output. In 1998 Sleeping sickness was estimated to infect approximately 300,000 to 500,000 people (unreported cases and therefore untreated) and threatened millions of others in 36 countries (Cattand *et al.*, 2001). According to WHO ~40 000 cases were reported and treated. In 2014 reported cases with diagnosed sleeping disease declined to 3796 due to various control efforts (WHO, 2016).

Substantial losses of cattle and other farm animals costing an estimated $ 1.2 billion per year, hence has profound impact on the agricultural development like crop farming and livestock keeping for nutritional resources (Shaw 2004). Several epidemics in Africa over the last century lead to an increased surveillance of infected populations and control measures.

1.2. Trypanosomes and tsetse fly biology

1.2.1. Trypanosomes

During blood meal intake by a tsetse from an infected host, trypanosomes are ingested and undergo cyclical development within the tsetse mid gut. Following morphological and physiological changes the parasite leaves the mid gut and enters the salivary gland, where transformation to the metacyclic stage occurs. This results in an infective parasite, which can be injected into a mammalian tissue by the next tsetse blood uptake (Vickerman *et al.*, 1988).

Trypanosomes evade the host immune system by synthesis of new surface glycoproteins, which provides antigenic variation. In addition, it has been shown that trypanosome infection leads to physiological alteration of the mammalian host, such as, blood pH, hormones and nutrient concentration (Seed, 2001), thus seemed to be more attractive to repeated tsetse bites, possibly due to trypanosome metabolites execrated in the host urine. This might suggest that a mammalian host is modified by trypanosomes to transfer the infection efficiently (Jenni *et al.*, 1980).

1.2.2. Vector

Tsetse flies belong to the order Diptera, family Glossinidae, with a single genus *Glossina*, in which over thirty species and subspecies have been described and classified based on the external morphological characteristics and geographical distribution in sub-Sahara Africa (Figure 1). Extension to the southern and northern parts of Africa is limited, due to low temperature (<20° C) and hydrometrical deficit

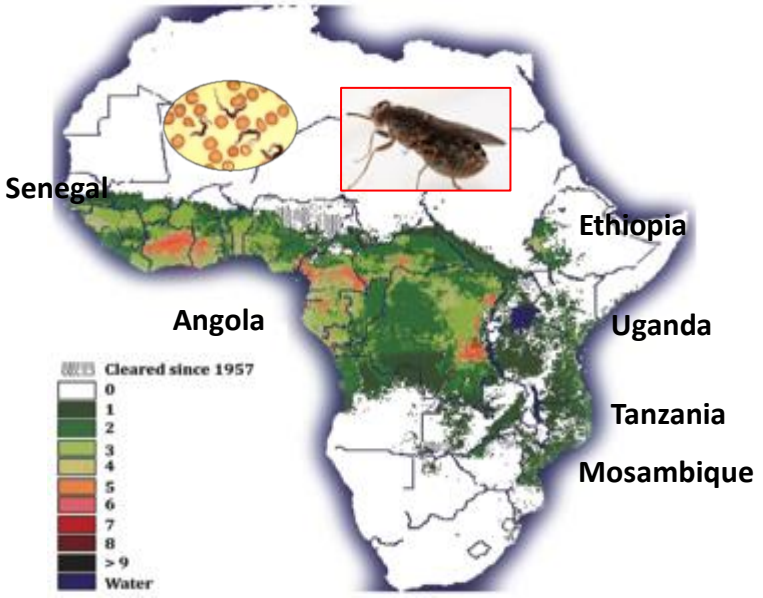

Figure 1: Distribution of tsetse flies in Africa. There are approximately 30 species of tsetse flies in sub-Saharan Africa, however only 8-10 species or subspecies are of economic importance: **G. pallidipes, G. morsitans, G. swynnertoni, G. brevipalpis, G. palpalis, G. fuscipes*. Numbers 0-9 refer to the number of different tsetse species infesting a given area (from http://www.tsetse.org/FAQ/africa.html***

(< 600 mm of annual rainfall) respectively, which do not correspond with the optimal tropical living conditions of the tsetse flies.

Tsetse flies, both male and female, are obligatory blood feeders and unlike most insect species, which produce large numbers of fertile eggs, tsetse females deposit a larva every nine to ten days. The larva grows in the modified common oviduct and feeds on the secretions of the uterine milk glands until maturity. Once the tsetse female deposits the larva, it moves actively for 15 to 30 minutes on a surface until pupation (Figure 2). The entire development of the metamorphosis into an adult fly occurs inside of the puparium (exterior shell of the pupa) (Moran *et al.*, 2016). This

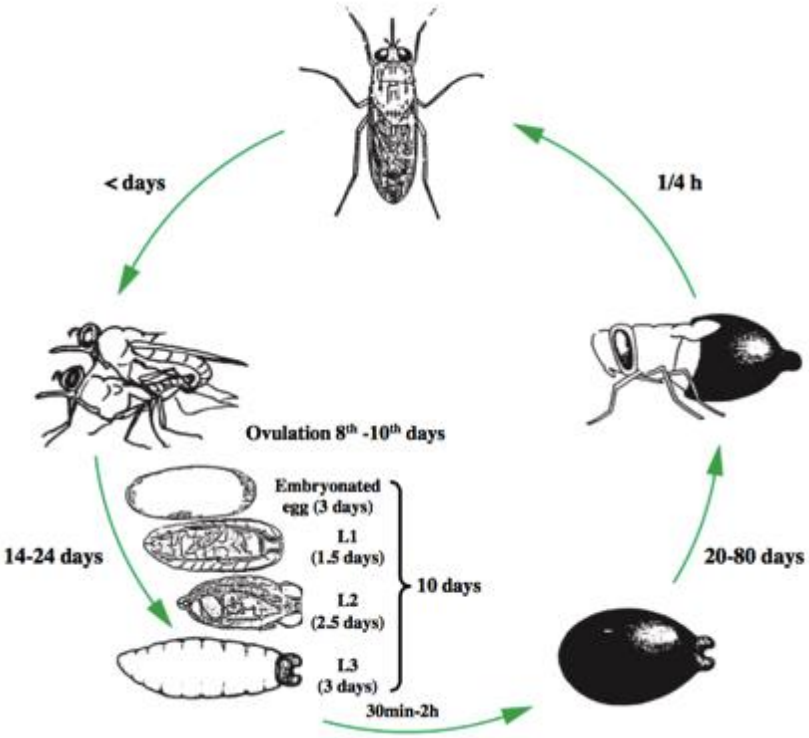

Figure 2: Tsetse fly life cycle (from Cuisance, 1989).

development process is variable and depends on the species, sex and climatic conditions (Solano *et al.,* 2010). Generally, female flies emerge 2-3 days earlier than the males. Emerged tsetse females are sexually mature and usually mate in the following hours. Mostly, the female accepts mating only once in her reproductive life cycle, this occurs for 30 minutes to 3 hours. The sperm is stored in the paired spermathecae and the inseminated female can produce progeny for up to 6 months (Sang *et al.*, 1997).

1.3. Application of sterile insect technique in tsetse fly control

During the last decades a lot of effort has been invested in studying vector and disease epidemiology aimed to optimize eradication programmes. Various vector control methods have been developed and implemented in order to eliminate trypanosomosis from sub-Saharan Africa. Some of the control methods are based on chemoprophylaxis or therapy with trypanocide compounds as well as suppressing vector populations by using insecticides, bush clearing and sterile insect technique (Du Toit, 1954; Allsopp, 1984; Vreysen *et al.*, 2000). However, some challenges are observed, for instance, trypanocidal compounds have been long-term used by the farmers and therefore led to the selection of drug resistant strains of trypanosomes in many African countries (Geerts *et al.*, 2001). Moreover, elimination of tsetse by aerial spraying of insecticides at a large scale, led to ecological concerns, like high mortality of non-target species causing biodiversity loss and environmental pollution. Hence it was essential to develop further tsetse selective techniques such as impregnated screens, traps and live baits (insecticide treated cattle). Screens (Figure 3A) include a piece of cloth, blue, black or a combination, with or without netting side panels, that is impregnated with insecticide (deltamethrin or cypermethrin). These are held on a frame and placed in the tsetse environment. Depending on the target tsetse species, the screens may also have odour baits (octanol, 4-methyl phenol, 3n-

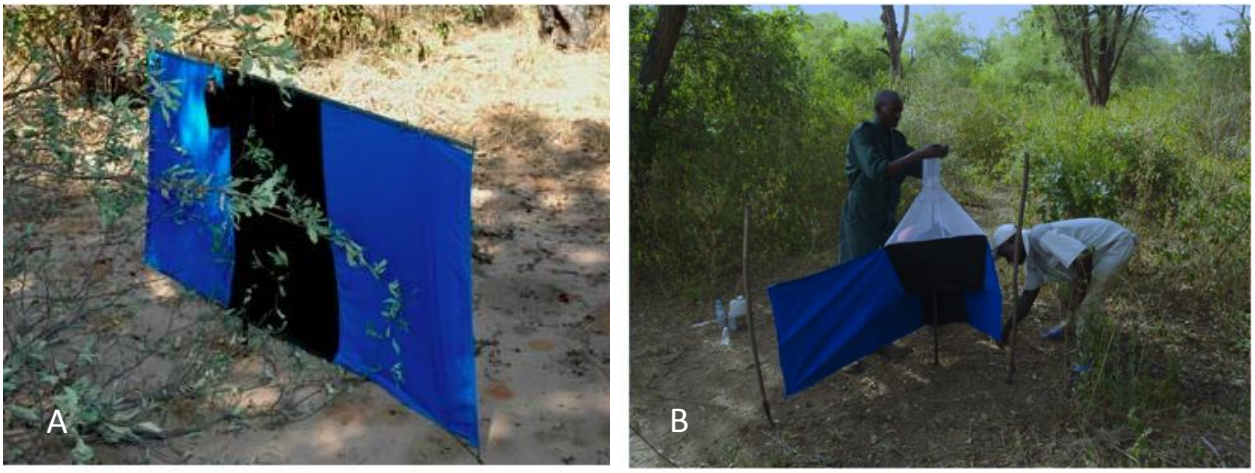

Figure 3: Traps for tsetse. A: Impregnated screen or target for tsetse control; B: NGU trap for tsetse monitoring (A, https://sparkuoguelph.wordpress.com/2011/08/11/tracking-the-tsetse-with-barcodes/; B, http://boards.cruisecritic.com/showthread.php?t=2088370.

propyl phenol or cow urine) that strongly attract some tsetse species. When they land (or with netting side panels, when they collide with the side panel) they pick up insecticide and die (Bauer *et al.*, 1999; WHO 2004). These techniques are effective

in reducing tsetse populations in infested areas and in preventing reinvasion of flies from neighbouring territories to previously cleared areas. Moreover, these techniques are essential prior to the application of the sterile insect technique (SIT) to lower the tsetse population densities. Subsequent application of SIT leads to complete eradication of a target population.

SIT may be used as the final component of area-wide integrated pest management (AW-IPM) campaigns as an environmentally-friendly and sustainable method, and is proven to be effective in eliminating major insect pest in general and tsetse fly populations in particular. It involves mass rearing of the target insect species and sterilization of the males by exposure to a specific dose of radiation (Robinson, 2002). Subsequently, the irradiated tsetse male flies are released by light aircraft in specific areas. Teneral tsetse females from wild population are coupled and inseminated by released sterile males, but do not produce progeny. Sterile males are released frequently, so they can outcompete the wild males for mating, and as the females normally mate only once in their life, the wild population eventually declines to extinction. For a successful SIT application, deep knowledge of ecology and biology of the target tsetse species is required. Tsetse species are mass-reared in facilities, such as Kality, Ethiopia and Burkina Faso, where competitive flies with similar behaviour to the wild type in a natural environment are produced.

The SIT method has been successfully applied to control several insect pests including the New World screwworm fly, Mediterranean fruit fly (Wyss, 2000) and tsetse species, such as *Glossina austeni* Newstead from the island of Ungunja, Zanzibar (Vreysen *et al*., 2000).

1.4. *Glossina fuscipes fuscipes* and Kality tsetse fly Rearing and Irradiation centre, Addis Ababa, Ethiopia

The Insect Pest Control Laboratory (IPCL) of the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria, has established colonies of several tsetse species, to support member states in providing the knowledge to combat the pest using SIT and therefore enhance food security and prevent diseases transmission. The eradication of *G. austeni*, as mentioned above, led to the development of several programs aimed to repeat the success in eliminating the trypanosomiasis in a vast area of sub-Saharan Africa. Such as one to control and

eradicate *Glossina fuscipes fuscipes* Newstead (*Gff)* in the Lake Victoria basin in south-eastern Uganda and western Kenya, where highly localized areas of *Gff* cause sleeping disease in humans and nagana in livestock (Welburn *et al.,* 2006; Waiswa *et al.*, 2003). In 2001 a new initiative was launched, the Pan African Tsetse and Trypanosomosis Eradication Campaign (PATTEC) involving the Program against African Trypanosomosis (PAAT) and supported by international organizations including the Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO) and International Atomic Energy Agency (IAEA) (Hyseni *et al.,* 2012).

Glossina fuscipes fuscipes is classified with the *palpalis* group (sub-genus *Nemorhina*, described by Robineau-Desvoidy in 1830) and found mainly in countries of central Africa, such as Cameroon, Gabon, Congo, Zaire, Central African Republic (CAR), Uganda and western Kenya.

The IPCL established a colony of *Gff* flies originating from the Central African Republic in 1986. It was transferred to Kality mass rearing facility in Ethiopia and Bratislava in 2005 to continue its growth. In early 2014 IPCL received material back from both daughter colonies in order to rebuild the *Gff* colony for experimental reasons (A. G. Parker, IPCL, personal communication). Mass rearing of *Gff* colonies in the mass-rearing facilities in Kality, Ethiopia experienced a steady reduction since 2008. Peak number reported was ~ 1.3 million females, however in 2015 it fell to 100 000 reproductive females and pupae per initial female of 0.2 (total pupae production/total female input), which may eventually lead to a colony collapse. Large-scale loses are not new to the mass rearing industry, such as the collapse of the *G. pallidipes* Austen colony in the IPCL mainly caused by the salivary gland hypertrophy virus, leading to testicular degeneration or ovarian abnormalities, which affects fertility and fecundity of naturally and experimentally infected flies (Abd-Alla *et al.,* 2007b; Lietze *et al.,* 2010). Efforts to identify the cause for the colony decline of *Gff* revealed high prevalence of *Spiroplasma* bacteria in male and female individuals, which is widely known as an insect symbiont and generally parasitic to their host causing negative fitness effects.

1.5. *Spiroplasma*: A common arthropod and plant pathogen

The genus *Spiroplasma* is a member of the Mollicutes class (Entomoplasmatales: Spiroplasmataceae). A wall-less microorganism, which is capable of maintaining a helical shape and exhibits translational movement in viscous media, related to Gram-positive bacteria. *Spiroplasma* has an evolutionary massive genome reduction approximately 530-2220 kb in size, 1-2 µm in diameter and 3-5 µm in length. Nevertheless, it belongs to one of the smallest and simplest free living and self-replicating cells (Gasparich 2002).

First observed in corn plants and described as a causative agent of corn stunt disease (Davis *et al*., 1972), since then they have been investigated intensively hence because of their unique features as microorganisms, but also the range of impact on their hosts. Some *Spiroplasma* species are found in the phloem of plants as well as flower surface and are horizontally transmitted to sap-sucking insects and ticks. Within a wide variety of insect taxa, *Spiroplasma* is considered as a symbiont and may live in the digestive tract, intracellular or via the haemolymph. They display diversity in the degree of pathogenesis and mortality. For example, large number of deaths of worker honey bees *Apis mellifera* L. (Hymenoptera: Apidae) colonies in USA and France (Clark *et al*., 1984; Mouches *et al*., 1984), tremor disease in crabs, *Eriocheir sinensis* H. Milne Edwards (Decapoda: Varunidae)(Wang *et al.,* 2004), lethargy disease in beetles, *Melolontha melolontha* L. (Coleoptera: Scarabaeidae)(Eskafi *et al*., 1987), and male specific mortality during embryogenesis and/or larva development in fruit flies (Williamson *et al*., 1999) (Figure 4). Much research has been conducted on *Spiroplasma* symbiotic involvement in the above-mentioned arthropods, frequency in natural populations, identity and their potential impact. However, there is no information about the impact induced by *Spiroplasma* in tsetse species in general and in *Gff* in particular in natural populations and laboratory colonies in IPCL and Kality. Therefore, the present study is aimed to survey the prevalence of infection in *Gff* and analyze its impact on the standard colony parameters of fecundity and mortality as well as behavioral impact such as mate choice and mating isolation.

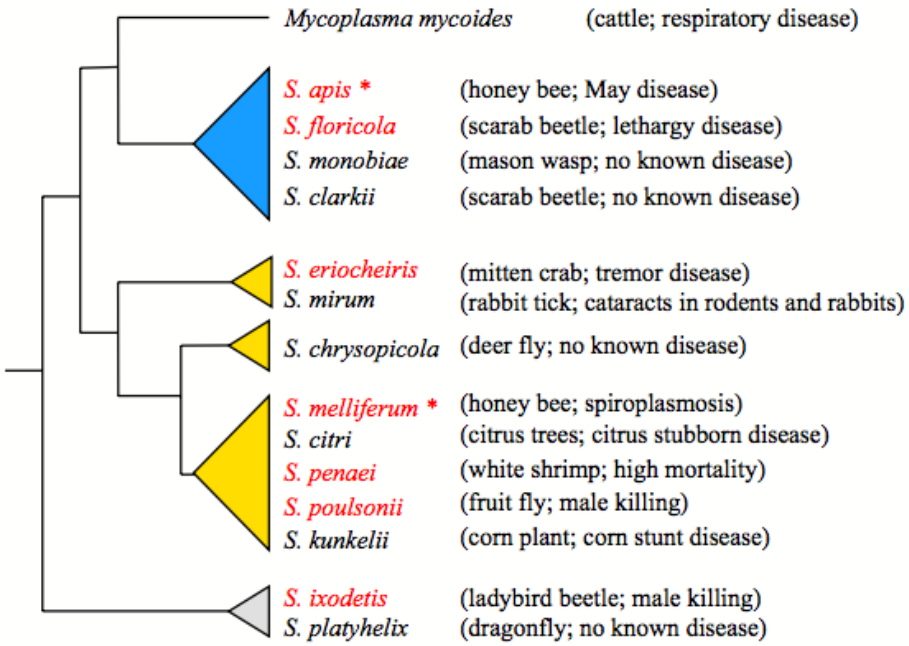

Figure 4: Phylogenetic tree of* Spiroplasma*. Three major clades of* Spiroplasma*; Blue: Apis clade; Yellow: Citri clade and Gray: Ixodetis clade. The red font indicates arthropod pathogens. Description of primary host and associated disease are given on the right. Phylogeny is based on 16S and 23S rRNA data (Gasparish 2004). Adapted from Schwarz et al., 2014.

1.6. Study hypothesis

The hypothesis of this study is that *Spiroplasma* infection of *Gff* exhibits pathogenic features, which might lead to the steady colony decline in the Kality mass rearing facility, Ethiopia. As *Spiroplasma* pathogenesis has been shown in other insect species, such as male-killing of embryos *Spiroplasma poulsonii* in fruit flies of genus *Drosophila* through possible apoptosis dependent epidermal cell death and neural malformation (Harumoto *et al.*, 2014) and *Spiroplasma apis* and *Spiroplasma melliferum* identified as causative agents of neurological disease in bees as previously described (Mouches *et al.*, 1983; Clark *et al.*, 1985). The infection could challenge the mass rearing production of *Gff* and reduce the availability of male flies for sterilisation for the application of SIT and therefore jeopardise tsetse control in sub-Saharan Africa.

1.7. Study approach and strategy

In attempt to gain insights into the mechanism underlying the symbiont-induced phenotype and to survey the prevalence of *Spiroplasma* infection in *Gff* colonies in

IPCL and Kality, two additional lines of *Gff* were established, a high prevalence line and one essentially with undetectable *Spiroplasma*. Using these lines we performed a detailed investigation on colony development parameters including emergence rate, mortality, male ability to participate in mating activities and productivity of females. Subsequently we were able to conduct behavioural studies on mate choice and mating isolation. Production was assessed in laboratory rearing cages and mating behaviour in field cages. Furthermore, two additional lines were established where only one of the parents was infected with *Spiroplasma* to determine its parental transmission to the progeny. Quantitative real time PCR was performed to reveal the load of *Spiroplasma* in individual infected *Gff* females form IPCL and Kality.

1.8. Expected impacts of the study

This study will reveal whether *Spiroplasma* infection of *Glossina* spp. in general and *Gff* in particular, might be a rising threat to the mass rearing production that could lead eventually to colony collapse. Additionally, results of this study will open novel avenues to: (i) Control the competence of tsetse flies in transmission of *Spiroplasma* and so impact on the improvement strategies for tsetse rearing facilities, thereby salvaging colonized tsetse for SIT application; (ii) Novel approach of using symbiont that may provide opportunities to reduce the vector competence of tsetse flies and therefore control of trypanosome transmission and infections in humans and farm animals due to their bites. These will significantly contribute to the efforts in tsetse and trypanosomosis control in sub-Saharan Africa.

2. MATERIALS AND METHODS

2.1. Laboratory and tsetse colonies

This laboratory work was conducted in The Insect Pest Control Laboratory (IPCL) of the Joint Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) laboratories, Seibersdorf, Austria.

Fly material from several laboratory colonies was used in this study: *Glossina* spp. maintained on in vitro rearing system at IPCL and Kality, Tsetse fly Rearing and Irradiation Center, Addis Ababa, Ethiopia (Table 1).

Table 1: Screening of *Spiroplasma* in different colonized tsetse species populations from IPCL and Kality.

No.	Species	Origin	*Spiroplasma* Screening		Total No.
			F	M	
1	*G. pallidipes*	IPCL	15	15	30
2	*G. morsitans morsitans*	IPCL	15	15	30
3	*G. morsitans centralis*	IPCL	15	15	30
4	*G. palpalis gambiensis*	IPCL	15	15	30
5	*G. palpalis gambiensis*	IPCL	15	15	30
6	*G. brevipalpis*	IPCL	15	15	30
7	*G. p. gambiensis BKF/SEN hybrid*	IPCL	15	15	30
8	*G. f. fuscipes*	IPCL	435	222	657
9	*G. f. fuscipes*	Kality, Ethiopia	344	150	494
10	*G. swynnertoni*	IPCL	6	6	12

2.2. Blood feeding

The experimental flies were maintained in a controlled insectary at 75-80% humidity and 24±1 ˚C. The adults were fed on warm (34-37 ˚C), defibrinated, bovine blood, for 15 min three times per week using an in vitro membrane feeding system (Feldmann, 1994).

2.3. Detection of *Spiroplasma*

To assess the prevalence of *Spiroplasma* in the colonized tsetse populations, all flies from Table 1 were tested. Males and females were selected randomly from each colony. Total DNA was extracted using DNeasy tissue kit (Qiagen Inc, Valencia, CA) according to the supplier's instructions. The DNA was eluted in 200 µl elution buffer, and the PCR was carried out using the primers and conditions described in section 2.4.3.

2.4. Establishment of *Spiroplasma* positive/negative *Gff* colonies

2.4.1. Detection of *Spiroplasma* in live flies

To study the impact of *Spiroplasma* infection on the *Gff* colony development, 481 teneral virgin females and males were selected randomly from the *Gff* colony in IPCL and maintained in individual numbered tubes, in order to identify them in subsequent steps. To detect flies that had *Spiroplasma* infection a non-destructive PCR test was used. The non-destructive PCR test enables the flies to stay alive and use them for subsequent mating and the establishment of four *Gff* colonies: (I) *Spiroplasma* positive *Gff*, (II) *Spiroplasma* negative *Gff*, (III) Spiroplasma positive males and negative females and (IV) Spiropplasma negative males and positive females.

2.4.2. DNA preparation from leg tissue for non-disruptive testing

The non-destructive PCR was conducted as previously described by Abd-Alla *et al*., (2007); in brief, one mesothoracic leg was cut from 302 *Gff* females and 179 *Gff* males under sterile conditions. Forceps and mini scissors were put briefly in 70% ethanol and then flamed after cutting each leg to avoid cross contamination. Each fly was returned to its tube back, whereas its leg tissue was placed in a microtube, which was labelled with the fly number. The total DNA was extracted from the leg using ZR DNA genomic kit (Zymo Research, California, USA) according to the suppliers' instructions. The crude extract was obtained by freezing the sample in liquid nitrogen and immediately grinding to a fine powder using a micropestle. Liquid nitrogen facilitates the disruption of the tissue samples and breaking of the cells. 500 µl of 2-betamercaptoethanol at 0.5% was added to the 100 ml Genomic lysis buffer. The powdered leg tissue and 500 µl Genomic lysis buffer were mixed with a micropestle, which was then removed and the microtube was sealed. The samples were centrifuged at 13 200 rpm for 3 minutes and the supernatant transferred to the

upper reservoir of a 96 well silicon-A plate. The silicon-A plate was placed on a collection plate for the collection of the filtrate. The plate was sealed with air pore tape sheets and centrifuged at 3 000 rpm for 5 minutes. The filtrate was discarded and the silicon-A filter plate was reconnected to the collection plate. 200 µl DNA pre wash buffer was added to the upper reservoir. The plates were sealed once again with air pore tape sheets and centrifuged at 3 000 rpm for 5 minutes. The filtrate was discarded and the DNA pellet was washed with 300 µl g-DNA wash buffer and centrifuged at 3 000 rpm for 5 minutes. The filtrate was discarded and the plate was centrifuged again to dry the filter from ethanol residues. The silicon-A plates were transferred onto the elution plates. 30 µl preheated DNA elution buffer was added in each well. The plates were incubated 5 min at room temperature and centrifuged at 3000 rpm for 5 min. The DNA samples were stored at -20 ˚C for further analysis. For PCR amplification 1.5 µl of DNA were used as a template.

2.4.3. Polymerase chain reaction (PCR)

In total 481 samples were screened for the presence of Spiroplasma. Screening was performed using bacterial species-specific 16S rRNA gene based PCR with the following primer pair:

63F (5'-3') GCCTAATACATGCAAGTCGAAC (Mateos *et al*., 2006)

TKSSspR (5'-3') TAGCCGTGGCTTTCTGGTAA (Fukatso *et al*., 2000)

For PCR amplification, the final reaction volume of 25 µl reactions each contained 22.5 µl of 1.1x pre-aliquoted PCR master mix, 1 µl of the forward and reverse primers (0.2 µM) and 1.5 µl of the DNA. This master mix provided a suitable chemical environment for optimum activity and stability of the Taq DNA Polymerase. Moreover, it increases the sample density allowing it to sink easily into the wells of an agarose gel and making it possible to load the reaction product directly onto a gel after amplification. The protocol included an initial 5 minutes denaturation at 94 ˚C, followed by 35 cycles of 45 seconds at 94 ˚C, 45 seconds at 58 ˚C as the annealing temperature, 1 minute at 72 ˚C and a final extension step of 10 minutes at 72 ˚C. The PCR products were separated electrophoretically on a 2 % agarose gel and stained with ethidium bromide in order to determine the presence and size of the fragments. Low range ladder from Thermofisher was used.

2.4.4. Mating post *Spiroplasma* screening

New colonies of *Gff* were established with four different mating possibilities and kept in standard colony holding cages (20 cm diameter x 5 cm height) at densities between 34 and 90 flies/cage and at a sex ratio of 1:3 (♂: ♀) (Table 2). All cages were maintained at a temperature of 24 ˚C and fed according to the protocol in section 2.2 with a slight modification. To avoid horizontal transmission of *Spiroplasma*, infected and non-infected flies were fed on separate blood trays. The mortality and productivity of the flies were recorded. The F1 pupae from the holding cages were collected in plastic tubes and labelled accordingly. Based on the *Spiroplasma* infection or non-infection of the parents, the pupae were divided into four groups, those from: (I) *Spiroplasma* positive *Gff* parents, (II) *Spiroplasma* negative *Gff* parents, (III) *Spiroplasma* positive males and negative females and (IV) *Spiropplasma* negative males and positive females. Pupae from the four groups were incubated at 24 ˚C for 30 days or until emergence. Further matings were conducted between F1 male and female flies within each group of pupae and treated as for the parents. The F2 pupae were collected from each group and incubated at 24 ˚C until emergence. *Spiroplasma* prevalence was tested in the F1 and F2 according to the protocol in section 2.3. and 2.4.3.

Table 2: Experimental set-up (fly densities and sex ratios) to assess the effect of *Spiroplasma* infection on colony development parameters, such as: mortality rate, fecundity and emergence rate.

Colony holding cage	*Spiroplasma* detection ♂ x ♀	No. of flies/cage	No. of males/cage	No. of females/cage	Ratio ♂: ♀
A	+♂ x +♀	90	24	66	1:3
B	-♂ x -♀	64	16	48	1:3
C	+♂ x -♀	57	14	43	1:3
D	-♂ x +♀	34	8	26	1:3

2.5. Purification and sequencing of PCR products

After PCR amplification and agarose gel analysis, 10 PCR samples with detected *Spiroplasma* were purified using a high pure PCR product purification kit (Qiagen). Briefly, 150 µl of binding buffer was added to the 20 µl PCR solution and mixed well. A high pure filter tube was inserted into a collection tube and the PCR product

sample was transferred to the upper reservoir of the filter tube and centrifuged at 14 000 rpm for one min at room temperature. The filtrate was discarded and the filter tube reconnected to the collection tube. 500 µl of wash buffer was added to the upper reservoir and centrifuged at 14 000 rpm for 1 minute. The filtrate was discarded and washing step was repeated with 200 µl wash buffer. The collection tube was discarded with the filtrate, centrifuged again for 1 minute to dry the filter from ethanol residues. The filter tube was connected to a sterile microtube. To elute the purified DNA, 50 µl of RNase free water was added to the upper reservoir of the filter tube, incubated for 1 minute in room temperature and centrifuged for 1 minute at 14 000 rpm. 15 µl of the purified DNA samples and 2 µl of the respective forward and reverse primers (63F and TKSSspR) were sent for sequencing. Nucleotides sequences were aligned using Bioedit program to determine differences or single nucleotide polymorphism and compared to those in the EzBioCloud (http://www.ezbiocloud.net) to identify the *Spiroplasma* strain. Conserved regions were determined for qPCR primers design.

2.6. Quantitative real-time PCR

We measured bacterial density of *Gff* in IPCL as well as Kality, using quantitative real-time PCR with the bacterial partial16S rRNA gene.

2.6.1. Design of primers for *Spiroplasma* DNA quantification

Based on the results from the sequenced PCR products, a pair of short specific primers located in the Spiroplasma 16S rRNA coding sequence was designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) as previously described (Thornton and Basu, 2011). These primers: SP_qPCR_F (5' AAACGACTGCTAATACCGCA 3'), SP_qPCR_R (5' ACTAACTAATACGCCGCACC 3') amplified a 100 bp fragment and synthesized by Eurofins genomics AT (Vienna, Austria). qPCR primers annealing temperature was optimized using the gradient PCR program.

2.6.2. Standard curve construction

To estimate the *Spiroplasma* titers, absolute 16S rRNA copy number was determined using a standard curve. PCR amplicon containing the *Spiroplasma* 100-nucleotide target sequence were used to create a calibration curve. The amplicon was obtained following PCR amplification of a ~450 nucleotide DNA fragment using

F63 and TKSSspR PCR primers and the DNA extracted from a *Spiroplasma* infected *Gff* was used as a template as described in section 2.4.3. and then purified as described above. The DNA concentration of the amplicon was measured using Nanodrop to prepare 10 fold-serial dilutions with known copy number of DNA molecules in DNA elution buffer.

2.6.3. *Spiroplasma* DNA preparation for qPCR testing

Twelve *Gff* females were collected randomly from the newly established positive/negative *Spiroplasma* colonies, as well as six *Spiroplasma* positive *Gff* females from Kality on different dates (May, September and November 2015). All flies were ~7 days old. The total DNA from whole body was extracted using DNeasy tissue kit (Qiagen Inc, Valencia, CA) according to the supplier's instructions. DNA was eluted in 200 µl elution buffer and quantified using Nanodrop. Samples were diluted separately to obtain the same DNA concentration (4 ng/µl). For qPCR amplification 5 µl of extracted diluted DNA was used as template.

2.6.4. Quantitative PCR

Quantitative PCR was performed using an iCycler IQ real time PCR detection system (Bio-Rad, Hercules, CA) in a final volume of 15 µl, containing 5 µl of DNA sample, 7.5 µl IQ™ SYBR green SuperMix 2X Reaction System® kit (Bio-Rad), 1 µl forward and reverse primers (0.2 µM) and ddH_2O. qPCR reactions were run on a programme of initial denaturation of 95 ˚C for 2 min, then 95 ˚C for 10 sec, 65 ˚C for 40 sec for 40 cycles. Three technical replicates were performed for each biological replicate and the amplification values were averaged and used for copy number calculations. Dissociation curves were constructed for verification of the target amplicon.

2.7. Mating Performance Field Cage Tests

2.7.1. Effect of *Spiroplasma* infection on mating behavior of *Gff*

Mating experiments were conducted to observe whether *Spiroplasma* infection affects the mating behaviour and interaction between *Gff* females and males. This refers to how *Spiroplasma* positive/negative females accept mating with positive/negative males. A walk-in field-cage was used in order to approximate as closely as possible the actual scenario in the natural environment. Cylindrical netted field cages, 2.9 m diameter and 2.0 m high, were placed in a laboratory at 24 ˚C and

60-65% humidity, and contained a potted tree in the centre. All experiments were carried out between 9.30 am and 12 noon as previously described (Mutika *et al*., 2001). The experiments were repeated six times. Each repeat is referred to as a replicate.

2.7.2. Fly collection

In each replicate, several dozens of pupae from the newly established *Spiroplasma* positive and negative *Gff* colonies were placed in the device used for emergence respectively. Within a few hours forty males and females from each device were randomly selected at a ratio of 1 female: 1 male and kept in separate cages to avoid sexual interaction. All 4 cages were labelled according to colony type (*Spiroplasma* positive/negative) and sex (Female/male) and maintained at temperature of 24 ˚C and fed according to the protocol in section 2.2. for approximate 5 days to reach sexual maturation. Before each group of flies was released in the field cage, they were chilled and marked with 4 different acrylic paints (a dot on the thorax), to distinguish to which group they belong.

2.7.3. Mating experiment

Eighty sexually mature, yet virgin females and males were released in cylindrical netted field cages (Figure 5A) under semi-controlled field conditions. The flies were observed for two and a half hours and copulating pairs were collected into individual tubes (Figure 5B), each tube being numbered to identify the individual male and female flies. After mating all pairs were removed, DNA extracted and PCR as previously described to determine *Spiroplasma* infection status.

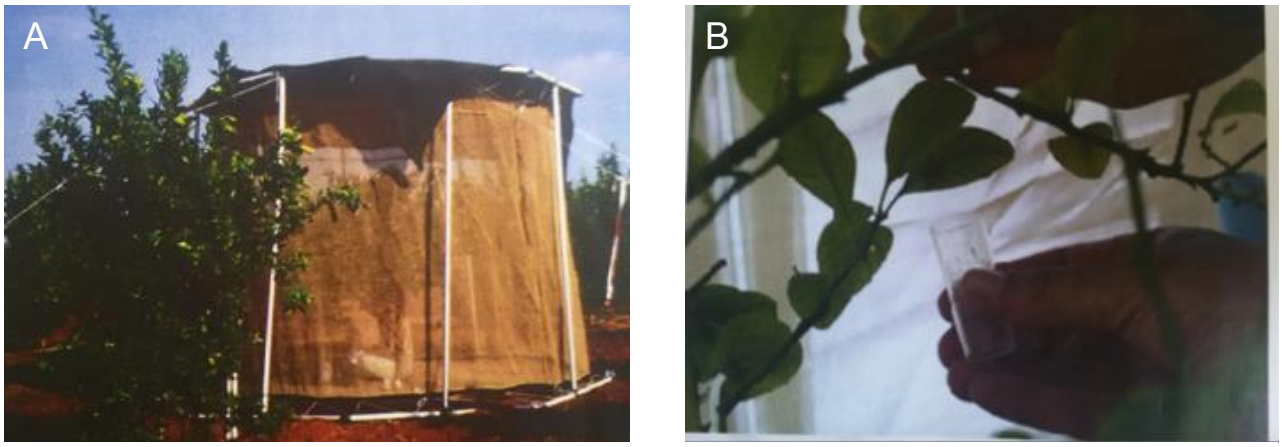

Figure 5: Mating experiment in walk-in field cage. A: Standard walk-in field cage used for the mating performance test; B: Collection of a* Gff *mating pair during field cage test (IPCL manuel)

2.8. Statistical data analysis

The mortality and productivity of the *Spiroplasma* positive and negative newly established *Gff* lines were recorded weekly. For statistical analysis mortality rate was angular transformed and productivity calculated as total pupae production/initial female input.

For quantitative PCR *Spiroplasma* titre was calculated as 16SrRNA *Spiroplasma* copies. Slope and efficiency values of the qPCR reaction were collected from iQ 5 software. Log-transformed values of the *Spiroplasma* copy number were used for statistical analysis.

For field cage test analysis the number of matings achieved was tested for equality of performance by *Spiroplasma* positive and negative *Gff* flies using the goodness of fit statistic G (Sokal & Rohlf, 1995).

Variation in values was analysed by One-Way ANOVA and means compared by t-test and Tuckey's HSD. Data are presented as mean value ± S.D. All statistical analysis was performed using Prism 7 software. *P* values <0.05 were considered as significant.

3. RESULTS

3.1. Comparison of *Spiroplasma* prevalence in Seibersdorf and Kality colonies

Can the decline of *Gff* colony in Kality be associated with a high prevalence of *Spiroplasma* infection? The first colony was established in 1986 at the IPCL and later transferred to Kality and Bratislava to continue its growth. In Kality, the colony expanded slowly to reach ~ 1.3 million of females however, it has experienced a steady decline since 2008 with low fecundity rates of 0.2 pupae per female per 10 days (A. G. Parker, FAO/IAEA Laboratories, personal communication). The colony status from 2015 showed 100 000 reproductive females, amount which could lead eventually to a colony collapse. *Spiroplasma* infection was detected in *Gff* males and females from Kality and IPCL.

A total of 1,373 tsetse flies were examined (657 *Gff* laboratory adapted from IPCL, 222 other tsetse species, laboratory adapted from IPCL and 494 *Gff* from Kality mass rearing and irradiation center, Ethiopia) to detect *Spiroplasma* infection. The overall prevalence of *Spiroplasma* infection in *Gff* of laboratory colonies in IPCL was 33% (218/657) and 55% (273/494) in the *Gff*, Kality colony (Table 3). This variation in prevalence of *Spiroplasma* infection in the two mass-rearing facilities was statistically significant (t = 5.236, d.f. = 5, p = 0.0034) (Figure 6). A statistically significant difference in prevalence of 31% (133/435) and 38% (85/222) in female and male *Gff* at IPCL respectively and 55% (192/344) and 56% (81/150) in female and male *Gff* at Kality respectively was observed (Table 4) (Figure 7). *Spiroplasma* prevalence in females and males in Kality is significantly higher than in IPCL (F = 12.41, d.f. = 3, p = 0.001). Means separation by Tukey's test showed the percentage of *Spiroplasma* prevalence in *Gff* females collected from IPCL as significantly lower than *Spiroplasma* prevalence in *Gff* females and males collected from Kality. *Spiroplasma* prevalence in *Gff* males from IPCL is significantly lower from the *Gff* females and males collected in Kality. There was no significat difference in prevalence between females and males within the same colony (Table 5). No *Spiroplasma* infection was detected in the 222 other examined laboratory adapted tsetse flies in IPCL.

Table 3: Prevalence of *Spiroplasma* infection (%) in *Gff*, laboratory adapted at IPCL and Kality, Ethiopia.

Run	*Glossina fuscipes fuscipes* IPCL			*Glossina fuscipes fuscipes* Kality		
	Examined	Positive	%	Examined	Positive	%
1	176	50	28%	244	136	56%
2	172	63	37%	111	52	47%
3	171	58	34%	139	85	61%
4	138	47	34%	-	-	-
Total	657	218	33%	494	273	55%

The prevalence of *Spiroplasma* infection differed significantly between the IPCL and Kality colonies (t = 5.236, d.f. = 5, $p = 0.0034$)

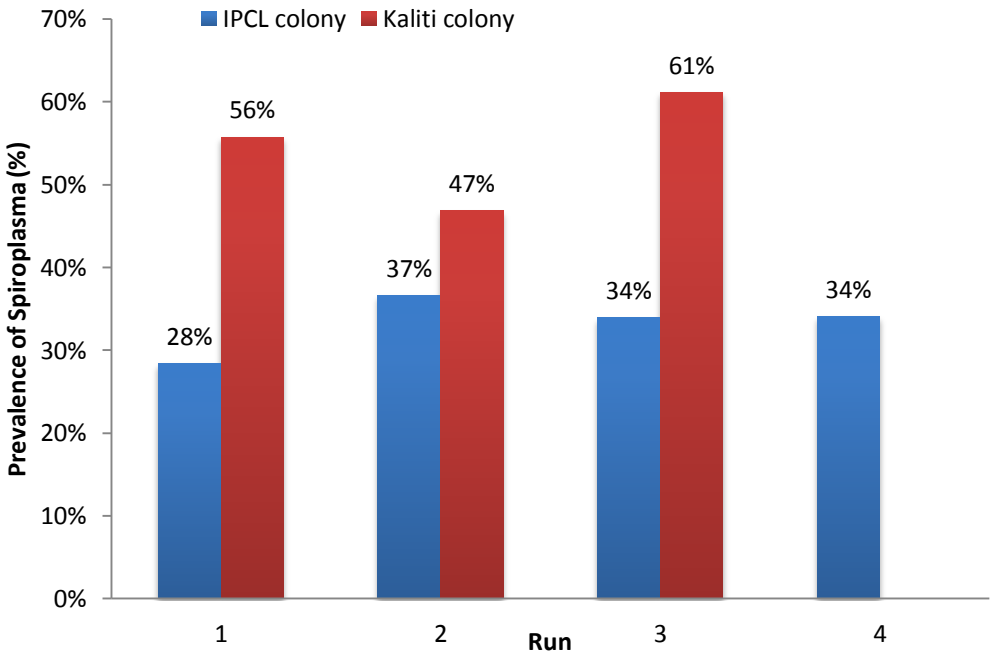

Figure 6: Prevalence of* Spiroplasma *in* Gff *in IPCL colony (Entomology Unit in Seibersdorf, Austria) and* Gff *colony maintained at Kality, Addis Ababa, Ethiopia. Variation is statistically significant (t = 5.236, d.f. = 5, p = 0.0034)

Table 4: Number of *Gff* females and males detected with *Spiroplasma* in IPCL vs. Kality

Run	*Glossina fuscipes fuscipes* IPCL		*Glossina fuscipes fuscipes* Kality	
	♀ positive	♂ positive	♀ positive	♂ positive
1	35/133 (26%)	15/43 (35%)	94/158 (59%)	42/86 (49%)
2	36/112 (32%)	27/60 (45%)	40/89 (45%)	12/22 (54%)
3	37/121 (31%)	21/50 (42%)	58/97 (60%)	27/42 (64%)
4	25/69 (36%)	22/69 (32%)	-	-
Total	133/435 (31%)	85/222 (38%)	192/344 (55%)	81/150 (56%)

The prevalence of *Spiroplasma* infection differed significantly between *Gff* females and males from IPCL and *Gff* females and males in Kality (F = 12.41, d.f = 3, p = 0.001).

Table 5: Tukey's test for the differences of the % mean *Spiroplasma* prevalence for positive *Gff* females and males from IPCL and positive *Gff* females and males from Kality. Above the diagonal is the difference between the means, below the significance.

			IPCL		Kality	
			♀	♂	♀	♂
			31,25	38,5	54,67	56
IPCL	♀	31,25		-7,25	-23,42	-24,75
	♂	38,5	ns		-16,17	-17,5
Kality	♀	54,67	**	*		-1,33
	♂	56	**	*	ns	

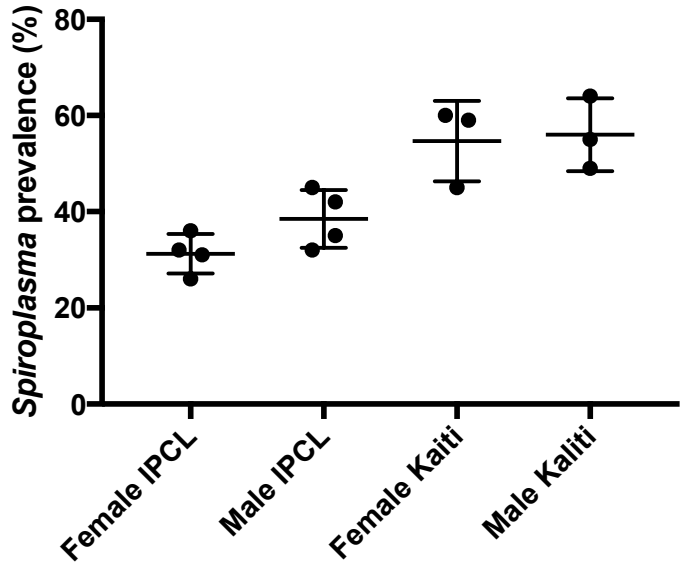

Figure 7: Scatter dot-plot of* Spiroplasma *prevalence between females and males in* Gff *in IPCL colony (Entomology Unit in Seibersdorf, Austria) and* Gff *colony maintained at Kality, Addis Ababa, Ethiopia. Values shown are mean and SD. Variation shown is statistically significant (F = 12.41, d.f. = 3, p = 0.001).

3.2. Detection of *Spiroplasma* in live flies and establishment of new *Gff* lines

The PCR products were obtained from the *Spiroplasma* 16S rRNA gene in the genomic DNA extracted from one mesothoracic leg in teneral *Gff* males and females. Products were run on 2% agarose gel and showed an amplicon size of ~450 bp (Figure 8). According to the presence of the band obtained, females and males were

divided into 4 groups of holding cages for further mating and establishment of new *Gff* lines (Table 2).

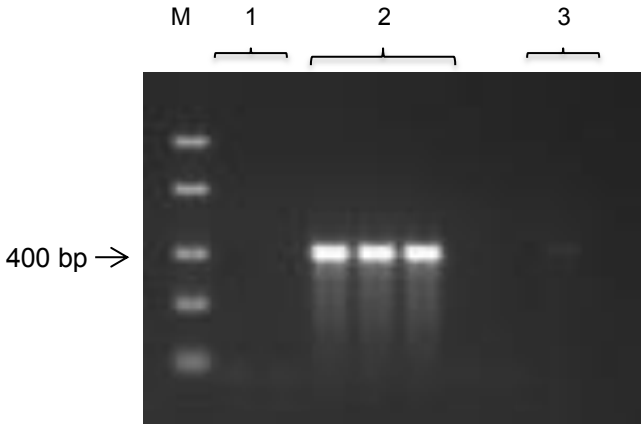

Figure 8: 2% agarose gel analysis of PCR amplification of 16S rRNA* Spiroplasma *gene, extracted from mesothoracic leg of* Gff *flies in IPCL colony, Austria. 1: Negative fly; 2: Positive fly, and 3: slightly infected fly. M: Low range ladder (Thermofisher).

3.3. *Spiroplasma* transmission and prevalence in progeny

Spiroplasma prevalence was tested by PCR in the first and second generations of the established colonies. All tested flies in F1 and F2 progeny of the negative colony (-♀ x -♂), showed undetectable amplification of the *Spiroplasma* 16S rRNA gene except one in the F1 progeny, hence 1.7% prevalence. However, in progeny F1 and F2 from the positive colony (+♂x +♀) 45% and 51% were positive, respectively (Table 6 and figure 9). In the positive colony we see an increase of *Spiroplasma* prevalence through the generations but the positive colony contains both *Spiroplasma* positive and negative flies. It is important to note that a negative individual (no band on agarose gel after PCR amplification) may not harbour *Spiroplasma* and thus not infected or infected, but with *Spiroplasma* load too low to be detected on the gel.

Table 6: Prevalence % of *Spiroplasma* in different generations of the newly established *Gff* lines

	Negative colony		Positive colony	
	Ratio	%	Ratio	%
F1	1/58	1.7	26/58	45
F2	0/82	0	36/71	51

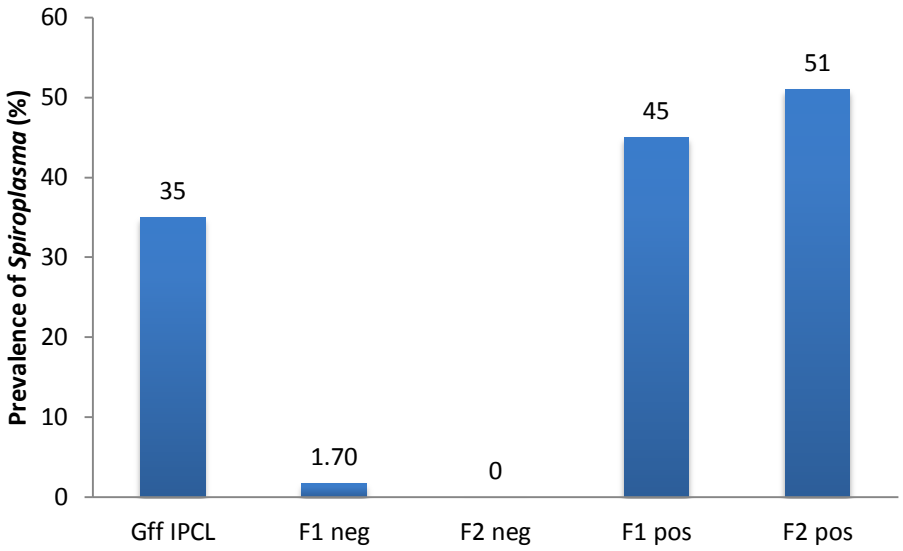

Figure 9: Prevalence of* Spiroplasma *infection in different generations of the newly established* Gff *lines. F1 and F2 neg are progeny of* Spiroplasma *negative parents. F1 and F2 pos are progeny of* Spiroplasma *positive parents.

F1 progeny from the colonies where one of the parents is *Spiroplasma* positive were tested as well (Table 2, holding cages C and D). Progeny of positive females (Cage D) produced a strong band (~450 bp) on the gel and were highly infected (Figure 10).

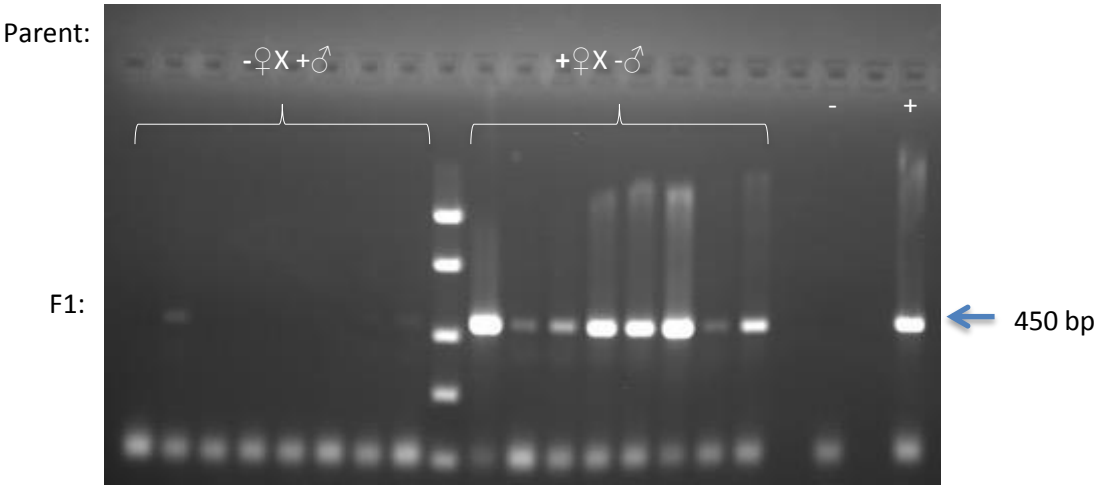

Figure 10: Detection by PCR of* Spiroplasma *16S rRNA. DNA extracted from F1 flies resulting from different mating combinations between* Spiroplasma *positive and negative* Gff *flies.

The result indicated that maternal transmission dominates. However, faint positive bands were also seen where only the father harbours *Spiroplasma*. The transmission of *Spiroplasma* is mainly vertical and stable over generations.

3.4. Impact of *Spiroplasma* prevalence on *Gff* colony development

This experiment (section 2.4.4.) was designed to test whether significantly higher *Spiroplasma* prevalence colonies, could affect the standard colony performance under laboratory conditions such as mortality and fecundity. The results are presented in Tables 7 and 8. PCR analysis of 71 females and males of the F2 generation from the *Spiroplasma* positive colony resulted in 36 infected, hence a *Spiroplasma* prevalence of 51%. However, undetectable amplification of *Spiroplasma* 16S rRNA was observed in 82 flies of the F2 progeny from the negative colony. The mortality and productivity of F2 in both *Spiroplasma* positive/negative colonies were recorded weekly. Both *Spiroplasma* positive and negative *Gff* colonies produced pupae with a slight variation that was not statistically significant ($t = 0.5122$, d.f. = 20, $p = 0.61$). The difference in mortality rate between the two groups was also statistically insignificant ($t = 0.9944$, d.f. = 20, $p = 0.33$) (Figure 11). The emergence of males and females was similar in both *Spiroplasma* positive and negative *Gff* colonies. No difference in sex ratio was observed.

Table 7: Mortality rate and productivity during 11 weeks observation of *Spiroplasma* positive *Gff* colony (F2)

Weekly Unit	Female Input	Total mortality	Mortality (ratio)	Mortality (Angular transformed)	Total pupae production	Pupae/initial female
43	12	6	0,50	0,79	46	3,83
42	9	5	0,56	0,84	28	3,11
41	11	9	0,82	1,13	21	1,91
40	17	16	0,94	1,33	25	1,47
39	22	7	0,32	0,60	72	3,27
38	34	17	0,50	0,79	104	3,06
37	23	8	0,35	0,63	78	3,39
36	11	4	0,36	0,65	48	4,36
35	21	11	0,52	0,81	80	3,81
34	32	21	0,66	0,94	120	3,75
33	12	4	0,33	0,62	39	3,25
Total	204	108	0,53	0,81	661	3,24

Table 8: Mortality rate and productivity during 11 weeks observation of *Spiroplasma* negative *Gff* colony (F2)

Weekly Unit	Female Input	Total mortality	Mortality (ratio)	Mortality (Angular transformed)	Total pupae production	Pupae/initial female
43	21	8	0,38	0,67	46	2,19
42	10	1	0,10	0,32	41	4,10
41	12	11	0,92	1,28	25	2,08
40	27	18	0,67	0,96	45	1,67
39	29	18	0,62	0,91	86	2,97
38	23	9	0,39	0,68	81	3,52
37	10	5	0,50	0,79	47	4,70
36	9	2	0,22	0,49	30	3,33
35	18	5	0,28	0,56	82	4,56
34	15	9	0,60	0,89	62	4,13
33	9	1	0,11	0,34	36	4,00
Total	183	87	0,48	0,76	581	3,17

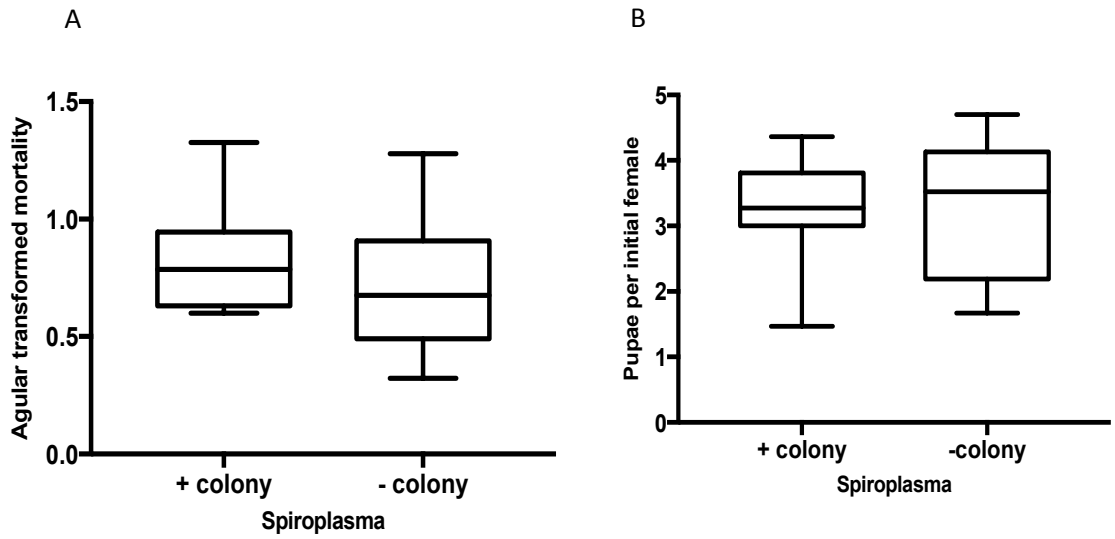

Figure 11: Box-plots graph of the effect of* Spiroplasma *infection on* Gff *colony development on A: Mortality and B: Pupae production. Values shown are mean (centre rules in boxes) with minimum and maximum. Variation in values is not statistically significant (t = 0.9944, d.f. = 20, p = 0.33 and t = 0.5122, d.f. = 20, p = 0.61 respectively).

3.5. Purification and sequencing

The 16S rRNA *Spiroplasma* PCR products obtained from extracted DNA throughout three generations (Parents, F1 and F2) of the *Spiroplasma* positive *Gff* colony in IPCL as well as infected *Gff* from Kality, mass rearing and irradiation center, were purified and sent for sequencing. The sequencing of all PCR products was successful. All obtained sequences were approximately 420 bp. All sequences were identical with no SNPs, therefore all Gff in IPCL and Kality harbour the same strain. Analysis based on partial 16S rRNA gene sequences identified *Spiroplasma penaei* SHRIMP(T), *Spiroplasma insolitum* ATCC 33502(T) (both 98.1% similarity), *Spiroplasma poulsonii* ATCC 43153(T), *Spiroplasma melliferum* BC-3(T) (both 97% similarity) and *Spiroplasma citri* ATCC 27556(T) (96.2%) as the next closest relatives, placing the *Spiroplasma* in the *citri* group.

3.6. Comparative analysis of *Spiroplasma* copy number in *Gff* from IPCL and Kality

3.6.1. Design of Primers and protocol

For qPCR, primers were designed to amplify a coding region of 100 bp in the middle of 16S rRNA *Spiroplasma* gene. Gradient PCR was performed and the annealing temperature was optimized to 65 ˚C. The *Spiroplasma* copy number in adult flies was estimated using the generated standard curve. Standard curve exhibited partial

linearity and correspond 2350 to 2.35 x 10^7 copies. Amplification efficiency, E, is calculated from the slope of the standard curve and indicates a PCR efficiency of 122.3% (y=-2.882+25.979) (Figure 12A and B). The specificity of qPCR amplicons was ascertained by checking their melting temperature (Figure 12C).

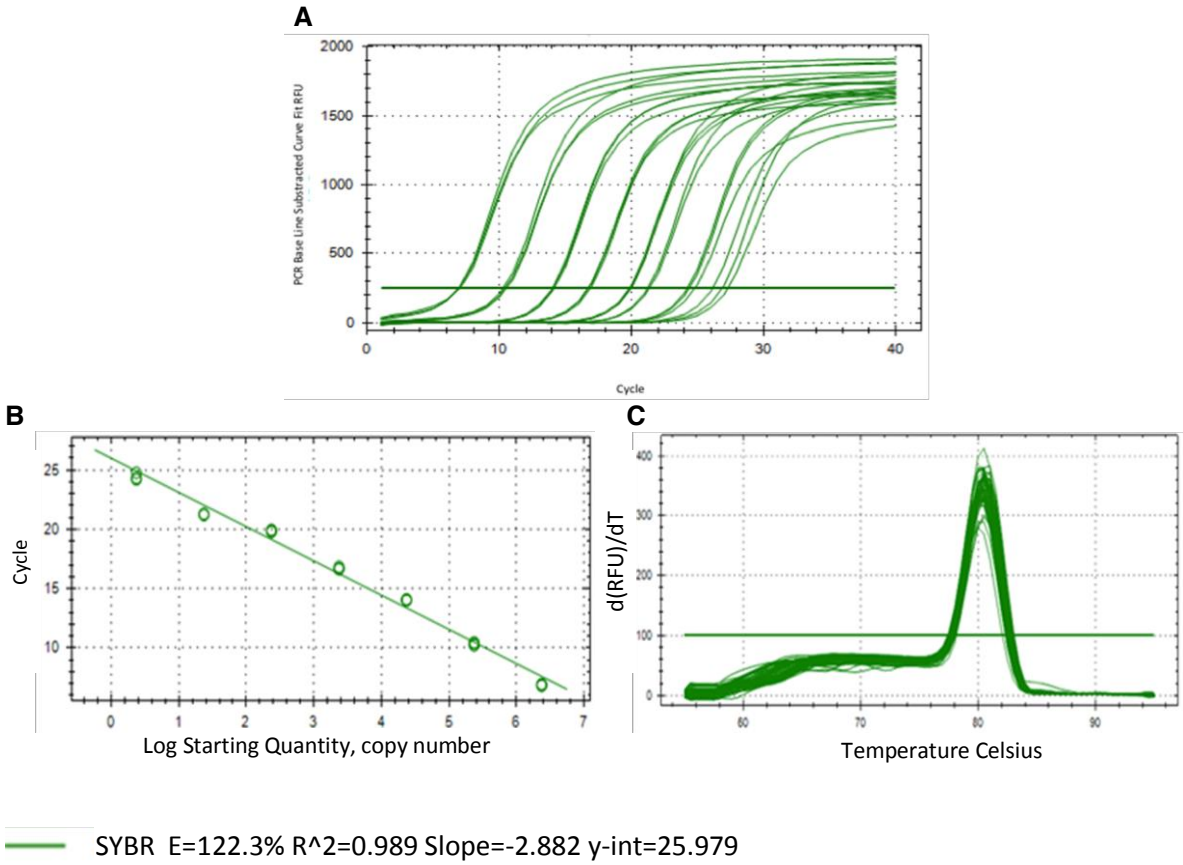

Figure 12: Quantitative PCR, generation of a standard curve for* Spiroplasma*. A: amplification curve of the dilution series. B: Standard curve with C_T plotted against the log of the starting quantity of template for each dilution and the equation for the regression line and r value. C: melt-curve analysis of reaction product from a SYBR Green I assay. The melt peak of 80.5 ˚C indicates the specificity of amplification reaction.

3.6.2. Quantification of *Spiroplasma* in infected and uninfected *Gff*

Using the above calibration *Spiroplasma* copy number was determined in the total DNA extracted from the whole body of six *Spiroplasma* positive G*ff* colony and six from *Spiroplasma* negative *Gff* colony following infection detection with PCR as described in section 2.4.3. As shown in table 8 the flies can be separated according to their *Spiroplasma* loads. The negative *Gff* had *Spiroplasma* loads averaging 2.77E+2 per individual whereas the positive *Gff* showed considerable variation between individuals and notably higher with average of 1.16E+4. General analysis

by t-test assessed *Spiroplasma* copy number between the two groups as statistically significant (t = 28.15, d.f. = 34, $p < 0.0001$) (Figure 13). *Spiroplasma* copy number with an average of 3,85E+3, 1,63E+4 and 1,95E+4 was detected from Kality colonies in May, September and November 2015 respectively (Table 10). Comparison of *Spiroplasma* loads between the three groups from Kality and IPCL was assessed by ANOVA (F = 5.544, d.f. = 68, $p = 0.0018$) (Figure 14). Means separation by Tukey's test showed the logarithm of the *Spiroplasma* copy number from *Gff* positive females collected from Kality in May and November 2015 was significantly different from the positive females from the newly established *Gff* lines in IPCL and Kality from September 2015 (Table 11).

Table 9: *Spiroplasma* copy number for the positive and negative *Gff* females from the newly established lines. DNA was extracted from whole body from 12 flies. The DNA concentration was determined using NanoDrop and diluted to 4 ng/µl. Each sample was assayed in triplicate.

	Spiroplasma copy number	
	Positive	Negative
1	15685,7	336,0
1	17449,0	346,9
1	16387,8	431,0
2	13497,4	167,2
2	13282,1	149,2
2	14220,5	245,4
3	6460,9	433,6
3	6490,1	484,8
3	7027,8	596,6
4	8598,1	107,2
4	9248,6	176,3
4	9390,7	189,9
5	11285,3	158,6
5	15328,0	274,1
5	13733,3	297,9
6	10118,8	156,8
6	10087,5	202,1
6	9734,4	231,8
Mean	11557,0	277,0

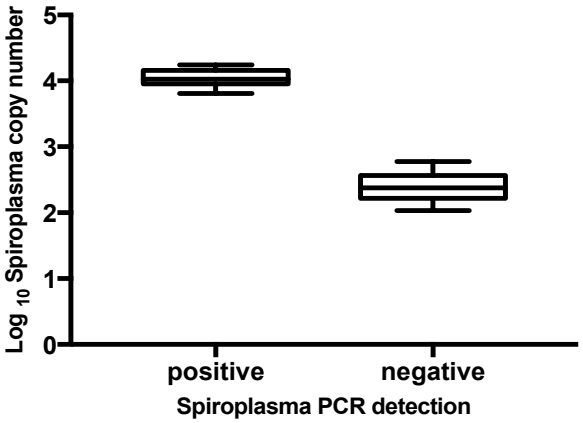

Figure 13: Box-plots graph of log_{10}* Spiroplasma *copy number for the positive and negative* Gff *colonies. Values shown are mean with minimum and maximum. Variation in values is statistically significant (t = 28.15, d.f. = 34, p < 0.0001).

Table 10: *Spiroplasma* copy number for 7 day-old females from the newly established *Spiroplasma* positive *Gff* line (F2) and Kality Tsetse fly rearing and irradiation centre, Addis Ababa, Ethiopia. Females from Kality were collected in three different occasions: May, September and November 2015. DNA was extracted from whole body. DNA concentration was determined using NanoDrop and diluted to 4 ng/µl. Each sample was assayed in triplicate.

	Spiroplasma copy number			
	IPCL	Kality May	Kality Sep	Kality Nov
1	15685,7	2747,4	10627,5	147,4
1	17449,0	2590,2	10580,4	142,0
1	16387,8	2500,7	9866,7	132,1
2	13497,4	3341,9	4387,8	5471,1
2	13282,1	3445,2	5795,9	5414,2
2	14220,5	3290,3	5722,4	5095,7
3	6460,9	5721,7	31245,4	14253,8
3	6490,1	6758,3	31736,1	13600,0
3	7027,8	7012,2	32804,1	12868,8
4	8598,1	1392,3	12029,6	27381,6
4	9248,6	1513,2	12437,0	26512,1
4	9390,7	1429,5	13966,7	25565,2
5	11285,3	3082,7	28071,1	33510,2
5	15328,0	2896,3	28266,7	32285,7
5	13733,3	3274,0	27462,2	31346,9
6	10118,8	5735,2	9752,5	36131,9
6	10087,5	6185,9	9890,6	41560,4
6	9734,4	6388,7	9220,1	40373,6
Mean	11557,0	3850,3	16325,7	19544,1

Table 11: Tukey's test for the differences of the log_{10} mean *Spiroplasma* copy numbers for positive *Gff* females from the newly established lines and positive *Gff* females from Kality. Above the diagonal is the difference between the means, below the significance.

		IPCL	Kality May	Kality Sep	Kality Nov
		4,043	3,531	4,129	3,922
IPCL	4,043		0,512	-0,086	0,121
Kality May	3,531	*		-0,598	0,391
Kality Sep	4,129	ns	**		0,207
Kality Nov	3,922	ns	ns	ns	

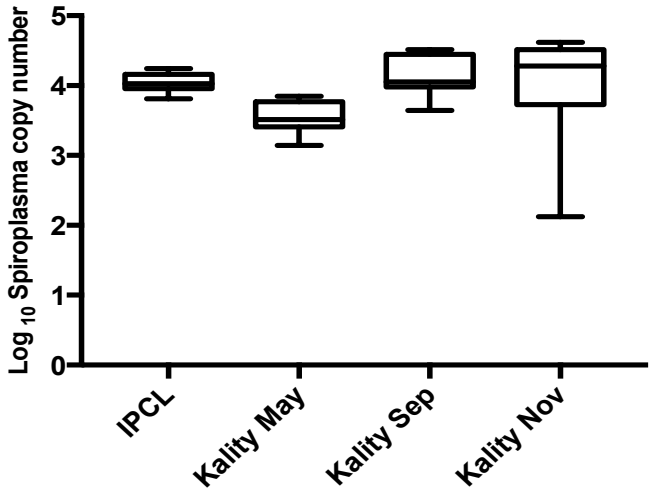

Figure 14: Box-plots of log_{10}* Spiroplasma *copy number for positive* Gff *females from the newly established lines and positive* Gff *females from Kality. Values shown are mean with minimum and maximum. Variation in* Spiroplasma *copy number in positive Gff from IPCL and Kality is statistically significant (F = 5.544, d.f. = 68, p = 0.0018).

3.7. Mating Performance Field Cage Tests

3.7.1. Effect of *Spiroplasma* infection on mating behaviour of *Gff*

Mating experiments were conducted to observe whether *Spiroplasma* infection affects the mating behaviour and interaction between *Gff* females and males. Total of 82 pairs with different mating combinations were collected during six replicates in field cage tests. PCR detection of *Spiroplasma* infection revealed 44 observed pairs of -♀ × -♂, 19 pairs of +♀ × -♂, 14 pairs of -♀ × +♂ and 5 pairs of +♀ × +♂ (Table 12). More than 50% of the pairs were *Spiroplasma* negative individuals. These results might suggest that *Spiroplasma* infection affects mating choice. However, one must not ignore that the *Spiroplasma* prevalence, from which the "positive" flies were collected, was 51% as shown previously in section 3.3. This creates a non-equal ratio between the positive and the negative individuals during the field cage test. To determine if these mating combinations were random or not, we determined *Spiroplasm*a prevalence in replicate 1 and 2 during the field cage tests and assumed

that this ratio maintains in subsequent replicates (Table 13). According to the actual ratio of positive and negative *Gff*, a contingency table with the expected ratios assuming random mating was calculated (Table 14). Multiplying this ratio by the total number of pairs collected during all replicates (82) resulted in the expected mating combination assuming random mating. The number of expected and observed matings was tested for equality of performance by *Spiroplasma* positive and negative *Gff* flies using the goodness of fit statistic G (Table 15). The observed pairing combinations didn't differ significantly from the expected values, therefore we don't reject the null hypothesis and therefore there is no tendency toward selective mating influenced by *Spiroplasma* infection. That means positive and negative females select positive or negative males randomly to mate (G = 1.39, d.f. = 3, *P* = 0.7).

Table 12: Mating performance of *Spiroplasma* positive and negative *Gff* flies from the newly established lines. Six replicates were performed. For each replicate the number of different mating combination pairs is listed.

	Replicate						
Mating combinations	1	2	3	4	5	6	Total
-♀ × -♂	4	12	6	10	5	7	44
+♀ × -♂	1	11	3	2	1	1	19
-♀ × +♂	0	5	3	1	2	3	14
+♀ × +♂	0	3	0	1	0	1	5
Total	5	31	12	14	8	12	82

Table 13: *Spiroplasma* prevalence ratio in replicate 1 and 2 during field cage tests. *Spiroplasma* detection with PCR was performed after the field cage test.

	Female		Male	
	-	+	-	+
Rep. 1 (PCR detection)	27	9	31	6
Rep. 2 (PCR detection)	17	14	23	8
Total	44	23	54	14
Ratio	0,657	0,343	0,794	0,206

Table 14: Contingency table of mating combination by random choice.

		-♀	+♀
		0,657	0,343
-♂	0,794	0,522	0,273
+♂	0,206	0,135	0,071

Table 15: Goodness of fit statistic G (Sokal & Rohlf, 1995) for random mating in the field cage tests.

Mating combinations	Expected $\hat{f}$	Observed f	f/ $\hat{f}$	f * ln (f /$\hat{f}$)	ln L	G
-♀ × -♂	42,764	44	1,029	1,254	0,693	1,386
+♀ × -♂	22,354	19	0,850	-3,089		
-♀ × +♂	11,087	14	1,263	3,266		
+♀ × +♂	5,795	5	0,863	-0,738		

4. DISCUSSION

The success of the SIT depends on efficient mass rearing production strategies and the release of sterile yet high quality and sexually competitive males of a target population. The threat of *Gff* colony collapse in the Kality tsetse mass rearing and irradiation centre in Ethiopia raised much concern and led to a thorough investigation of potential causes. The gram-positive bacteria from the genus *Spiroplasma* was suspected to be the causal agent of the severe decline in the *Gff* colony in Kality as a high prevalence of this infection was shown in male and female individuals.

Spiroplasma is widely known to be harbored by most insect species, including important disease vectors and crop pests. Studies reported that in arthropods some strains of *Spiroplasma* lead to male killing in the early embryonic stages and some are generally parasitic to their hosts causing negative fitness effects (Harumoto *et al*., 2014; Maudlin 1991). A similar scenario in tsetse flies would propose improved strategies on controlled tsetse fly competency in transmission of *Spiroplasma* for rearing facilities and thereby salvaging colonized tsetse for SIT application in sub-Saharan Africa. In this study we intended to verify whether the *Spiroplasma* strain infecting *Gff* effects colony development parameters, such as productivity, mortality and mating choice or isolation, which could lead to insufficient mass production and hence increase costs of the SIT program.

The prevalence of *Spiroplasma* was tested in the tsetse colonies listed in Table 1. Screening of *Spiroplasma* in ten different colonized tsetse populations in IPCL and Kality was negative for all colonies except *Gff*. The infection prevalence in the IPCL *G. f. fuscipes* colony stands at approximately 33% whereas in Kality it averaged significantly higher at 55%.

Using sensitive specific and non-destructive PCR assay enabled flies that gave the same PCR amplicon with a size ~450 bp to be grouped to four different colonies. The establishment of *Gff* new colonies in compliance with the parental status of *Spiroplasma* infection was attempted to gain insights into the symbiont-induced phenotype and investigation of their influence on fly longevity. One of the difficulties in establishing new lines is the low fecundity of the female tsetse flies combined with reproductive period spread over several months. This prolongs the time required to

establish pure lines over several generations. Therefore to speed the establishment of the new lines, individuals who showed a fine band after PCR amplification (DNA extracted from one mesothoracic leg) were included in the matings of the positive colony. Fine bands might refer to a low load of *Spiroplasma* infection. F2 of the negative colony showed no *Spiroplasma* infection (No band on the agarose gel). This implies that F2 generation of the *Spiroplasma* negative colony is not infected or harbours low *Spiroplasma* load and is undetectable on agarose gel (no band). However in the positive colony there was a stable and increasing level of infection prevalence over three generations.

Spiroplasma studies in *Drosophila* show that transmission efficiency might be affected by the paternal host immune system, age, temperature (high temperature may lower transmission efficiency) and the host titre loads (Haselkorn *et al*., 2013). The full role of these factors in *Spiroplasma* infections in tsetse flies is yet unclear, however our results show that *Spiroplasma* is inherited through a maternal lineage. End-point PCR amplification of F1 progeny from the newly established *Gff* lines where only the mother was infected showed a band on the agarose gel, however F1 progeny where only the father was infected showed either no bands or very faint ones. As previously described in the literature, while many lineages exhibit transovarial transmission in *Drosophila spp.* (Herren *et al*., 2013) some others are transmitted horizontally (Gasparish, 2002). We suspected that horizontal transmission of *Spiroplasma* in tsetse occurred in the laboratory colony fed on the *in vitro* membrane system through contamination of the blood by the release of saliva during feed. Multiple feedings on the same blood meal might be a reason for a stable and significantly higher *Spiroplasma* prevalence in Kality laboratory colonies.

The development of a qPCR protocol allowed a more precise quantification of *Spiroplasma* copy number from female DNA extracts in both colonies. These results showed flies with extremely low titre *Spiroplasma* infection averaging 277 copies detectable in 7-day old females, and flies with *Spiroplasma* titres higher than 6000 copies. The detection of *Spiroplasma* in negative flies may not be due to *Spiroplasma* presence, but typical SYBR binding during amplification reaction to any dsDNA, such as primer dimers. These titres correspond to flies giving no band (*Spiroplasma* negative) and a strong band (*Spiroplasma* positive) in the end-point PCR, respectively. *Spiroplasma* loads in females collected from Kality *Gff* colonies in

September and November 2015 didn't show a significant difference from those in the IPCL *Gff* positive colony. *Spiroplasma* loads in positive *Gff* from Kality collected in May were significantly lower than the positive *Gff* in IPCL and Kality in September, but not different from Kality in November. The results of sample number 1 from Kality November (Table 9) were not consistent. This might have been due to pipetting mistake while loading the DNA to the reaction sample and SYBR binding to primer dimers during PCR reaction as we see in negative flies. The figures of this sample are reducing the average of Kality in November, which might explain why it is statistically insignificant from the positive flies from Kality May. There is no significant different in *Spirplasma* loads between Kality September and November, this might suggest that decreased *Spiroplasma* copy number in Kality May might be due to collection of younger *Gff* females (less than ~7 days) (*Spiroplasma* population didn't grow much).

The individual variation in *Spiroplasma* copy number of randomly sampled flies supports the results in section 3.3, where we suggest that the transmission is vertical and reflects differences in *Spiroplasma* copy number acquired by each fly from the mother.

Results from sequence analysis of the partial 16S rRNA gene of *Spiroplasma* from all newly established *Gff* lines in IPCL and Kality, Ethiopia, clearly demonstrate a member of the genus *Spiroplasma*, however very high similarities (>97%) between members of different species indicate that the partial sequences of the 16S rRNA is not useful for identification at the species level. However, the fact that the Kality *Gff* colony was originally transferred from IPCL strengthens the suggestion that this is the same strain. Isolation and further detailed physiological and biochemical characterization of the *Spiroplasma* strain found in *Gff* should be performed.

Demonstrating purity of both newly established *Gff* colonies (*Spiroplasma* positive and negative) and the significant difference in *Spiroplasma* prevalence (F2 of the positive colony, achieved a *Spiroplasma* prevalence averaging 51% in comparison to 0% in the F2 of the negative colony) allowed us to verify whether high infection prevalence affects fly productivity, mortality and mating behaviour.

The weekly data collection (pupae production and female mortality) from the *Spiroplasma* positive and negative lines (F2 generation) in IPCL permitted us to

compare colony development parameters and to determine whether a high prevalence of *Spiroplasma* can reduce healthy colony performance. The results demonstrate no significant difference between *Spiroplasma* positive and negative *Gff* colonies. Positive and negative colonies exhibited similar mortality (angular transformed 0.81 and 0.76, respectively) and pupae production per initial female (3.24 and 3.17, respectively). *Spiroplasma* infection screening of 139 randomly collected *Gff* females and males from Kality colonies in November 2015, resulted in a total of 85 infected individuals (61%). Although the F2 of the *Spiroplasma* positive *Gff* in IPCL exhibits a prevalence of 51%, it remains a productive colony with pupae per initial female of 3.24.

Field cages, set up to mimic as closely as possible the natural environment for sexual compatibility studies between strains (mass reared laboratory strains, wild populations, different fly species or hybrids and genetic sexing strains), are generally used to monitor quality of tsetse for use in the SIT. We used the field cage to capture the actual scenario in the small laboratory cages and determine if *Spiroplasma* infection leads to mating isolation and so affects the normal growth of a mass reared colony. The data from our field cage tests indicated that there is no tendency towards assortative mating, that is *Spiroplasma* infected/non-infected female do not select a mating partner according to *Spiroplasma* infection. Both types of males are as competitive in mating regardless of *Spiroplasma* infection status.

5. CONCLUSION AND PERSPECTIVES

The SIT is important as final component implemented in area-wide pest management worldwide. The success of SIT is achieved by the release of mass-produced sterile males into the environment. Maintaining a high ratio of sterile to wild males within an area leads to wild females mating mostly with sterile males, thereby lowering their fertility. This success primarily depends on the efficient mass production of a specific species, thus any reduction in mass rearing or poor colony development will increase the labor and attendant costs and reduce the efficiency of SIT.

The near collapse of the *Gff* colony in Kality was associated with a high prevalence of *Spiroplasma* infection. This prompted the establishment of new *Gff* lines after a sensitive assay, which identified flies carrying *Spiroplasma* infection. Monitoring the prevalence and bacterial load of *Spiroplasma* through PCR and qPCR in these lines demonstrated no significant difference to the *Gff* colony in Kality. However, in contrast to the Kality colonies, the newly established *Spiroplasma* positive and negative *Gff* lines in IPCL exhibited stable development and normal productivity. There is no clear correlation between high prevalence of *Spiroplasma* infection in Kality and the reduction of colony productivity. This may be due to: (1) A better colony handling in IPCL due to its proportional small size, and so exposure to less stress, this leads to a healthy immune system, which lowers or keeps *Spiroplasma* pathogenic nature under control, (2) *Spiroplasma* infection does not affect tsetse colony performance.

Additional factors to the poor colony development in Kality cannot be ruled out as no further description were given related to colony management strategy and its deviation from standard procedures. *Gff* colony decline could have been associated with inefficient colony management and bad handling, such as multiple feeding on one blood meal, suboptimal blood temperature during feeding, low blood quality, low humidity and in general low hygiene conditions. Reason for the decline could also be a combination of ineffective colony handling and of pathogens, which might thrive when flies are exposed to additional stress, resulting in a weak immune system.

These research results were aimed to determine whether high prevalence of *Spiroplasma* infection in *Gff* affects colony development and partially serve for the establishment of effective improvement strategies for tsetse rearing factories in Kality.

Based on the results form the Spiroplasma positive and negative newly established Gff colonies in IPCL, we recommend regular monitoring of *Spiroplasma* prevalence in the next five generations and its impact on colony development. Furthermore, to determine *Spiroplasma* opportunistic feature, positive and negative colonies should be observed under stressful conditions.

For the Kality *Gff* colony we suggest better feeding management protocols, which could be a key factor to reduce bacterial and viral horizontal transmission in the blood and, therefore, maintain a stable and productive mass rearing for the application of SIT strategies for the control of tsetse and trypanosomosis in sub-Saharan Africa.

7. REFERENCE LIST

Abd-Alla A, Bossin H, Cousserans F, Parker AG, Bergoin M and Robinson A (2007a). Development of a non-destructive PCR method for detection of the salivary gland hypertrophy virus (SGHV) in tsetse flies. J. Virol. Methods.139 (2): 143-149, doi: 10.1016/j.jviromet.

Abd-Alla A, Cousserans F, Parker AG, Parker N, Robinson A and Bergoin M (2007b). Characterization of a novel virus from the mass-reared tsetse fly *Glossina pallidipes*. In J. C. Van Lenteren, P. DeClerq, & M. W. Johnson (Eds.), *Bulletin IOBC Global: Vol. 3* (pp. 1-4). Precedings of 11th meeting of the Working Group Arthropod Mass Rearing and Quality Control, Wageningen, The Netherlands: International Organization for Biological Control of Noxious Animals and Plants.

Allsopp R (1984). Control of tsetse flies (Diptera: Glossinidae) using insecticides: a review and future prospects. Bull. Entomol. Res. 74:1-23.

Bauer B, Amsler–Delafosse S, Clausen P.–H, Kabore I and Petrich–Bauer J (1995). Successful application of deltamethrin pour–on to cattle in a campaign against tsetse flies (Glossina spp.) in the pastoral zone of Samorogouan, Burkina Faso. Trop. Med. Parasitol. 46:183-189.

Cattand P, Jannin J and Lucas P (2001). Sleeping sickness surveillance: an essential step towards elimination. Trop. Med. Int. Health. 6(5):348-61.

Clark TB and Whitcomb RF (1984). Pathogenicity of mollicutes for insects: possible use in biological control. J. Ann. Microbiol. 135A:141-150.

Clarck TB, Whitcomb RF and Tully JG (1985). *Spiroplasma melliferum,* a new species from the honeybee (*Apis mellifera)*. Int. J. Syst. Bacteriol. 35:296-208.

Cuisance D (1989). Glossines et trypanosomes. Master international Erasmus/Comett/Alfa sur les Maladies parasitaires tropicales. Valençia (Spain). 115 p.

Davis R, Worley J, Whitcomb R, Ishijima R and Steere R (1972). Helical filaments produced by a mycoplasmalike organism associated with corn stunt disease. Science 176:521-523.

Du Toit R. (1954). Trypanosomosis in Zululand and the control of tsetse flies by chemical means. J. Vet. Res. 26:317-387.

Eskafi FM, McCoy RE and Norris RC (1987). Pathology of *Spiroplasma* floricola in *Galleria mellonella* larvae. J. Invertebr. Pathol. 49:1-13.

Feldmann U (1994). Some quality control parameters used in the rearing of tsetse flies. In J.P.R. Ochieng'-Odero (Ed.), Techniques of insect rearing for the development of integrated pest and vector management strategies.

Fukatsu T and Nikoh N (2000). Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera). Appl. Environ. Microbiol. 66**:**643-650.

Gasparish GE (2002). *Spiroplasma*: evolution, adaptation and diversity. Front. Biosci. 7:d619-d640.

Gasparich GE (2004). *Spiroplasmas* and phytoplasmas: microbes associated with plant hosts. Biologicals. 38(2):193-203.

Geerts S, Holmes PH, Diall O and Eisler MC (2001). African bovine trypanosomosis: the problem of drug resistance. Trends Parasitol. 17:25-28.

Harumoto T, Anbutsu H and Fukatsu T (2014). Male-killing *Spiroplasma* induces sex-specific cell death via host apoptotic pathway. PLoS. Pathog. 13:10(2), doi: 10.1371/journal.ppat.1003956.

Haselkorn TS, Watts TD and Markow TA (2013). Density dynamics of diverse *Spiroplasma* strains naturally infecting different species of *Drosophila*. Fly (*Austin*) 7(3): 204-210. doi: 10.4161/fly.25469

Herren JK, Paredes JC, Schüpfer F and Lemaitre B (2013). Vertical transmission of a *Drosophila* endosymbiont via cooption of the yolk transport and internalization machinery. MBio. 4(2), doi: 10.1128/mBio.00532-12.

Hyseni C, Kato AB, Okedi LM, Masembe C, Ouma JO, Aksoy S and Caccone A (2012). The population structure of *Glossina fuscipes fuscipes* in the Lake Victoria basin in Uganda: implications for vector control. Parasit Vectors. 5:222, doi: 10.1186/1756-3305-5-222.

Jenni L, Molyneux D, Livesey JL and Galun R (1980). Feeding behavior of tsetse flies infected with salivarian trypanosomes. Nature 283:383-385.

Lietze VU, Abd-Alla AM, Vreysen MJ, Geden CJ and Boucias DG (2010). Salivary gland hypertrophy viruses: a novel group of insect pathogenic viruses. Annu. Rev. Entomol. 56:63-80. doi: 10.1146/annurev-ento-120709-144841.

Maudlin I (1991). Transmission of African Trypanosomiasis: Interactions among tsetse immune system, symbionts and parasites. Inc. Advances in Disease Vector Research 7:117-148

Mateos M, Castrezana SJ, Nankivell BJ, Estes AM, Markow TA and Moran NA (2006). Heritable endosymbionts of *Drosophila*. Genet. 174:363–376, doi: 10.1534/genetics.106.058818.

Moran ZR and Parker AG (2016). Near Infrared Imaging as a Method of Studying Tsetse Fly (Diptera:Glossinidae) Pupal Development. J. Insect Sci. 16(1), doi: http://dx.doi.org/10.1093/jisesa/iew047 72.

Mouches C, Bové JM and Albisetti J (1984). Pathogenicity of *Spiroplasma apis* and other spiroplasmas for honeybees in southwestern France. Ann. Microbiol. (Inst. Pasteur) 135A:151-155.

Mouches C, Bové JM, Tully JG, Rose DL, McCoy RE and Carle-Junca P (1983). *Spiroplasma apis*, a new species from the honey-bee *Apis mellifera*. Ann. Microbiol. (Inst. Pasteur) 134A:383–397.

Mulligan HW (1981). Tropical medicine and research in British territories overseas. Trans. R. Soc. Trop. Med. Hyg. 75:9-11.

Mutika GN, Opiyo E, Robinson AS (2001). Assessing mating performance of male *Glossina pallidipes* (Diptera: Glossinidae) using a walk-in field cage. Bull. Entomol. Res. 91(4):281-8.

Robinson AS (2002). Mutations and their use in insect control. Mutat. Res. 511:113-132.

Sang RC, Jura WG, Otieno LH, Mwangi RW and Ogaja P (1997). Effects of tsetse DNA virus infection on the survival of a host fly *Glossina morsitans centralis* (Diptera: Glossinidae). J. Invertebr. Pathol. *69*(3): 253-260.

Schwarz RS, Teixeira ÉW, Tauber JP, Birke JM, Martins MF, Fonseca I and Evans JD (2014). Honey bee colonies act as reservoirs for two *Spiroplasma* facultative symbionts and incur complex, multiyear infection dynamics. doi: 10.1002/mbo3.172

Seed JR (2001). African trypanosomosis research: 100 years of progress, but questions and problems still remain. Int. J. Parasitol. 31:434-442.

Shaw APM (2004). Economics of African trypanosomosis. In: Maudlin I., Holmes P.H. & Miles M.A. (eds.). The trypanosomosis. Wallingford: CABI Publishing. pp. 369-402.

Sokal RR and Rohlf FJ (1995). Biometry: the principles and practice of statistics in biological research. New-York: Freeman and Company. 703 p.

Solano P, Bouyer J, Itard J, and Cuisance D (2010). Cyclical vectors of trypanosomosis, pp. 153-181. In: Infectious and parasitic diseases of livestock (vol. 1), Chapter 012, P.-C. Lefèvre, J. Blancou, R. Chermette and G. Uilenberg Editors. Lavoisier (Tec & Doc), Paris.

Thornton B and Basu C (2011). Real-time PCR (qPCR) primer design using free online software. Biochem. Mol. Biol. Educ. 39(2):145-54, doi: 10.1002/bmb.20461.

Vickerman K, Tetley L, Hendry KA and Turner CM (1988). Biology of African trypanosomes in the tsetse fly. Biol. Cell 64:109-119.

Vreysen MJ, Saleh KM, Ali MY, Abdulla AM, ZhuZ.-R and Juma KG (2000). *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. J. Econ. Entomol. 93(1):123-135, doi:10.1603/0022-0493-93.1.123.

Waiswa C, Picozzsi K, Katunguka-Rwakishaya E, Olaho-Mukani W, Musoke RA and Welburn SC (2009). *Glossina fuscipes fuscipes* in the trypanosomosis endemic areas of southeastern Uganda: apparent density, trypanosome infection rates and host feeding preferences. Acta Trop. 99:23–29.

Wang W, Wen B, Gasparich GE, ZhuN, Rong L, Chen J and Xu Z (2004). A *Spiroplasma* associated with tremor disease in the Chinese mitten crab (*Eriocheir sinensis*). Microbiology. 150(Pt9):3035-40.

Welburn SC, Coleman PG, Maudlin I, Fevre EM, Odiit M, Eisler MC (2006). Crisis, what crisis? Control of Rhodesian sleeping sickness. Trends Parasitol. 22:123-128.

WHO (2004) http://www.who.int/tdr/publications/documents/tsetse_traps.pdf

WHO (2016) http://www.who.int/trypanosomiasis_african/diagnosis/en/ and http://www.who.int/mediacentre/factsheets/fs259/en/

Williamson DL, Sakaguchi B, Hackett KJ, Whitcomb RF, Tully JG and Carle P (1999). *Spiroplasma poulsonii sp*. nov., a new species associated with male-lethality in *Drosophila willistoni,* a neotropical species of fruit fly. Int. J. Syst. Bacteriol. 49:611-618.

Wyss JH (2000). Screwworm eradication in the Americas. Ann. NY Acad. Sci. 916:186-193.